

# **Poisonous Plants and Related Toxins**

*Edited by*

**T. Acamovic, C.S. Stewart and T.W. Pennycott**

*Animal Biology Division SAC  
AYR KA6 5HW Scotland UK*

*Gut Microbiology and Immunology Division  
Rowett Research Institute Bucksburn  
ABERDEEN AB21 9SB Scotland UK*

*Veterinary Sciences Division SAC  
AYR KA6 5HW Scotland UK*

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Tel: +44 (0)1491 832111  
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Fax: +1 617 354 6875  
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# Preface

The papers in this book are the majority of those that were presented at the Sixth International Symposium of Poisonous Plants (ISOPP6) held in Glasgow, Scotland, August 2001. The papers have been subjected to refereeing and editing. The editors are grateful to the referees and to the authors, who responded in a timely manner in the format requested. Although the refereeing process has delayed publication we hope that the changes made will help make the volume accessible to the more general reader. Due to limitations of space, some submissions have been shortened considerably. We hope that the resulting loss of content does not detract from the authors' intended message. Where animal studies were conducted, authors are responsible for meeting the ethical and legal requirements of their institutes and countries. A number have made explicit statements in this regard.

The decision to hold ISOPP6 in Glasgow, the first ISOPP venue outside the USA or Australia, was made by delegates at ISOPP5 in Texas in 1996. The organizing committee (T. Acamovic, C.S. Stewart, T. Pennycott, L. Berretti and P. Sharland) maintained the format of previous meetings with a few modifications. The meeting was dedicated to four scientists, Peter Dorling, John Edgar, Fanie Kellerman and Barry Smith, who have made outstanding contributions to the development and understanding of natural plant toxins and their effects in animals. In addition to the offered papers, there were, for the first time, five invited papers on various aspects of plant, fungal and microbial toxicology and these have been allocated extra space within this book. The editors are especially grateful to the invited speakers for their authoritative contributions.

The symposium brought together scientists from around the world with expertise in many disciplines demonstrating the global and multidisciplinary nature of the topic as well as the impact of plant toxins on a wide range of farmed and other animal species.

The symposium was held during the period of the UK foot and mouth outbreak of 2000–2001. This was a difficult time for British agriculture, and it also affected the willingness and ability of some potential participants to travel to the UK. Despite this problem, we hope that the readers of this volume will agree that the decision to press on with the meeting, in spite of the lower than anticipated number of participants, was the right one.

The next symposium will be held in Logan, Utah, USA, in 2005 under the chairmanship of Dr Kip Panter. The organizers of ISOPP6 wish Kip and colleagues well in the planning and organization of ISOPP7.

The Editors

*T. Acamovic, C.S. Stewart and T. Pennycott  
Scotland, UK*

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The organizers wish to thank the Royal Society of Chemistry and Dow AgroSciences Ltd for their financial sponsorship of the meeting and the Glasgow Tourist Board for their help with accommodation and the civic reception. We are grateful for the support provided from the Scottish Executive Environment and Rural Affairs Department, channelled through our respective organizations. The editors are indebted to Liz Berretti for her invaluable secretarial and organizational help and to Dr Priscilla Sharland for copy-editing and preparing the camera-ready copy of this book. We are also most grateful to Prof. Alan Seawright, one of the originators of this series of symposia, for his support and for his contribution.

The editors are especially grateful to the numerous referees who devoted their time to critically reviewing and commenting on the manuscripts. We are also extremely grateful to Dr Nicholas Sparks for his help in the preparation of the final version of this work.

## Dedications

The organizers of the Sixth International Symposium on Poisonous Plants, held in Scotland in 2001, were pleased that the symposium was dedicated to the following, who have been prominent in studies on plants which have toxic effects in animals.



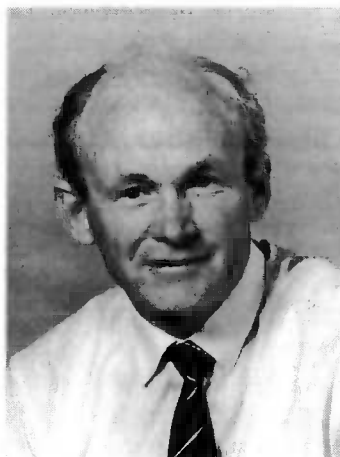
Peter R. Dorling



John A. Edgar



T.S. (Fanie) Kellerman



Barry L. Smith

**Peter R. Dorling**

Peter has contributed to the field of poisonous plants research as few have. Trained in biochemistry (PhD, University of Melbourne), he is also skilled in chemical pathology, pathology, pharmacology, toxicology, botany and organic chemistry. Truly, Peter embodies the multidisciplinary outlook essential to solving plant-associated poisoning problems.

Peter formed the plant toxins research group at Murdoch University, and has been its leader since 1976. At Murdoch, he held several appointments, including the head of the Veterinary Biological Sciences Division, and acting Dean of the School of Veterinary and Biomedical Sciences, Chair of Postgraduate Studies, and Pro-vice Chancellor for Research.

(Abstract of the dedication by Roger Coulombe)

**John A. Edgar**

In 1967, John joined the Division of Applied Chemistry of the Commonwealth Scientific and Industrial Research Organization (CSIRO) in Australia to work as an organic chemist with Dr Claude Culvenor, a doyen of plant-associated toxin research and an expert in pyrrolizidine alkaloid chemistry. In 1972, John moved with Dr Culvenor to the CSIRO Division of Animal Health to participate in the Biological Chemistry Group, a multidisciplinary team charged with investigating many economically important livestock poisoning diseases in Australia. In about 1986, John became manager of the Plant-associated Toxins Program for CSIRO's Division of Animal Health, now CSIRO Livestock Industries.

Faced with the downturn in funding and management support for plant-associated toxin (PAT) research relating solely to animal health and productivity, John recognized the need to address human health aspects of PATs, especially the potential for low level, long-term exposure to these toxins in the human food supply. Consequently, the Plant Toxins Research Group, as it is presently constituted, directs a major portion of its research effort towards addressing human health-related issues such as development of sensitive screening and validation of analytical techniques for quantifying the presence of PATs in food material.

(Abstract of the dedication by Steven M. Colegate)

**T.S. (Fanie) Kellerman**

T.S. (Fanie) Kellerman was affiliated to the Division of Toxicology of the Onderstepoort Veterinary Institute from 1970 up to his retirement in 1998, when he held the posts of Assistant Director and Head of the Division of Toxicology. During his tenure, his research centred mainly on the plant poisonings and

mycotoxicoses of livestock in southern Africa, particularly on those affecting the central nervous system and liver.

(Abstract of the dedication by Christo Botha)

### **Barry L. Smith**

Barry completed a degree in agriculture in New Zealand, then graduated in veterinary science from Brisbane and then gained a PhD from the University of Waikato. He is currently a senior scientist (Veterinary Pathology and Toxicology) in the Ruakura Research Centre in New Zealand, where he continues his work on veterinary pathology. He is one of few who have attended all the poisonous plant symposia and has contributed papers on bracken toxicosis and pitho mycotoxicosis to the various symposia and other scientific meetings.

(Abstract of the dedication by Alan Seawright)

# Chapter 1

## **Evolution of Toxins and Anti-nutritional Factors in Plants with Special Emphasis on Leguminosae**

M. Wink

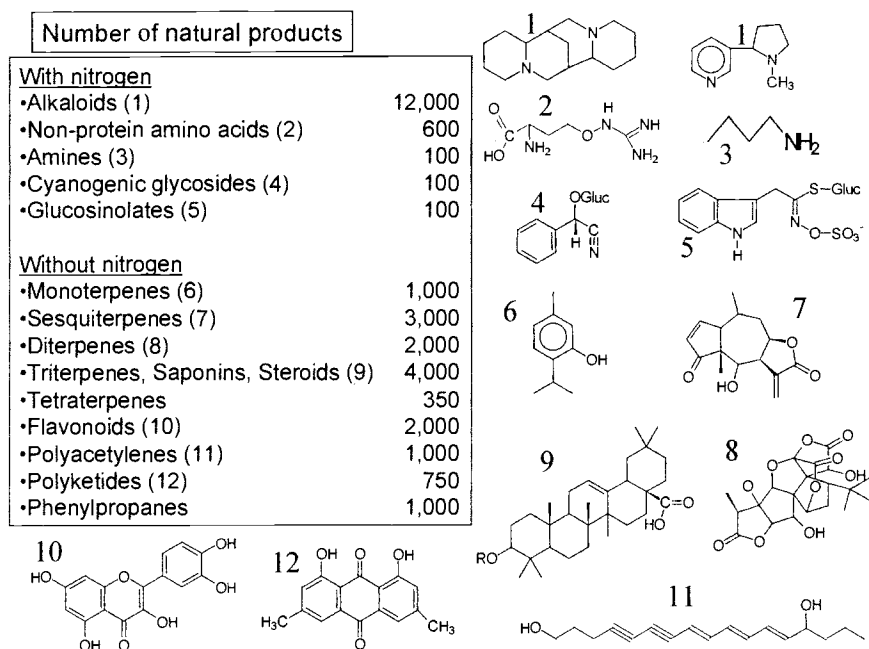
*Universität Heidelberg, Institut für Pharmazeutische Biologie, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany*

### **Introduction**

Secondary metabolites (SM) represent a diverse group of natural products. More than 80,000 natural compounds have been described from plants, over 20,000 from microorganisms and fungi, more than 20,000 from amphibia, reptiles, arthropods and marine organisms. These compounds have been isolated and their structures determined by mass spectrometry (EI-MS, CI-MS, FAB-MS), nuclear magnetic resonance ( $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ ) or X-ray diffraction (Harborne, 1993; Wink, 1999a, b). It is likely that many more compounds will be found in the future, as only a small proportion of existing organisms has been thoroughly analysed. Fig. 1.1 represents an estimate of known secondary metabolites and illustrates chemical structural examples for some of the classes of these natural products.

### **Biosynthesis, Transport, Storage, Turnover and Costs of Secondary Metabolites in Plants**

Some SM are produced in all plant tissues, but generally formation is organ-, tissue-, cell- and often development-specific. It is also assumed that the corresponding genes associated with production of these SM are regulated in a cell-, tissue- or development-specific fashion. Cellular sites of biosynthesis are compartmentalized in the plant cell and most biosynthetic pathways proceed (at least partially) in the cytoplasm. However, there is evidence that some alkaloids, such as coniine, quinolizidines, caffeine and some terpenes are synthesized in the chloroplast (Roberts, 1981; Wink and Hartmann, 1982). Coniine and amine formation has been localized in mitochondria (Roberts, 1981; Wink and Hartmann, 1981) and biosynthetic steps of protoberberine occur in the vesicles (Amann *et al.*, 1986). Hydroxylation steps are often membrane-bound in the endoplasmic reticulum where synthesis of other lipophilic compounds also



**Fig. 1.1.** Estimated number of described secondary metabolites from plants and illustration of a few representative structures.

occurs.

In many instances the site of biosynthesis is restricted to a single organ, such as roots, leaves or fruits, but an accumulation of the corresponding products can be detected in several other plant tissues. Therefore, long-distance transport must take place in these instances and occurs via xylem, phloem and sym- or apoplastic transport.

Storage of SM may be tissue- or cell-specific (Guern *et al.*, 1987). Idioblasts have been identified in many plants which contain tannins, alkaloids, or glucosinolates. More often SM are concentrated in trichomes or glandular hairs (many terpenoids in Labiatae, Asteraceae), stinging hairs (many amines in Urticaceae), or the epidermis itself (many alkaloids, flavonoids, anthocyanins, cyanogenic glycosides, coumarins, etc.) (Wiermann, 1981; Wink, 1993a, 1997; Wink and Roberts, 1998). Flowers, fruits, and seeds are usually rich in SM, especially in annual plants. In perennial species, SM are high in bulbs, roots, rhizomes and bark of the roots and stems. Water soluble secondary compounds are usually stored in the vacuole (Matile, 1978, 1984; Boller and Wiemken, 1986) whereas hydrophilic substances are sequestered in resin ducts, laticifers, glandular hairs, trichomes, thylakoid membranes, or on the cuticle (Wiermann, 1981).

Several SM are not end products of metabolism but are turned over at a regular rate (Barz and Köster, 1981). N-containing SM such as alkaloids, non-protein amino acids (NPAA), cyanogenic glycosides and protease inhibitors are metabolized at germination and serve as a nitrogen source for growing seedlings (Wink and Witte, 1985). Carbohydrates (e.g. oligosaccharides and lipids) are also turned over during germination. Concentrations of some SM vary diurnally, such as quinolizidine alkaloids, nicotine, atropine, monoterpenes and phenylpropanoids; an active interplay between synthesis and turnover is involved in these instances. Turnover of SM is also readily seen in cell suspension cultures (reviews in: Barz and Köster, 1981; Wink, 1997). Enzymes of SM biosynthesis and transport also show a regular turnover, suggesting that mRNA must be transcribed and translated into proteins regularly, even for constitutive compounds.

Both transcription and translation require substantial energy input utilizing ATP or reduction equivalents (i.e. NADPH<sub>2</sub>). For defence or signal compounds to exhibit their function, allelochemicals need to be concentrated at the right place and time. Many SM are synthesized in the cytoplasm or in cell organelles, but are stored in vacuoles. Energy for the uphill transport across the tonoplast and/or for trapping the metabolite in the vacuole is provided by H<sup>+</sup>-ATPase. When special anatomical differentiations (ducts, gland cells, trichomes) are needed, the formation and maintenance of these structures are produced at a cost to the plant. Thus, the biosynthesis and sequestration events to provide defence and signal compounds for protection to the plant comes at a substantial energy cost.

## **Evolution and Function of Secondary Metabolites**

Although several SM have been used by mankind for thousands of years as dyes (indigo, shikonine), flavours (vanillin, capsaicin, mustard oils), fragrances (rose oil, lavender oil and other essential oils), stimulants (caffeine, nicotine, ephedrine), hallucinogens (morphine, cocaine, mescaline, hyoscyamine, scopolamine, tetrahydrocannabinol), insecticides (nicotine, piperine, pyrethrin), vertebrate and human poisons (coniine, strychnine, aconitine) and even as therapeutic agents (atropine, quinine, cardenolides, codeine, etc.), their putative functions remain somewhat controversial (Mann, 1992; Roberts and Wink, 1998).

### **Defence or signal compounds versus waste theory for SM**

Secondary metabolites provide mechanisms of defence for plants and can be compared to the ability of animals and birds to evade danger through speed, flight, body armour or internal immune responses to invading microbes or parasites (Edmunds, 1974). Stahl argued in 1888, in Jena (Germany), that secondary metabolites serve as defence compounds against herbivores. Surprisingly, the defence function of SM in plants still arouses controversy.



The defence hypothesis has been elaborated during recent decades (Fraenkel, 1959; Ehrlich and Raven, 1964; Levin, 1976; Swain, 1977) and a large body of experimental evidence (reviews in: Wink, 1988, 1992, 1993c; Harborne, 1993; Bernays and Chapman, 1994) supports the following functions of SM:

1. Defence against herbivores, insects and vertebrates;
2. Defence against fungi, bacteria and viruses;
3. Defence against other plants competing for light, water and nutrients;
4. Signal compounds to attract pollinating and seed dispersing animals;
5. Signals for communication between plants and symbiotic microorganisms (N-fixing Rhizobia or mycorrhizal fungi); and
6. Protection against UV-light or other physical stress.

A number of plants employ defensive methods using mechanical and morphological protection, such as thorns, spikes, glandular and stinging hairs (often filled with noxious chemicals), or develop an impenetrable bark (especially woody perennials); these features can be interpreted as antipredatory defences (in analogy to weapons and shells in animals). Sessile or slow moving animals, such as sponges, slugs, corals and amphibia (e.g. salamanders, poison frogs, toads) are infamous for their ability to produce a wide range of usually toxic chemicals (reviews: Braekman *et al.*, 1998; Proksch and Ebel, 1998). Some insects either produce SM themselves or sequester them from their host plants (overviews in Duffey, 1980; Blum, 1981; Bernays and Chapman, 1994). In contrast to most higher animals, plants can replace various parts which have been diseased, wounded or browsed. This capacity for open growth and regeneration, is most prominent in perennials and allows for a certain tolerance toward herbivores and microbes.

In most plants, synthesis and accumulation of SM is regulated in space and time. As a rule, vulnerable tissues are defended more than old senescing ones. For example, it is usually observed that seeds, seedlings, buds and young tissues either sequester large amounts of a compound or actively synthesize them. Organs that are important for survival and multiplication, such as flowers, fruits and seeds, are nearly always a rich source of defence chemicals.

The specific localizations of SM make sense if we accept their role as defence and/or signal compounds: trichomes and glandular hairs are always on the surface of the plant; a herbivore cannot avoid a direct contact with them if it tries to feed on this plant. If membrane-active terpenes reach their lips, tongue or mandibles, many herbivores can be deterred before they actually start feeding on the plant. Another example is the sequestration of high concentrations of SM in vacuoles which are often positioned in a favourable site for defence, as many of them are stored in epidermal and subepidermal cells (Saunders and Conn, 1978; Kojima *et al.*, 1979; Matile, 1984; Wink *et al.*, 1984; Werner and Matile, 1985; Gruhnert *et al.*, 1994; Wink, 1992, 1997). If a small herbivore or microbe attacks such a plant, it will encounter a high concentration immediately at the periphery when wounding or entering the tissue which might deter further feeding. Compounds which are sequestered in resin ducts or laticifers are often under high pressure and readily squirt out when these elements become wounded. For a small insect

herbivore, this is a dangerous situation, since these effluents make their mandibles sticky. However, a few 'clever' beetles and caterpillars cut the veins of leaves upstream of the area that they want to feed on. The fluids emerge from the cuts, but cannot reach the parts downstream and thus allow consumption (Dussourd and Eisner, 1987; Becerra, 1994).

Defence against herbivores and pathogens is not necessarily constitutive. Recent research has shown that wounding and infection triggers several events in plants. For example, wounding leads to tissue breakdown thus releasing prefabricated defence chemicals (such as glucosinolates, cyanogenic glycosides, bidesmosidic saponins, alliin, ranunculin, coumarylglycosides) and mix them with hydrolysing enzymes, such as  $\beta$ -glycosidase, myrosinase, nitrilase, or alliinase (Matile, 1980). Active allelochemicals are the result. In other instances it has been shown that the level of existing defence chemicals is increased substantially within hours or days after wounding or infection, for example nicotine in *Nicotiana tabacum* (Baldwin, 1994), or lupin alkaloids in *Lupinus polyphyllus* (Wink, 1983). Especially after infection, new compounds are made and sequestered with antifungal, antibacterial or herbivore deterring activities; phytopathologists have termed these compounds 'phytoalexins'. These compounds include several isoflavones, pterocarpan, furocoumarins, chalcones, stilbens and others; many of these metabolites have antifungal properties, so that they are sometimes considered to be part of a specific antimicrobial defence system of plants. However, since most of these compounds also affect herbivores, the induced plant defence appears to be a more general phenomenon.

Recent research has shown that elicitors, receptors and ion channels and the pathways leading to jasmonic acid and salicylic acid are important elements in converting the external signal into a cellular response (Creelman and Mullet, 1997). The defence system by SM works in general, but a number of herbivores and microorganisms evolved that have overcome the defence barrier (similar to the situation of some viruses, bacteria or parasites which outcompete our immune system). In these organisms a series of adaptations can be observed, allowing them to tolerate or even use the dietary defence chemicals (reviews in: Ahmad, 1983; Brattsten and Ahmad, 1986; Rosenthal and Berenbaum, 1991, 1992; Wink, 1993c; Bernays and Chapman, 1994; Brown and Trigo, 1995; Hartmann and Witte, 1995).

Several volatiles are produced by plants when wounded, including aldehydes, esters, amines or ethylene. It has been proposed that some of these volatiles can alert the defence system of neighbouring plants. In addition, these volatiles can attract predatory arthropods. A well-studied example is spider mites (*Tetranychus urticae*) on *Phaseolus lunatus* leaves. Volatiles from infested plants attract predatory mites (*Phytoseiulus persimilis*) which prey on the spider mites that induced the reaction in the first place (Dicke *et al.*, 1990; De Moraes *et al.*, 1998). It is likely that more tritrophic systems work in this way; many of them still await to be discovered.

While zoologists have never doubted that many SM serve as chemical defence against predators, it has been argued that SM were only waste products and had

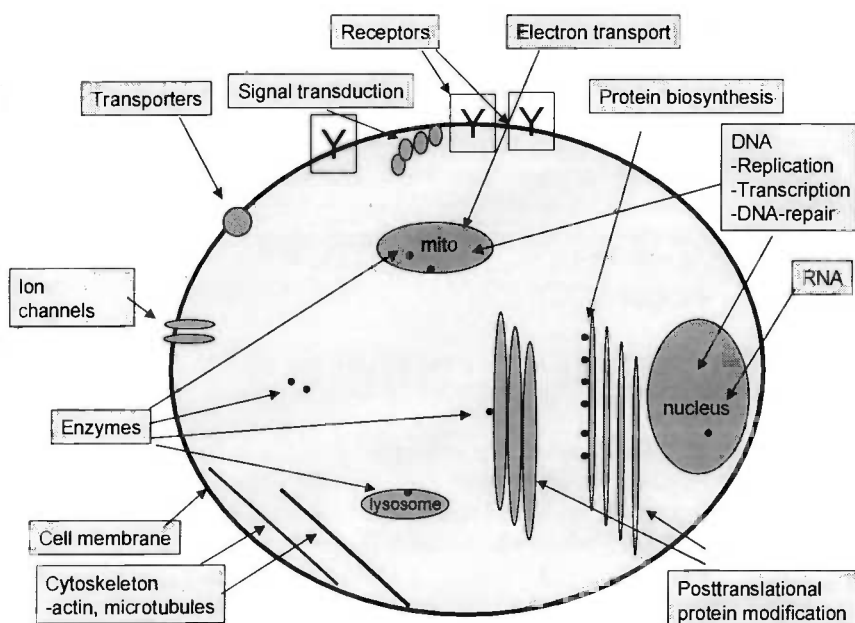
no beneficial function at all. However, this theory failed to explain several observations including:

1. Waste products are characteristic and necessary for heterotrophic animals that cannot degrade their food completely for energy production. These organisms excrete waste products that are often rich in nitrogen (i.e. urea, uric acid). However, plants are essential autotrophs and therefore do not need elaborate excretory activities. Furthermore, nitrogen is a limiting nutrient for plants. Consequently, the production of nitrogen-containing excretions, such as alkaloids, would be difficult to explain. In addition, alkaloids are often found in young or metabolically active tissues, but not in dying or senescing cells as would be expected according to the waste product hypothesis.
2. Secondary metabolites are often not inert end products of metabolism (an expected trait of waste products) but many of them can be metabolized by plant cells. For example, nitrogenous secondary metabolites, such as alkaloids, NPAA and cyanogenic glycosides are often stored in considerable quantities in legume seeds. During germination a degradation of these nitrogenous compounds occurs providing essential nitrogen sources for the growing seedling.
3. Secondary metabolism is energetically costly, often very complex and regulated in a tissue and developmentally specific manner, which would be surprising for a waste product having no inherent function.

In several instances SM provide both defensive and attractant activities such as anthocyanins or monoterpenes which are insect attractants in flowers, but serve an insecticidal and antimicrobial role in leaves. In addition, some SM concomitantly exhibit physiological functions, for example they can serve as mobile and toxic nitrogen transport and storage compounds or UV-protectants (such as flavonoids or tannins). These multiple functions are typical in plants and do not contradict the main role as chemical defence and signal compounds. If a trait can serve multiple functions and conserves energy, it is more likely that it is maintained through natural selection.

## **Toxicology of Secondary Metabolites**

If defence compounds should inhibit the growth of microbes or herbivores or are otherwise toxic to them, they must interfere with the physiology and biochemistry of these organisms. A large body of pharmacological and toxicological literature clearly documents that these activities do exist (Teuscher and Lindequist, 1994; Wink, 1993c, 1998, 1999a, b, 2000). Typical organ systems that are often affected by SM in animals include organ development, reproduction and fecundicity, respiration, digestion, muscles and motility as well as the nervous system including signal transduction, perception and processing. In order to interfere with these systems, the structures of secondary metabolites have been shaped during evolution so that they can closely interact with molecular targets in cells and tissues or other physiological features in animals or microorganisms.



**Fig. 1.2.** Illustration of potential molecular targets of cells which are affected by secondary metabolites.

Quite often structures of SM resemble endogenous substrates, hormones or neurotransmitters and can thus mimic a response at the corresponding molecular targets. There is hardly any target in animals or microorganisms for which a natural product does not exist that might modulate it. Thus, plants provide a wide array of bioactive substances. This is why so many natural products can be used in biotechnology, pharmacy, medicine and agriculture in many different ways. We use known substances or look for new, hitherto undiscovered substances or the corresponding genes encoding for their biosynthesis in plants living in deserts, rain forests or other ecosystems (so called bioprospection or gene prospection). In many instances the mechanisms that underlie these effects have been elucidated; often specific interactions with one or several of the molecular targets shown in Fig. 1.2 can be observed. We have argued that defence compounds have been shaped during evolution to specifically interact with particular targets in a process termed 'evolutionary molecular modelling' (Wink, 1997) (Figs 1.3 and 1.4).

Secondary metabolites often interfere with more than a single molecular target, which is advantageous for the producer, as a toxin might be more efficient if it inactivates two targets instead of a single target. Furthermore, it will be more difficult for a herbivore or microbe to develop resistance to such a compound, as concomitant resistance at two targets would be required. Plants usually produce a

complex mixture of compounds, each of which has its own set of biological activities, which make these mixtures even more powerful as means of defence and protection.

Because of this evolutionary logic, most plants are able to withstand various threats from herbivores, microbes and the physical environment. Exceptions are many agricultural crops, which have been optimized for yield and quite often, their original lines of defence have been selected out, as the underlying metabolites were unpalatable or toxic for humans or livestock. An example of this using quinolizidine alkaloids and plants that contain them follows.

### Quinolizidine alkaloids

Several defence compounds are transported via the phloem from the site of synthesis to other plant organs. Since the phloem is a target for many sucking insects, such as aphids, these insects encounter a high load of alkaloids in the plants producing them. Lupins, for example, produce quinolizidine alkaloids (QAs) in leaf chloroplasts and export them via the phloem all over the plant where they accumulate in epidermal tissues and especially in reproductive organs (Fig. 1.5). For lupins, in which alkaloid-rich (bitter lupins) and almost alkaloid-free varieties ('sweet' lupins) are known, it could be shown that aphid generalists (e.g. *Myzus persicae*) only sucked on 'sweet' lupins but never on alkaloid-rich varieties with high alkaloid contents in the phloem (Wink, 1992). Also, many other animals from leaf miners (*Agromyzidae*) to rabbits (*Oryctolagus cuniculus*)

### Evolutionary molecular modelling

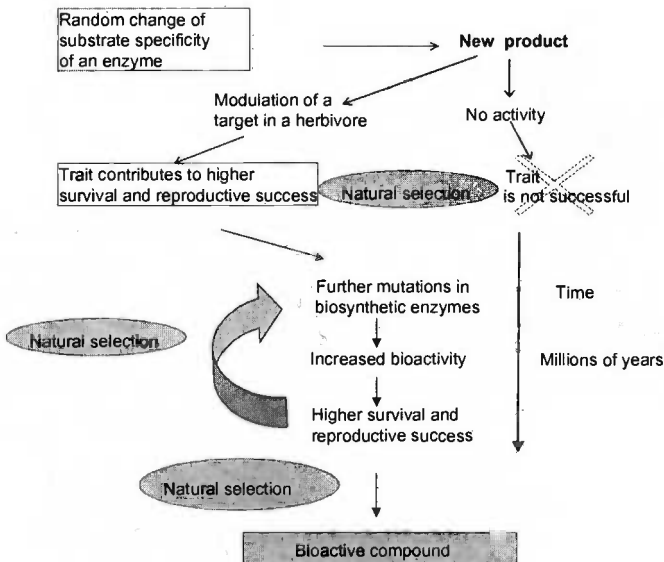
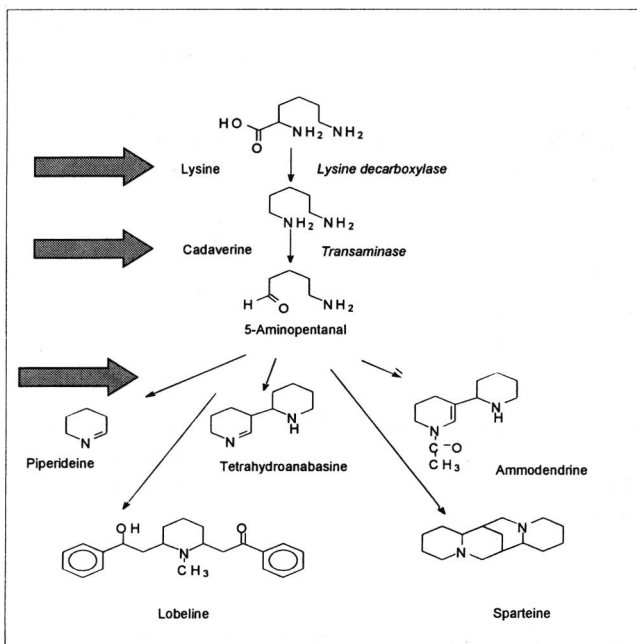


Fig. 1.3. Schematic illustration of 'evolutionary molecular modelling'.



**Fig. 1.4.** Mutations can modify existing enzymes so that new biosynthetic reactions are realized leading eventually to pharmacologically active secondary metabolites. Few mutations in enzymes of primary metabolism might generate new enzymes that can produce new natural products.

showed a similar discrimination, in that alkaloid-rich plants were left alone, while 'alkaloid-free' cultivars were highly predated (Fig. 1.6). The only exception is a specialized aphid, *Macrosiphum albifrons*, which lives on bitter lupins and sequesters the dietary alkaloids and uses them for its own defence against predators (Wink and Römer, 1986). The defence potential of lupin alkaloids is plausible as these alkaloids are toxic to animals (Fig. 1.7), interfere with the nervous system, affecting mainly nicotinic and muscarinic acetylcholine receptors (Fig. 1.8) and inhibiting  $\text{Na}^+$  and  $\text{K}^+$  channels. Minor targets are: dopamine receptors, GABA receptors, NMDA receptors, alpha 2 receptors, membrane permeability, protein biosynthesis and DNA (anagyrine, ammodendrine).

In general, we find a series of related compounds in each plant; often a few major metabolites and several minor components that differ in the position of their substituents (Fig. 1.8). The profile usually varies between plant organs, within developmental periods and sometimes even diurnally (e.g. in lupin alkaloids; Wink and Witte, 1984). Also, marked differences can usually be seen between individual plants of a single population, even more so between members of different populations. This variation, that is part of the apparent evolutionary arms race between plants and herbivores, makes adaptations by herbivores more

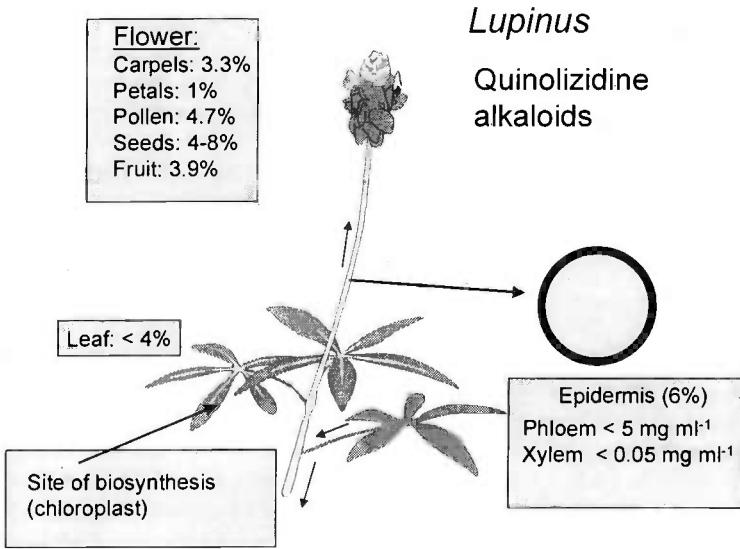


Fig. 1.5. Biosynthesis, transport and storage of quinolizidine alkaloids in lupins.

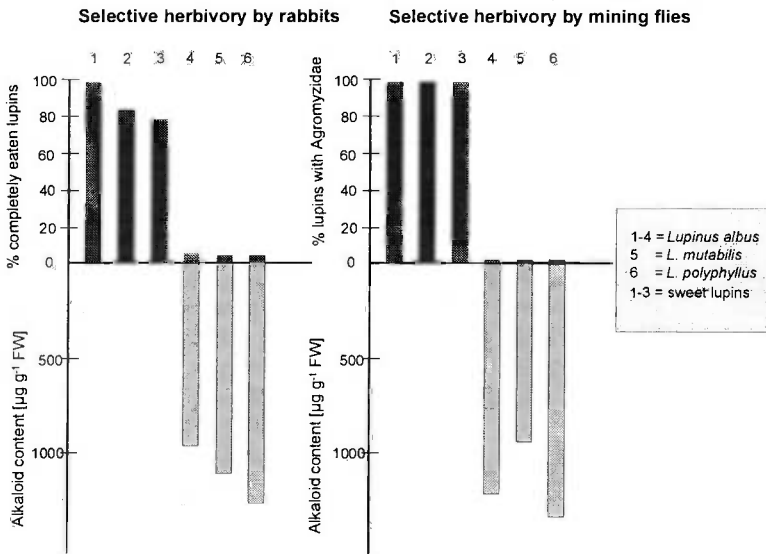
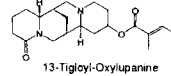
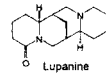
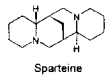


Fig. 1.6. Selective advantage of lupins with alkaloids as compared to 'sweet lupins'. Alkaloid concentrations are given as % fresh weight.

Quinolizidine alkaloids



<b>Vertebrate toxicity (LD<sub>50</sub>)</b>		
(i.v., i.p.)	20-70 mg kg <sup>-1</sup>	20-200 mg kg <sup>-1</sup> n.d.
(p.o.)	300-500 mg kg <sup>-1</sup>	410-1464 mg kg <sup>-1</sup> n.d.
<b>Insect toxicity (LD<sub>100</sub>)</b>		
Ceratitis	0.2%	0.07% 0.2%
Phaedon	1%	0.3% 0.2%
Plutella	1%	0.3% 0.2%
Dysdercus	0.9%	0.3% 0.2%

- Toxicity also found in worms, molluscs and other animals
- QAs exhibit some
- antimicrobial
- antiviral
- phytotoxic properties

Fig. 1.7. Overview of toxic properties of quinolizidine alkaloids.

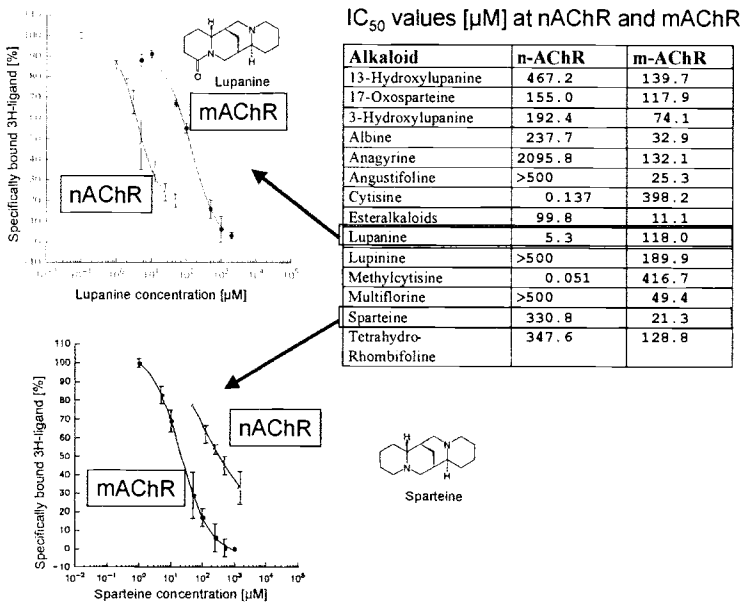


Fig. 1.8. Binding of quinolizidine alkaloids to acetylcholine receptors. Binding was analysed using radio receptor assays (after Schmeller *et al.*, 1994).



difficult, since even small changes in chemistry can be the base for new pharmacological activities.

## Evolution of Chemical Defence in the Leguminosae

We have selected the Leguminosae as a specific example to research the evolution of chemical defence, since this very large plant family with 650 genera and more than 18,000 species has been extensively studied phytochemically. Several types of alkaloids, NPAAAs, amines, flavonoids, isoflavones, coumarins, anthraquinones, di-, sesqui- and triterpenes, cyanogenic glycosides, protease inhibitors and lectins have been described in this family, most of which function as defence chemicals or as signal compounds (see reviews and compilations in Harborne *et al.*, 1971; Polhill *et al.*, 1981a, b; Kinghorn and Balandrin, 1984; Stirton, 1987; Wink, 1993a, b; Hegnauer and Hegnauer, 1994; Southon, 1994; Sprent and McKey, 1994; Wink *et al.*, 1995; Wink and Waterman, 1999). Furthermore, *rbcL* sequences of over 300 legumes have been sequenced (Käss and Wink, 1995, 1996, 1997a, b; Wink *et al.*, unpublished data) which for the first time provide the phylogenetic framework to analyse the distribution of SM in legumes. We have selected about 100 genera which cover most tribes of the Leguminosae. In several cases, *rbcL* sequences of these genera cluster in a way that is consistent within their traditional grouping in tribes and subfamilies (Polhill, 1994) but there are also significant differences. Members of the Caesalpinioideae cluster at the base of the legume tree which agrees with the fossil record (Herendeen and Dilcher, 1992). Members of the Mimosoideae unambiguously derive from the Caesalpinioideae (Doyle, 1994; Käss and Wink, 1995, 1996, 1997a) and are not ancestral, as sometimes had been assumed. The Papilionoideae form a monophyletic clade, starting with Sophoreae at the base and leading to Genisteae as the more advanced tribes.

Quinolizidine alkaloids figure as the most prominent group of alkaloids in legumes, present in members of the subfamily Papilionoideae in the tribes Genisteae, Crotalariaeae, Podalyrieae, Thermopsidaeae, Lipariaeae, Euchresteae, Bossiidaeae and Sophoreae (Kinghorn and Balandrin, 1984; Wink, 1993a, b). Also, dipiperidine alkaloids of the ammodendrine type, which also derive from lysine as a precursor, exhibit a comparable distribution pattern (Fig. 1.9). These tribes, except the Sophoreae, are apparently monophyletic and nearly all taxa in this assemblage accumulate QAs. Obvious exceptions are members of the large genus *Crotalaria* that either sequester pyrrolizidine alkaloids (PAs) or NPAAAs (Figs 1.9 and 1.10). In *Lotonis* some taxa produce QAs and others PAs. Since *Crotalaria* and *Lotonis* derive from ancestors that definitely produced QAs but not PAs, the general ability to make QAs must have been present. But corresponding genes are either lost or more likely completely turned off in *Crotalaria* and partially in *Lotonis*. The formation of PAs instead appears to be a new acquisition for chemical defence, which evolved convergently; the occurrence of simple PAs in *Laburnum* and *Adenocarpus* might be interpreted

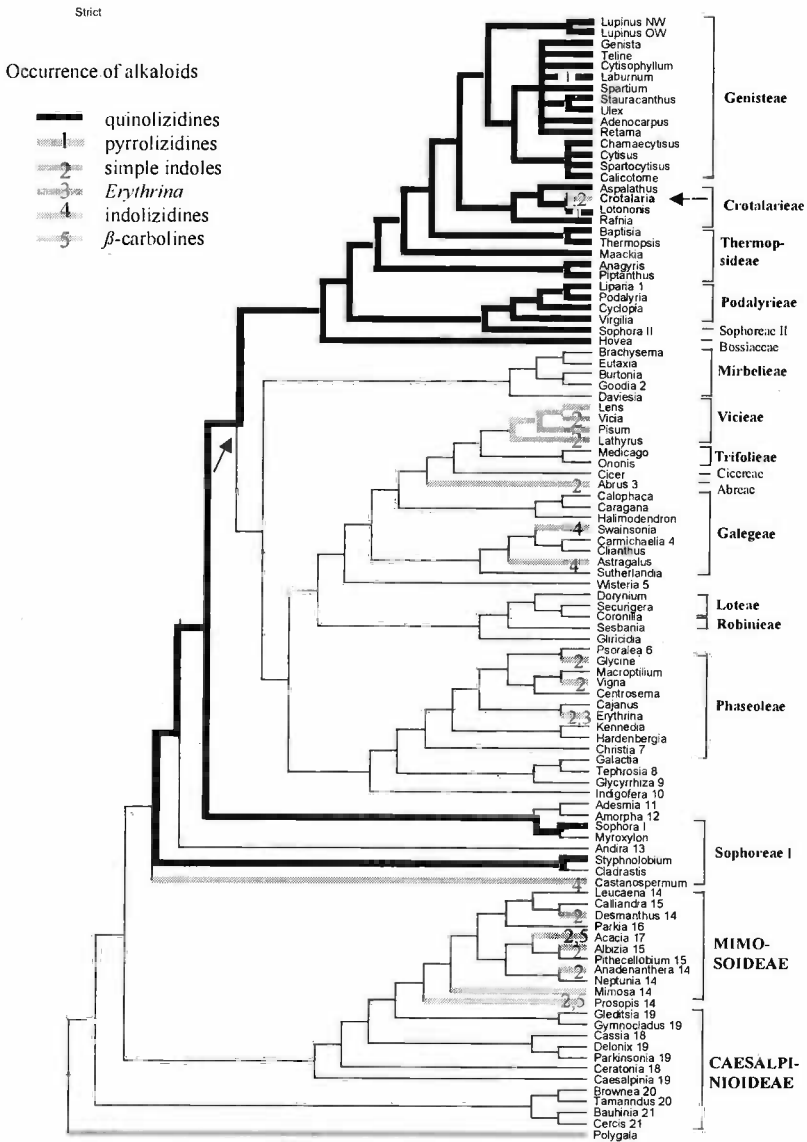


Fig. 1.9. Molecular phylogeny of legumes inferred from *rbcL* sequences and the distribution of alkaloids in legumes.

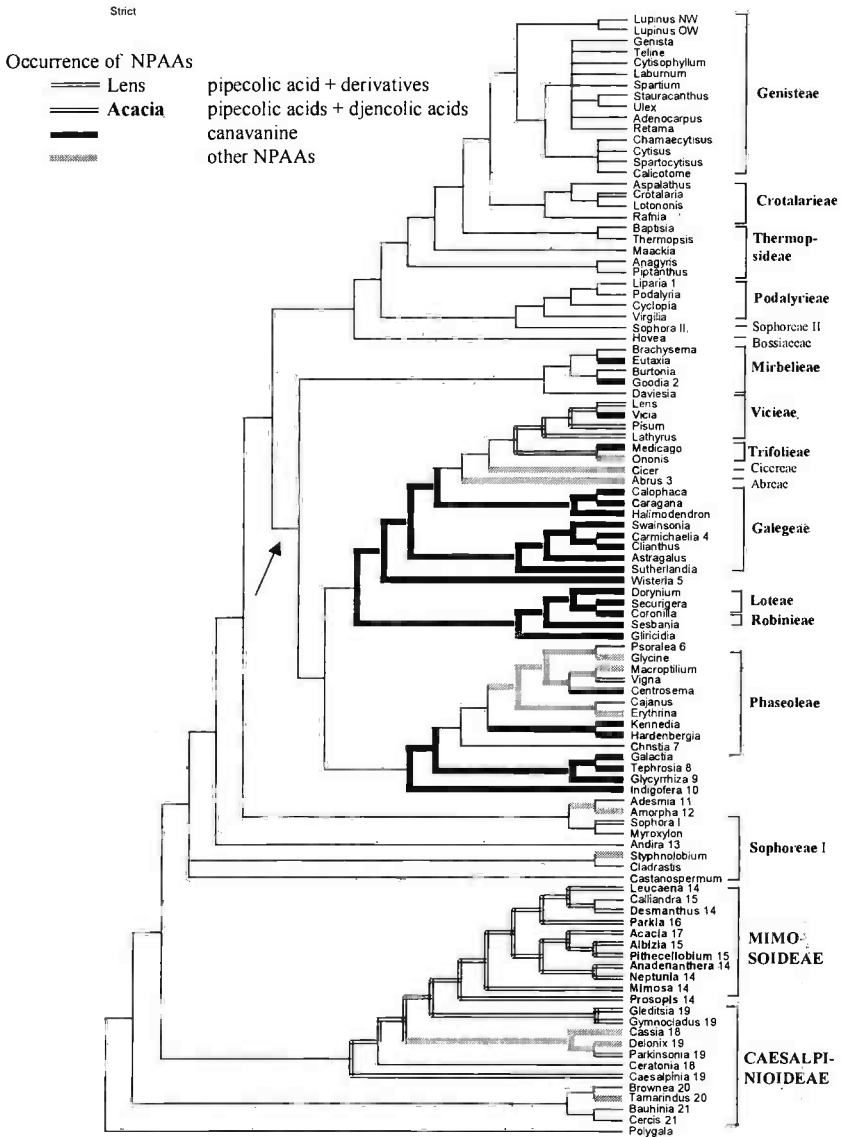


Fig. 1.10. Distribution of NPAA's in legumes.

accordingly. In a few taxa which cluster within QA-accumulating genera, QAs are hardly detectable or levels are very low, such as in *Ulex*, *Calicotome* or *Spartocytisus*. These taxa have in common extensive spines, which apparently have substituted chemical defence; in this case the presence or absence of QAs is clearly a trait reflecting different ecological strategies rather than taxonomic relationships.

Quinolizidine alkaloids have also been found in a few unrelated genera of Chenopodiaceae, Berberidaceae, Ranunculaceae, Scrophulariaceae and Solanaceae (Teuscher and Lindequist, 1994). Since traces of QAs could be detected in plants and cell cultures of even more taxa, we have postulated (Wink and Witte, 1983) that the genes which encode the basic pathway leading to lupanine, must have evolved early during evolution, but that these genes are turned off in most instances, but turned on in plants which use the alkaloids as chemical defence substances (Wink, 1992, 1993c, d).

Besides QAs, legumes accumulate a wide range of other alkaloids, deriving from different precursors. Most of them show occurrences which are restricted to a few, often non-related taxa (Fig. 1.9). For example, *Erythrina* alkaloids, which derive from tyrosine as a precursor, are typical for members of the large genus *Erythrina* and have not been found elsewhere in the plant kingdom. Indolizidine alkaloids, which inhibit hydrolytic enzymes, have been reported in *Swainsonia*, *Astragalus* (tribe Galegeae) and *Castanospermum* (Sophoreae).  $\beta$ -Carboline alkaloids have been detected in a few mimosoid taxa of the tribes Mimoseae and Acacieae. Also a number of simple phenylethylamine or simple indole alkaloids have been found, usually in taxa which do not accumulate QAs (Fig. 1.9). Interestingly, the occurrence of quinolizidines and other alkaloids is usually mutually exclusive, indicating the parsimonious utilisation of chemical defence strategies.

The pattern of NPAA accumulation (Fig. 1.10) is almost complimentary to the distribution of QAs, if all NPAAs with different structures and activities are grouped together. Similar to the functions of QAs and other nitrogenous compounds, NPAAs serve at least two purposes: both as chemical defence compounds and as mobile nitrogen storage compounds in seeds which are used as a nitrogen source for the germinating seedling. Considering different types of NPAAs, a more differentiated picture becomes apparent. At least three groups of NPAAs are common in legumes, such as canavanine, pipecolic acid and derivatives, and the sulphur-containing djenkolic acids. Canavanine is common in the tribes Galegeae, Loteae, Tephrosieae, Robinieae and some Phaseoleae. It could be assumed that the trait of canavanine accumulation was acquired by an ancestor (see arrow in Fig. 1.10) from which all the other tribes derived, but that the canavanine genes are turned off in Viciaeae, Trifolieae, Cicereae and Abreae which produce pipecolic acids instead. Whether pipecolic acid biosynthesis was independently invented in Caesalpinioideae/Mimosoideae and in the papilionoid tribes Viciaeae and Trifolieae, or whether the canavanine genes were only inactivated in Viciaeae and Trifolieae is open to debate, similar to the situation of protease inhibitors (PIs). Several other NPAAs have been described from legumes (Harborne *et al.*, 1971; Polhill *et al.*, 1981a, b; Stirton, 1987; Hegnauer

and Hegnauer, 1994; Southon, 1994; Sprent and McKey, 1994); most of them have a more restricted occurrence, and presence or absence in phylogenetically related taxa is a common theme.

Cyanogenic glycosides, which release the toxic cyanide upon activation, appear to be more common in the more ancestral than the more developed legume tribes (Fig. 1.11). Whether the occurrences of cyanogenic glycosides are based on common genes which are turned off in most instances and turned on in a few places cannot be answered yet; a convergent and independent evolution might also be possible.

The distribution of PIs (i.e. trypsin and chymotrypsin inhibitors) exhibits an almost complementary pattern (Fig. 1.12) to QAs. Members of the Caesalpinioideae and many Mimosoideae accumulate PIs in their seeds, where they serve concomitantly as chemical defence and nitrogen storage compounds. It is not clear whether some genera of the Mimosoideae have secondarily lost this trait, or whether they have not been studied in sufficient detail. Within the Papilionoideae, PIs are prominent in the tribes Viciae, Trifolieae, Cicereae, Abreae, Galegeae, Loteae, Phaseoleae and Tephrosieae, but have not been described in the Mirbelieae. According to Fig. 1.12, PI formation in Caesalpinioideae/Mimosoideae and Papilionoideae could be based on common ancestry, which would mean that this trait is turned off in a number of papilionoid tribes that produce QAs and other secondary metabolites instead. Alternatively, formation of PIs evolved independently in these legume subfamilies. Since the genes for PIs are known, it would be challenging to analyse whether PI genes are present or absent in PI non-producing taxa.

In conclusion, the numerous nitrogen-containing metabolites appear to function both as chemical defences and nitrogen storage compounds in legumes and are thus open to natural selection. Although they appear as plausible taxonomic markers in a few places, they fail to demonstrate this taxonomic reliability in other parts of the legume tree. Thus, their occurrence probably reflects different evolutionary and life strategies rather than taxonomic stringency. In many cases their occurrence appears to be mutually exclusive; tribes which produce NPAAAs do not accumulate alkaloids and vice versa (Figs 1.9 and 1.10).

Flavonoids are found in all three subfamilies and isoflavones are obviously restricted to the subfamily Papilionoideae (Fig. 1.13). Except for a few tribes and genera, among which are several Australian taxa, all taxa accumulate isoflavones and derivatives, including several phytoalexins of the pterocarpan type. Catechins and proanthocyanins, or galloylcatechins, occur in all three subfamilies; their occurrence reflects life style, i.e. growth as trees, rather than taxonomic relatedness. In Caesalpinioideae and Mimosoideae, both traits are almost congruent, since woody life style dominates in both subfamilies.

Coumarins and furanocoumarins, which serve as potent defence compounds in Apiaceae, occur in a few, mostly unrelated species. Only in the genus *Psoralea* do they have a wider distribution. Anthraquinones, which are potent Na<sup>+</sup> and K<sup>+</sup>ATPase inhibitors and strong purgatives, ubiquitously occur in the genus *Cassia*, but only occasionally in *Andira* and *Abrus*.

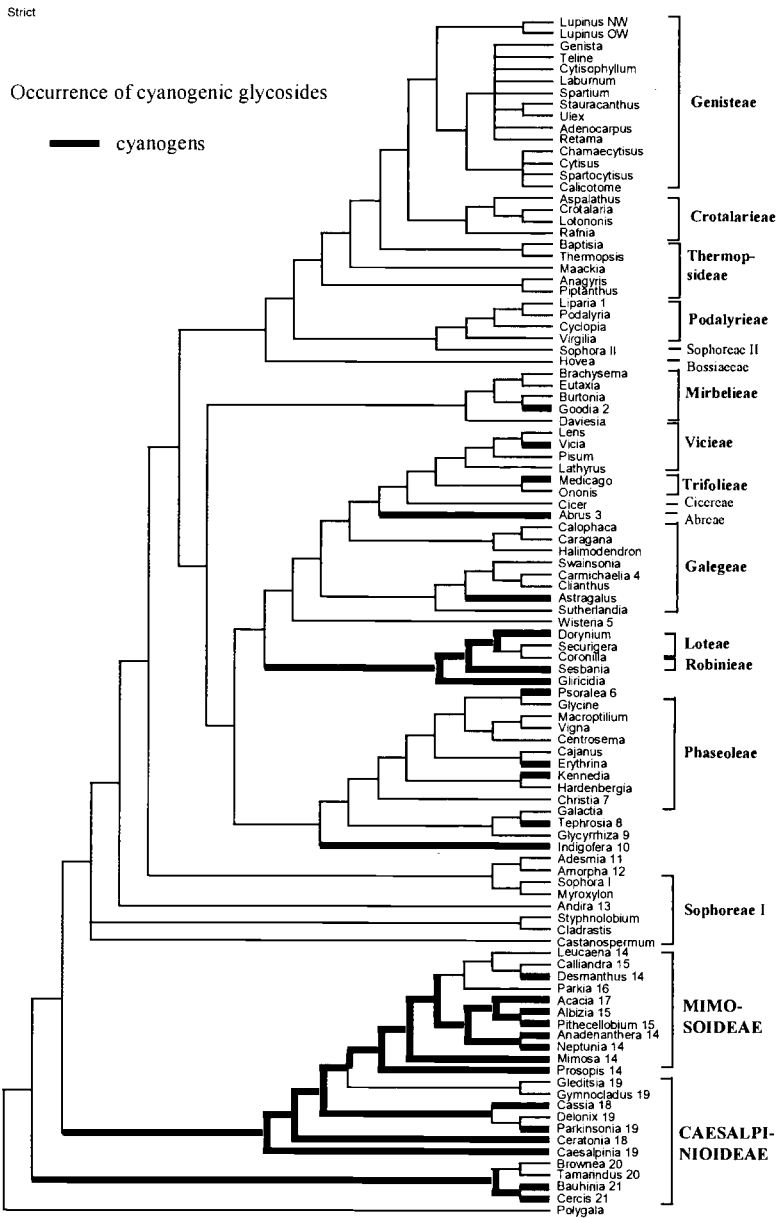


Fig. 1.11. Distribution of cyanogenic glycosides in legumes.

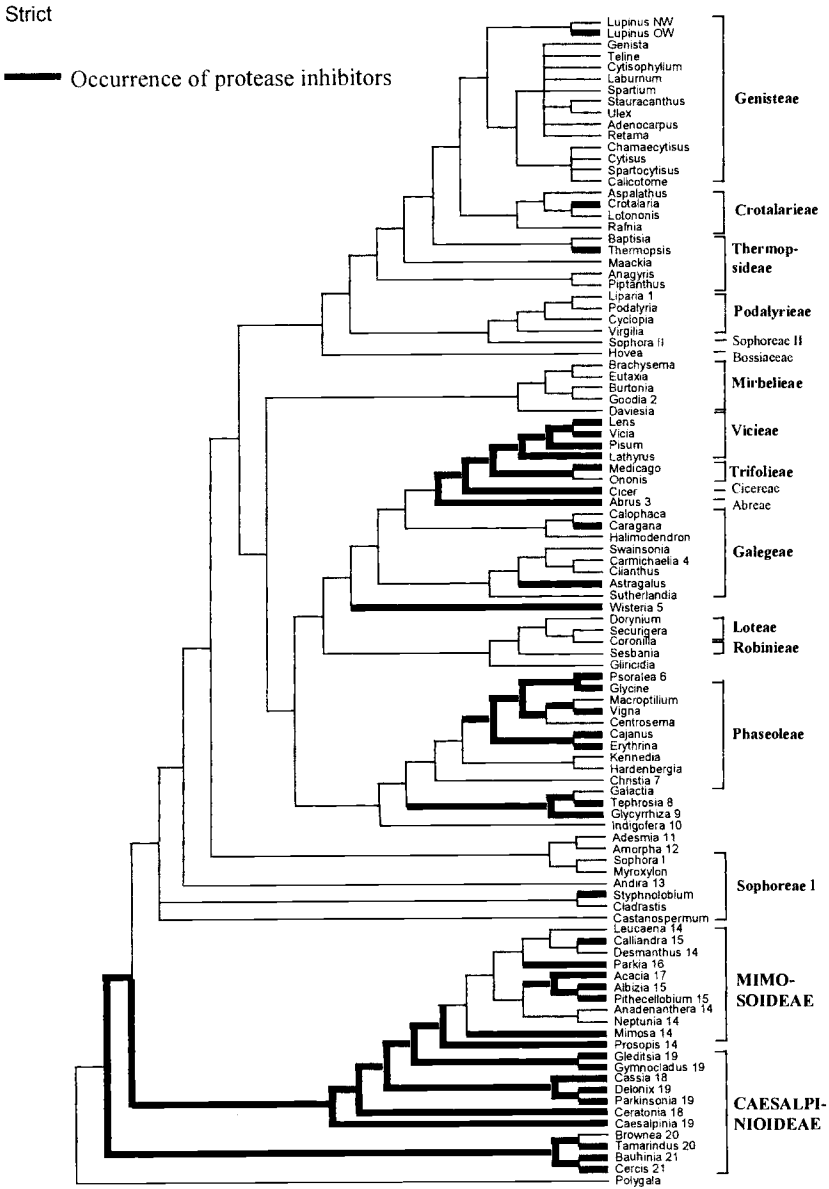


Fig. 1.12. Distribution of protease inhibitors in legumes.

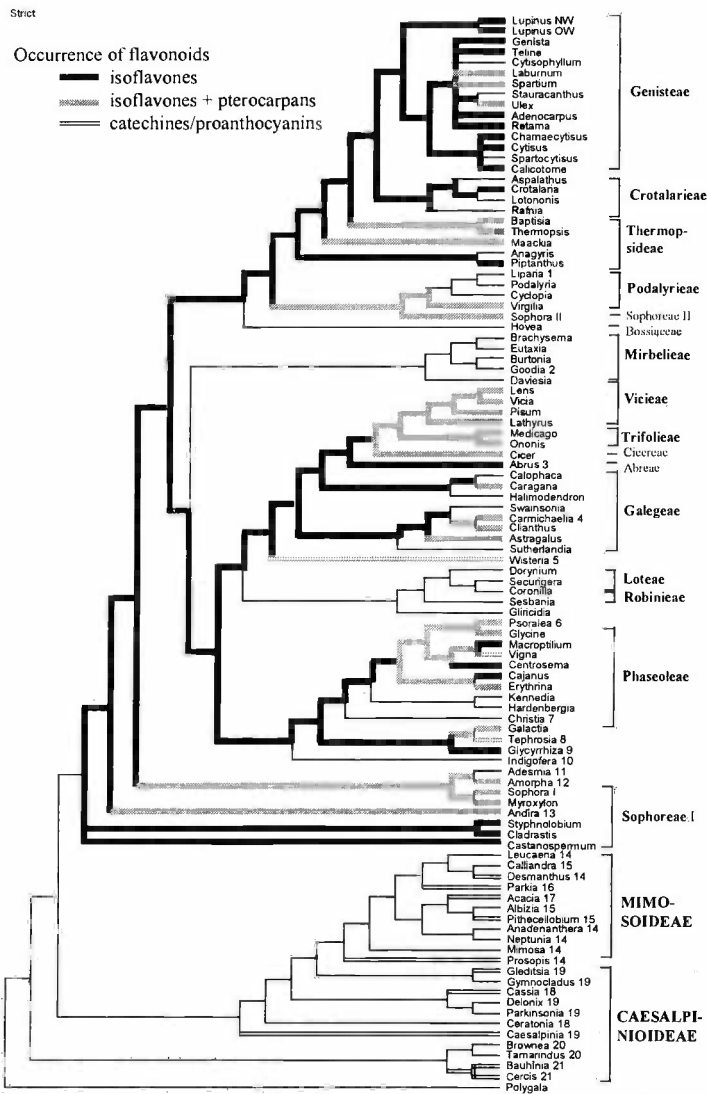


Fig. 1.13. Distribution of isoflavones in legumes.



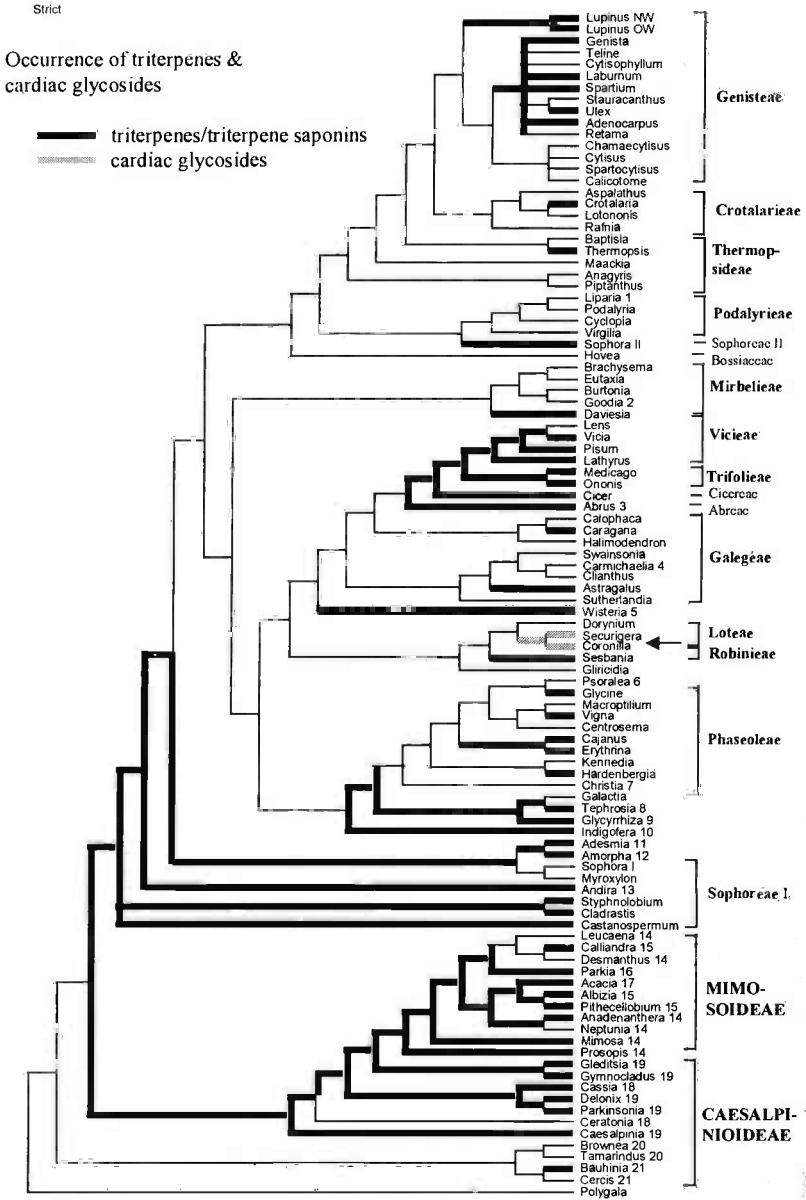


Fig. 1.14. Distribution of triterpenes and saponins in legumes.

Among terpenoids, all classes of terpenes have been found in legumes. The occurrence of triterpenes and triterpene and steroidal saponins (including cardiac glycosides in *Securigera* and *Coronilla*, both Loteae) is illustrated in Fig. 1.14. Triterpenes and saponins, which are powerful defence compounds against microbes and herbivores, are more common in the ancestral Caesalpinioideae/Mimosoideae and in the basal tribes of the Papilionoideae, but also in Viciaeae, Trifolieae, Cicereae and Phaseoleae. Whether they have appeared independently in the tribes seen, which are sometimes not related by a common link, or whether the genes have evolved at the beginning of legume evolution, but switched on or off according to ecological needs, cannot be answered with certainty. The wide distribution of triterpenes and triterpene saponins in the plant kingdom and their common basic structures favours the latter possibility.

When analysing the alkaloid profiles within the genus *Lupinus*, we observe the same phenomenon as found for other secondary metabolites at the tribe or family level. In some instances, all members of a monophyletic clade share a chemical characteristic; in other instances not (details in Wink and Waterman, 1999). The main question is: What were the selective forces to activate the corresponding genes in one taxon and to turn them off in another? Since SM play a vital role as defence or signal compounds, their occurrence apparently reflects adaptations and particular life strategies rather than taxonomic relationships. In general, if a certain defence trait is lost, a gain of another defence trait can be observed. For example, the sudden occurrence of PAs in the Crotalariaeae and the silencing of QAs which are present in all the other members of the genistoids. Thus, studying the distribution of SM in plants as compared to their molecular phylogeny offers information on the underlying evolutionary, ecological and systematic processes and strategies.

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## Chapter 2

# Lack of Apparent Base Sequence Preference of Activated Pyrrolizidine Alkaloid Cross-links with DNA

R.A. Coulombe Jr and W.K. Rieben Jr

*Graduate Program in Toxicology and Department of Veterinary Sciences, Utah State University, Logan, UT 84322-4620 USA*

### Introduction

Upon ingestion, pyrrolizidine alkaloids (PAs) are oxidized by cytochromes P-450 (CYPs) to reactive, pyrrolic bifunctional electrophiles that readily cross-link DNA, a reputed event critical in PA-induced toxicity. Our laboratory has demonstrated that PAs form nearly equal proportions of both DNA interstrand and DNA-protein cross-links *in vitro* (Hincks *et al.*, 1991), and that the cytotoxic, antimitotic and megalocytic activity of PAs correlate with cross-linking (Kim *et al.*, 1993). Structural features, most notably the presence of  $\alpha,\beta$ -unsaturation and a macrocyclic diester, confer potent cross-link activity to PAs in cellular systems (Kim *et al.*, 1999). We have recently shown that actin is the major protein involved in cellular PA-induced DNA-protein cross-links (Coulombe *et al.*, 1999). Dehydrosenecionine (DHSN) and dehydromonocrotaline (DHMO) have also been shown to inhibit amplification of a segment of pBR322, implying that cross-linking by activated PAs is functionally significant (Kim *et al.*, 1999).

Activated PAs share a common pyrrolic substructure with reductively activated bifunctional mitomycins, such as mitomycin C, which preferentially cross-link 5'-CG sequences within DNA (Woo *et al.*, 1993). Several DNA bases have been shown to be involved in covalent interactions and/or cross-links by pyrrolic PAs, such as the N<sup>2</sup> of deoxyguanosine (dG) and N<sup>6</sup> of deoxyadenosine (dA), and O<sup>2</sup> of uridine and deoxythymidine or thymidine (dT) have also been identified as targets (Robertson, 1982; Wickramanayake *et al.*, 1985). Dehydromonocrotaline and dehydroretorsine preferentially cross-link dG-to-dG at a 5'-CG sequence in synthetic duplex DNA (Weidner *et al.*, 1990). Recently, DHMO was shown to cross-link at the N7 position of guanine in a 35 bp fragment of pBR322 with a preference for 5'-GG and 5'-GA sequences (Pereira *et al.*, 1998). The purpose of this study was to determine whether an activated PA shows sequence specificity in cross-linking defined 14-base poly AT oligonucleotides which differ only by the two central bases. Similar defined oligonucleotides are frequently used tools to discern sequence specificity of various cross-linking agents such as mitomycin C.

## Materials and Methods

Dehydromonocrotaline (DHMO) was prepared by chemical oxidation of monocrotaline using *o*-chloranil; yield and purity was checked by  $^1\text{H}$  NMR (Kim *et al.*, 1995). DHMO was stored in anhydrous dimethylsulphoxide (DMSO).

Cross-linking of oligonucleotides was analysed by gel-shift analysis (Kim *et al.*, 1999). Three duplex 14-mer oligonucleotides which differed by two central bases, 'CG oligo': 5'-ATATATCGATATAT-3'; 'GC oligo': ATATATGCATATAT; and 'TA oligo': ATATATTAATATAT were end-labelled with [ $\gamma$ - $^{32}\text{P}$ ]ATP according to manufacturers specifications (MBI Fermentas, Inc., Amherst, NY). Oligos were annealed (Williams *et al.*, 1997) to ensure they were in duplex form (confirmed by thermal denaturation,  $\lambda = 260$  nm) under cross-linking conditions. The reactions (100  $\mu\text{l}$  total in  $\text{H}_2\text{O}$ ) were begun by first adding  $\text{H}_2\text{O}$ , DNA and DMSO (1% of total volume included to insure solubility of the pyrrole), followed by DHMO at varying (DNA:DHMO) molar ratios at 4°C for 4 h and allowed to warm to room temperature overnight. Lanes containing equal dpm of cross-linked DNA were separated from free DNA by denaturing 25% PAGE (acrylamide:bis-acrylamide 19:1, 7 M urea, 0.025% TEMED, 0.07% APS) with 1X TBE (pH 8.3) and exposed to X-ray film. Autoradiographs were computer-archived, and band intensities were computed with a Nucleovision 920 Imaging Workstation (NucleoTech Corporation, San Carlos, CA).

Oligonucleotides were incubated with DHMO and  $\sim 7.4 \times 10^5$  dpm of  $^{32}\text{P}$ -labelled at 37°C for 1.75 h as above with varying amounts of aspartate (Asp), cysteine (Cys), glutathione (GSH), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser), threonine (Thr) or tyrosine (Tyr). The order of reagents added in the 100  $\mu\text{l}$  reaction was:  $\text{H}_2\text{O}$ , DNA, DMSO, competitor, then DHMO. Each tube was briefly mixed, then aliquots of equal dpm per well were electrophoretically separated and gels were exposed to X-ray film. Electrophoretic migration of oligonucleotides was not affected by the addition of those nucleophiles alone (data not shown).

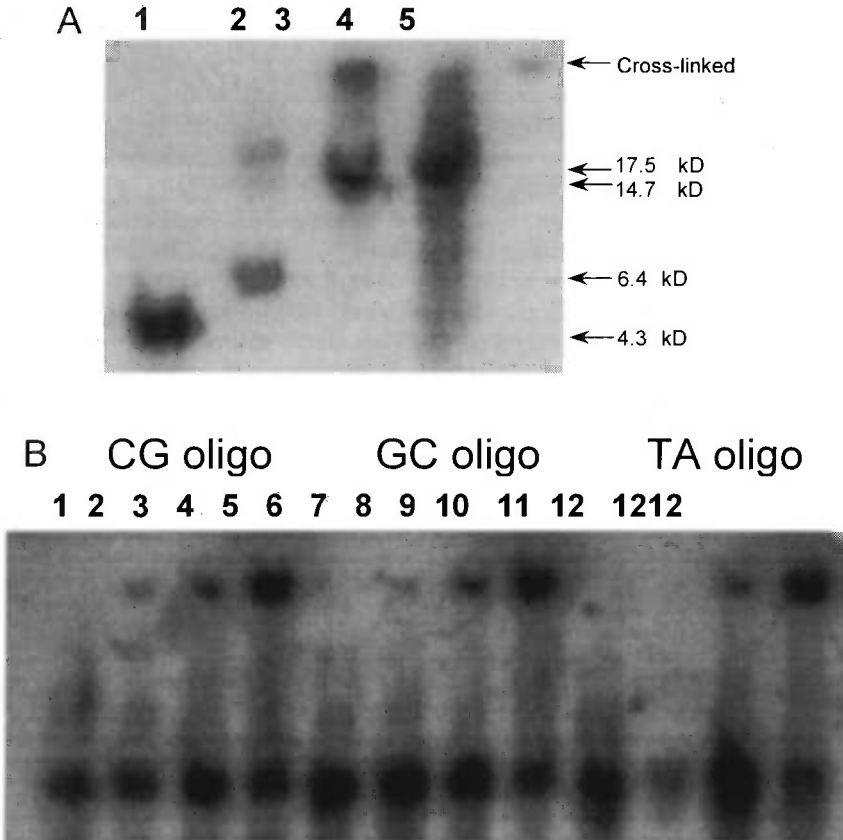
## Results

In the gel-shift assay, DNA cross-linking agents created larger molecular weight, slowly migrating complexes apparent near the sample well at the top of the gel compared to native, untreated DNA (lane 5, Fig. 2.1A). Using the set of unmodified, single-stranded oligonucleotides as molecular weight markers, it is clear that the pyrroles induced a complex with a  $M_r$  substantially greater than 17.5 kD, the approximate size of a pyrrole-DNA cross-link of two duplex 14-mers (Fig. 2.1A). Thus, it is likely that DHMO caused some combination of intra- and interstrand DNA cross-links in our system. In each cross-linking experiment, unmodified DNA was well-resolved from the presumed pyrrole-DNA cross-links (Fig. 2.1B). At the three molar ratios examined, cross-linking was dependent upon the amount of DHMO added to the reactions; in all cases, DNA cross-

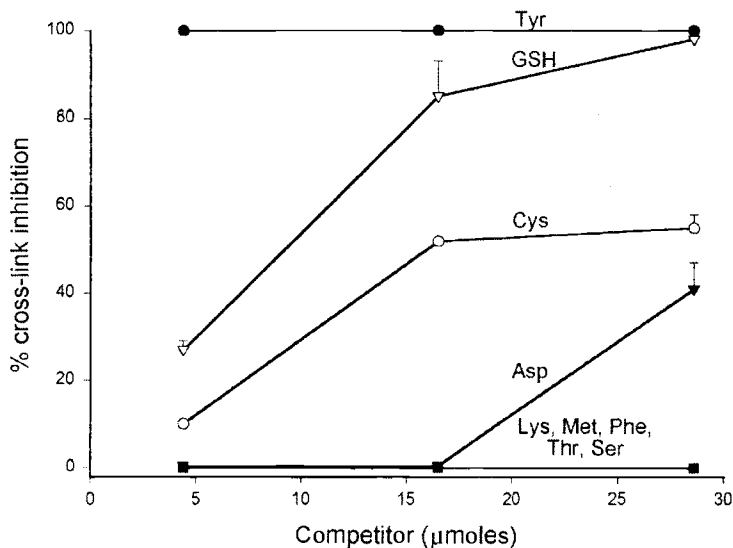


linking was dose-dependent (Fig. 2.1B). However, DHMO did not appear to cross-link any one oligonucleotide preferentially ( $P < 0.05$ ) (Fig. 2.1B).

We used the standard gel-shift assay to determine which cellular nucleophiles, if any, compete with DNA for DHMO-induced cross-links. In this assay, competition can be seen as a sequential disappearance of the large molecular weight cross-linked complex with a concomitant reappearance of the native DNA. For example, additions of GSH caused a sequential disappearance of the



**Fig. 2.1.** Autoradiographs of denaturing PAGE analysis of DHMO-induced cross-linking of duplex  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -radiolabelled DNA. (A) Native DNAs for molecular weight references: CG oligo (4.3 kD; lane 1), 21-mer (6.4 kD; lane 2), 48-mer (14.7 kD; lane 3) and 57-mer (17.5 kD; lane 4), and DHMO-induced DNA-interstrand cross-linking of CG oligo (1:600, DNA:pyrrole; lane 5). (B) DNA-interstrand cross-linking by DHMO of duplex CG, GC and TA oligos. Lanes 2-4, 6-8, and 10-12 are DNA:pyrrole molar ratios of 1:200, 1:600 and 1:1000, respectively. Controls (native, untreated CG, GC and TA oligo) are lanes 1, 5 and 9, respectively.



**Fig. 2.2.** Comparative inhibitory effect of various alternate nucleophiles on cross-linking of CG oligo by DHMO. This graph shows that tyrosine (Tyr), glutathione (GSH), cysteine (Cys) and aspartate (Asp), but not lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr) or serine (Ser) compete with DHMO for cross-linking of CG oligo. Data is derived from digital densitometry of gel shift analyses,  $\pm$  SE,  $n \geq 2$ .

large molecular weight cross-linked complex with a concomitant reappearance of the original native oligonucleotide while Lys did not affect the extent of DHMO-induced cross-linking (Fig. 2.2). Tyrosine was the most effective competitor of cross-linking, followed by GSH, then Cys and Asp. Lysine, Met, Phe, Thr and Ser did not compete at any concentration.

## Discussion

The interactions of cross-linking agents with DNA are complex, giving rise to lesions that can underlie mutagenesis and carcinogenesis. One of the potential variables in such interactions is the sequence of bases in available regions of the target DNA. Bifunctional pyrrolic (dehydro) derivatives, pyrrolizidine alkaloids readily cross-link cellular nucleophiles such as amino acids, proteins and DNA (Hincks *et al.*, 1991; Kim *et al.*, 1995; Coulombe *et al.*, 1999).

Here, we present evidence that pyrrolic PAs do not appear to have a strong base sequence preference when they cross-link a set of self-complementary 14-mer poly AT oligonucleotides that contained central 5'-d(CG), 5'-d(GC) or 5'-d(AT) sequences. The degree of cross-linking was only affected by the amount of pyrrole added to the reactions, and not the central base sequence of the

oligonucleotide DNA targets.

Our results contrast with two previous studies showing some sequence preference involving dG by PA pyrroles or similar bifunctional cross-linkers. For example, DHMO and dehydroretorsine preferentially formed dG-to-dG cross-links at 5'-d(CG) to 5'-d(GC) sequences in 17-mer synthetic duplex oligonucleotides (Weidner *et al.*, 1990), but possible cross-linking by these agents of duplexes comprised of only dA and dT was not examined. The synthetic pyrrolic analogue, dehydroretronecine acetate, was found to be non-reactive toward a duplex containing only dA and dT (Weidner *et al.*, 1990). In another study, DHMO cross-linked 5'-d(GG) and 5'-d(GA) sequences in a 375 bp fragment of an *EcoRI* digest of BR322 (Pereira *et al.*, 1998). However, since those investigators relied on a protocol utilizing DMSO and hot piperidine that detects alkylation only at guanyl residues, cross-linking at other non-guanyl sites was not evaluated. Thus, while these two prior studies have argued for a sequence preference in PA-induced cross-linking involving dG, there is contradiction with respect to the actual sequence involved. While there is precedence for indiscriminate DNA cross-link formation, the mechanism for the apparent lack of base pair preference by DHMO is unclear, and is the subject of current investigations using a larger number of oligonucleotides of varying sequences and lengths.

We also show that certain cellular nucleophiles such as tyrosine (Tyr), glutathione (GSH), cysteine (Cys) and aspartic acid (Asp) can inhibit DNA cross-linking by pyrroles, presumably by providing an alternate nucleophilic cross-link target. Previous data from our laboratory suggests that reduced thiols such as GSH and Cys have a strong affinity for pyrroles (Coulombe *et al.*, 1999; Kim *et al.*, 1999). One possible significance of this finding is that these molecules may act as protective scavengers of reactive pyrroles in the cell. Indeed, animals given dietary supplements of Cys showed reduced symptoms of PA-induced hepatotoxicity compared to control animals (Miranda *et al.*, 1982). Alternatively, proteins especially rich in Tyr, Cys, and Asp may be preferential cellular targets for pyrrolic PAs.

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## Chapter 3

# Correlation of an Endophytic Fungus (*Alternaria* spp.) with the Presence of Swainsonine in Lambert Locoweed (*Oxytropis lambertii*)

D.R. Gardner<sup>1</sup>, J. Romero<sup>2</sup>, M.H. Ralphs<sup>1</sup> and R. Creamer<sup>2</sup>

<sup>1</sup>Poisonous Plant Research Laboratory, ARS, USDA, Logan, Utah, USA; <sup>2</sup>New Mexico State University, Las Cruces, New Mexico, USA

Locoweed poisoning is the most widespread poisonous plant problem in the western USA (Kingsbury, 1964; James and Nielsen, 1988). The term 'locoweed' is defined and used as a general name for those *Astragalus* and *Oxytropis* species known to contain toxic levels of the trihydroxy indolizidine alkaloid swainsonine (Molyneux and James, 1982). The presence of swainsonine in locoweeds is believed to be the result of secondary plant biosynthesis (Harris *et al.*, 1988a). Swainsonine is also known to be produced by the plant fungal pathogen *Rhizoctonia leguminicola* (Harris *et al.*, 1988b), often associated with red clover; *Metarhizium anisopliae* (Hino *et al.*, 1985), a known insect pathogen; and most recently *Alternaria* sp. *nova* (Braun, 1999), a new endophytic fungus associated with locoweeds. Braun (1999) isolated *A.* sp. *nova* from several *Astragalus* and *Oxytropis* species and found a strong relationship between locoweeds and presence of the endophytic fungus. In addition, *A.* sp. *nova* was found to produce swainsonine *in vitro*. From these findings questions are being asked as to the possible correlation between endophyte infection, swainsonine concentration and toxicity of locoweed plants (Molyneux and James, 1991; Braun and Liddell, 1999).

*Oxytropis lambertii* or Lambert locoweed is one of the *Oxytropis* species incriminated in livestock poisonings (Kingsbury, 1964; Molyneux and James, 1991) and previously shown to contain the locoweed toxin swainsonine (Molyneux *et al.*, 1989, 1991). *Oxytropis lambertii* is found in at least eight different western states and taxonomically it is divided into three varieties (*O. lambertii* var. *lambertii*, var. *bigelovii*, and var. *articulata*) (Barneby, 1952). It was surprising, when in some preliminary swainsonine analyses of *O. lambertii* samples, no swainsonine was found in populations previously assumed to be toxic. In the work presented here we examined 16 different populations of *O. lambertii*, measured the swainsonine content and tested for the presence or absence of the endophyte *A.* sp. *nova*.

## Materials and Methods

### Plant materials

Lambert locoweed (*Oxytropis lambertii* Pursh.) samples (n = 10/location) were collected from 16 different locations throughout its known geographic distribution (Table 3.1). Ten individual plants were collected at each location and physically separated in half. One half was used for swainsonine analysis and the corresponding second half was used to screen for the presence of *A. sp. nova*. In addition, at each location voucher specimens were collected, pressed and deposited in the Monte L. Bean Herbarium (Brigham Young University, Provo, Utah). All plant materials used for swainsonine analyses were dried in a forced air oven at 60°C for 48 h, ground to pass through a 1 mm screen, and stored in a plastic bag at ambient temperature.

### Swainsonine assay

Plant samples were analysed for swainsonine (% dry weight) using the method described in detail by Gardner *et al.* (2001). In brief, samples (100 mg of dry, ground plant material) were extracted with a mixture of chloroform (4 ml) and 2% acetic acid (5 ml) for 16 h (overnight). The upper acetic acid solution was then removed to a prepared ion-exchange extraction tube (a 5 ml pipette tube containing ~ 0.7 g of Dowex 50WX8-100 resin) and mixed for 15 min to retain the swainsonine. After washing twice with deionized distilled water the swainsonine was eluted from the resin by extraction with 1N ammonium hydroxide solution (5.00 ml) for 15 min. An aliquot of the extract was then analysed by LC/MS with a quantitation level of 0.001% swainsonine (dry weight). For samples that were negative for swainsonine using this methodology a second aliquot was concentrated and analysed again by LC/MS, and checked qualitatively by GC/MS, for the presence of swainsonine with a detection limit of ~ 0.0001% (1 ppm).

### Endophyte assay

From each plant, four leaf pieces, four stem pieces, and (if present) four flower pieces were harvested. Pieces were approximately 0.5 to 1 cm in length and were surface sterilized by immersion in 70% ethanol for 30 s, then 1% sodium hypochlorite for 3 min, and then washed with sterile water for 30 s. The samples were then dried on sterile paper towels and placed on to a water agar media (four pieces per plate). The plates were checked for fungus growth 1 week after culturing and any suspected endophytic fungus was transferred to PDA (potato dextrose agar). The original plates were re-checked for additional fungal growth approximately 1 week later and again any suspected fungus was transferred to PDA. The PDA plates were then placed on racks at room temperature with natural and artificial room lighting during the day.

Samples were determined to be endophyte positive (E+) if any of the assayed pieces (leaf, stem, flower) were positive. The per cent endophyte infection was determined as number of E+ plant pieces/total number of plant pieces cultured. For samples containing sufficient leaf, stem, or flower material the assay was run in duplicate.

## Results

*Oxytropis lambertii* samples were collected from 16 different locations in eight different states (Table 3.1). These specific sites were chosen to collect from the three known varieties (vars *lambertii*, *bigelovii* and *articulata*). Significant concentrations of swainsonine (> 0.001%) were found in five of the 16 populations with two from New Mexico, two from Arizona, and one population from Utah (Fig. 3.1). The average swainsonine content varied from a low of 0.008% (Kanab, UT) to a high of 0.054% (Flagstaff, AZ). Populations containing significant levels of swainsonine were restricted to *O. lambertii* var. *bigelovii*

**Table 3.1.** *Oxytropis lambertii* populations, mean swainsonine level and endophyte presence (+) or absence (-).

Location	<i>O. lambertii</i>	Mean <sup>a</sup> (% dry weight ± SD)	Endophyte
Meade, KS	var. <i>articulata</i>	< 0.0001	-
Knowles, OK	'	< 0.0001	-
Buffalo, OK	'	< 0.0001	-
Flagstaff, AZ	var. <i>bigelovii</i>	0.054 ± 0.027	+
Springerville, AZ	'	0.026 ± 0.021	+
Kingston, NM	'	0.016 ± 0.013	+
Winston, NM	'	0.038 ± 0.035	+
Kanab, UT	'	0.008 ± 0.016	+
Ferron, UT	'	< 0.0001	-
Fort Collins, CO	'	0.0002	-
Ocate, NM	'	0.0006	-
Capulin, NM	'	0.0001	-
Sophia, NM	'	< 0.0001	-
Sidney, NE	var. <i>lambertii</i>	0.0007	-
Hot Springs, SD	'	0.0001	-
Lusk, WY	'	< 0.0001	-

<sup>a</sup>For those samples with initial swainsonine levels at < 0.001%, a separate bulk sample was analysed with quantitation down to 0.0001% (1 ppm) and the presence of swainsonine confirmed by GC/MS.

and geographically found in the most southern and western portion of its distribution. Swainsonine was detected in the other 11 populations of *O. lambertii*, although the levels were extremely low ( $< 0.001\%$ ) with some concentrations estimated to be below 1 ppm ( $< 0.0001\%$ ).

The endophyte (*A. sp. nova*) was cultured from five *O. lambertii* populations (Kanab, UT; Flagstaff, AZ; Springville, AZ; Kingston, NM; Winston, NM). These five populations were the same as those found to contain significant levels of swainsonine ( $> 0.001\%$ ). The endophyte was absent from those populations where the swainsonine concentrations were found to be  $< 0.001\%$  (Table 3.1). The correlation between endophyte and swainsonine, presence or absence, for all 16 populations was high ( $r = 0.92$ ) given a swainsonine concentration greater than 0.01%. The correlation coefficient was  $r = 0.84$  for a swainsonine level greater than 0.001%. Of the 55 plants from the five E+ populations, 40 plants were E+, and of those 38 had a swainsonine concentration greater than 0.01%. Of the 15 plants that were endophyte negative (E-), none had a measured swainsonine concentration greater than 0.01%, seven had a measured swainsonine concentration between 0.001% and 0.01% and the other eight plants contained only trace ( $< 0.001\%$ ) levels of swainsonine.

For the 40 E+ plant samples an estimated quantitative value of per cent endophyte infection was compared to swainsonine concentration. The correlation coefficient between the per cent endophyte infection and swainsonine concentration was  $r = 0.46$  which appeared random (Fig. 3.2).

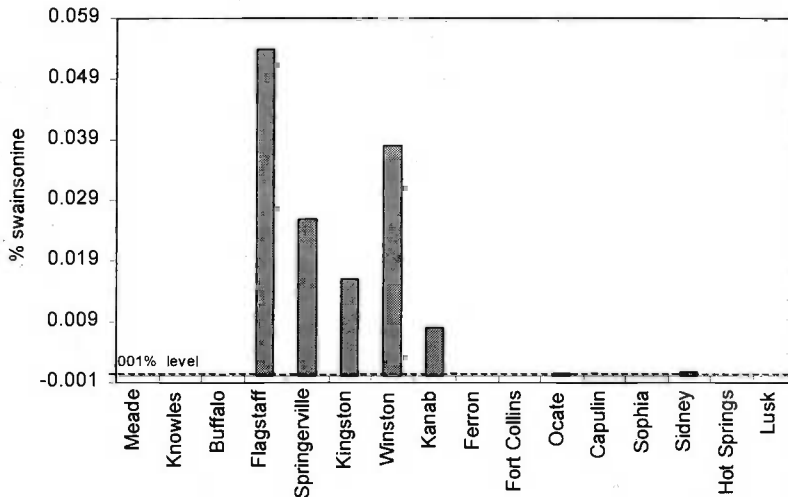
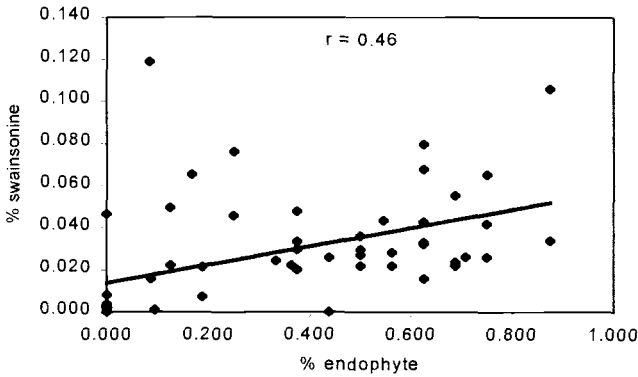


Fig. 3.1. Mean swainsonine concentration measured at 16 different locations for *Oxytropis lambertii* populations.





**Fig. 3.2.** Plot of per cent endophyte infection versus swainsonine concentration for all samples from E+ populations.

## Conclusions

All 16 populations of *O. lambertii* sampled were found to contain swainsonine at some level, however, there was a clear distinction in that some populations contained a significant toxic ( $> 0.001\%$ ) level and some clearly did not (Fig. 3.1). A threshold level for toxicity has been conservatively suggested to be around  $0.001\%$  swainsonine (dry weight) (Molyneux *et al.*, 1994). All of the *Oxytropis* samples in this investigation that contained significant levels of the alkaloid ( $> 0.001\%$ ) exceeded that value by at least an order of magnitude and clearly should be considered to be toxic and a threat to livestock grazing the plant over an extended time period. Of the *O. lambertii* plants found to contain significant levels of swainsonine all were of the var. *bigelovii*. However, not all var. *bigelovii* populations were found to contain high levels of swainsonine (five of ten populations).

The correlation between *O. lambertii* populations that contained significant levels of swainsonine and the presence of the *Alternaria* endophyte was very high and suggests a causal effect of the endophyte with toxic levels of swainsonine in the plant and supporting the earlier findings of Braun (1999). Equally important were the 11 populations of *O. lambertii* that contained only trace levels of swainsonine ( $< 0.001\%$ ) and those populations were all found to be endophyte free. A quantitative link between the swainsonine concentration in the plant and the corresponding level of endophyte infection was not as evident (Fig. 3.2). Braun (1999) did, however, observe a much higher correlation ( $r = 0.94$ ) between swainsonine concentration and degree of endophyte infection.

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## Chapter 4

# Delphinium Alkaloid Toxicosis in Cattle from Switzerland

B. Puschner<sup>1</sup>, M.C. Booth<sup>1</sup>, E.R. Tor<sup>1</sup> and A. Odermatt<sup>2</sup>

<sup>1</sup>California Animal Health and Food Safety Laboratory System – Toxicology Laboratory, One Shields Avenue, University of California, Davis, CA 95616, USA; <sup>2</sup>Tierärztliche Gemeinschaftspraxis Lindenmatte AG, Obere Bahnhofstrasse 19, Frutigen, 3714, Switzerland

### Introduction

Between 1995 and 1999 there were a number of unexplained deaths in cattle in Switzerland. A total of five animals died, but only one animal was necropsied. No significant lesions were found post-mortem. One animal was removed from the pasture after clinical signs of weakness, ataxia, respiratory distress and diarrhoea were observed. The animal recovered with supportive treatment without sequelae. All deaths occurred on the same pasture at 1500 m elevation in the Swiss Alps between 9 and 15 July of each year. Every year, a group of approximately 80 heifers grazed this particular pasture for only several weeks before being moved to a higher altitude around 20 July. Plants collected from the pasture in late July 1999 were identified as *Aconitum vulparia*, *Aconitum napellus* and *Delphinium elatum*. In addition, the pasture contained various grasses and bushes commonly encountered in alpine zones in the Alps.

*Aconitum napellus*, *A. vulparia* (Tutin *et al.*, 1993) and *D. elatum* (Seitz, 1969) are widely distributed in alpine regions, especially in the western and central Alps. In the USA, *Delphinium* spp. (larkspur) poisoning is the most serious poisonous plant problem on mountain and high plain rangelands in the West (Pfister *et al.*, 1999). Interestingly, there are few reports of larkspur poisoning in livestock outside of north western USA. *Aconitum napellus* is native to Europe, but also grows wild in North America. It is considered one of Europe's most poisonous plants although cases of livestock poisonings are rare. *Aconitum vulparia* is commonly found in central and southern Europe, and it is considered as toxic as *A. napellus*. Plants of the *Aconitum* (monkshood) and *Delphinium* genera are recognized as rich sources of biologically active and structurally complex diterpenoid alkaloids. Over many years, the diversity of alkaloids present in the seeds and whole plants of monkshoods and larkspurs have been studied, including those found in *A. napellus* (Liu and Katz, 1996) and *D. elatum* (Park *et al.*, 1995). The mixture of alkaloids varies among species (Ralphs *et al.*, 1997), locations, years (Ralphs *et al.*, 1988), individual plants (Ralphs *et al.*,

1998), during the growing season and among plant parts. In general, the total alkaloid concentrations are high in early growth and decline as the season progresses (Ralphs *et al.*, 1997). In larkspurs, alkaloid concentrations are highest in new growth of leaves, flowering racemes, and in seed pods (Pfister *et al.*, 1994a). Roots, leaves, seeds and honey containing pollen of *Aconitum* spp. have also been reported to be poisonous (Puri, 1974).

The larkspur alkaloids act as potent competitive antagonists of acetylcholine receptors in the muscle and brain leading to neuromuscular paralysis (Dobelis *et al.*, 1993; Kukel and Jennings, 1994). Clinical signs of larkspur toxicosis include muscle tremors, ataxia, weakness, muscle twitching, respiratory distress and collapse into sternal recumbency (Nation *et al.*, 1982). In addition, bloat is often reported in larkspur fatalities. The signs usually occur within a few hours of exposure and death may result. The clinical outcome depends on the concentration of toxic alkaloids and the amount and rate of ingestion. Aconitine and related alkaloids of *Aconitum* spp. are known neurotoxins and cardiotoxins and exert their effect by increasing the permeability of sodium ions in excitable membranes (Catterall, 1980). Clinical signs of aconitine poisoning in humans include vomiting, diarrhoea, perioral numbness and paresthesia, weakness and paralysis in all four limbs, hypotension, and cardiac arrhythmias (Chan *et al.*, 1994). Reports of aconitine poisonings in animals have not been published.

Sudden death or non-specific illness in cattle can result from the ingestion of diterpenoid alkaloid-containing plants. In fatal cases, diagnosis may be facilitated by finding *Delphinium* or *Aconitum* plant parts in the ingesta or in the environment. However, leaves may be macerated beyond identification, or a complete history of potentially toxic plants present in the environment of the animal may not be provided. Toxic plant material was not identified in the ingesta of the case presented in this paper. The objective was to determine whether the sudden death described in these cattle was caused by the ingestion of numerous alkaloid-containing plants. Gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) was used for analysis of the gastrointestinal samples submitted to the laboratory.

## Materials and Methods

A multi-residue alkaloid screen (Holstege *et al.*, 1995) using GC/MS was employed for the detection of *Delphinium* alkaloids in rumen, abomasal, small intestine, and cecal contents. Samples (5 g) were extracted with 100 ml 5% ethanol in ethyl acetate (v/v) after the addition of 1 ml of 10 M NaOH and 50 g of Na<sub>2</sub>SO<sub>4</sub>. An aliquot (40 ml) was transferred to a separating funnel containing 100 ml of hexane and extracted with 15 ml of 0.5 M HCl. The aqueous extract (bottom layer) was sparged with nitrogen to remove trace organic solvents, the pH was increased to > pH 10 with 10 M NaOH, and the extract was adsorbed on a POLYSorb<sup>®</sup> ACT-1 RP-C18 polymeric SPE column (Transgenomic, Omaha, NE). The alkaloids were eluted with 2 ml of ethyl acetate and analysed semi-

quantitatively by GC/MS (Model HP 5890 with HP 5970 MSD, Hewlett Packard) using a 12m x 0.2mm x 0.33  $\mu\text{m}$  HP-1 column (Hewlett Packard). Deltaline, delpheline, lycoctonine and methyllycaconitine (MLA) standards were provided by Dr G.D. Manners. Alkaloids for which standards were not available were quantified using the response factor of similar compounds.

For aconitine evaluation, an aliquot of the extract was injected into a Hewlett-Packard Model 1050 HPLC coupled with a Finnigan LCQ ion trap mass spectrometer. The analytical column was a Luna C18(2), 150 mm x 4.6 mm x 5  $\mu\text{m}$  (Phenomenex<sup>®</sup>) with a C18 guard column. The mobile phase was an isocratic acetic acid water/methanol (5:95 v/v) mixture (1 ml glacial acetic acid + 99 ml water methanol; 5:95), at a flow rate 0.5 ml min<sup>-1</sup>. The instrument was tuned by optimizing the response of m/z 646 while infusing 10  $\mu\text{g ml}^{-1}$  of aconitine standard at 10  $\mu\text{l min}^{-1}$  into a mobile phase of 1% acetic acid in water/methanol (5:95) at 0.5 ml min<sup>-1</sup>. MS data were acquired in the positive ionization ESI with the following scan event: (646)->(586)->(300-600); MS<sup>2</sup> conditions: collision energy 24.0%, isolation window 1.5; MS<sup>3</sup> conditions: collision energy 23.0%, isolation window 1.5. ESI parameters: capillary temperature 230°C, capillary voltage 46 V, spray voltage 4.75 kV, tube lens voltage 55 V, sheath gas flow 95, auxiliary gas flow 5. Quantification: external calibration, three point calibration curve of analytical standard prepared in matrix to be analysed, using the response of the ions at m/z 526 and m/z 554. Aconitine standard was purchased from Sigma (St Louis, MO).

## Results and Discussion

Deltaline, a good marker compound for exposure to *Delphinium* spp. was the primary alkaloid found in the gastrointestinal samples submitted for analysis (Holstege *et al.*, 1996). This was consistent with previous findings for *D. elatum* (Pelletier *et al.*, 1989). In the rumen contents, the following diterpenoid alkaloids were detected: 18 ppm deltaline, 1.4 ppm deltamine and 4.4 ppm lycoctonine. The abomasal contents contained 7 ppm deltaline, 0.6 ppm delpheline and 0.6 ppm lycoctonine; and 3 ppm deltaline and 1.1 ppm lycoctonine were detected in the small intestine contents. In the cecal contents: 8 ppm deltaline, 1.6 ppm delpheline and 1.3 ppm lycoctonine were detected. The results were semi-quantitative based on the response factor of deltaline. The presence of lycoctonine in samples using the described method may be indicative of MLA, which is readily hydrolysed to lycoctonine at high pH (Majak, 1993). Delpheline and lycoctonine have also been identified in *D. elatum*. Thus, the GC/MS results confirmed the exposure of cattle to *D. elatum* in the pasture.

Aconitine was positively identified in rumen and abomasal contents of the cattle by a highly specific technique of LC/MS<sup>3</sup>. Aconitine is a norditerpenoid alkaloid that has been found in *A. napellus* (Liu and Katz, 1996). Most research has focused on the detection of diterpenoid alkaloids in the seeds of *Aconitum*

spp., but it is known that the whole plant contains the toxic alkaloids, with seeds and roots containing the highest concentrations.

In the cattle poisoning case reported here, the finding of *D. elatum*, *A. napellus* and *A. vulparia* in the pasture and detection of *Delphinium* and/or *Aconitum* alkaloids in gastrointestinal samples suggested *Delphinium* and/or *Aconitum* poisoning. The typical height of *D. elatum* at maturity is 40–200 cm (Tutin *et al.*, 1993) and the flowering season is June and July in the western and central Alps of Europe. It is well known that highest concentrations of toxic alkaloids are found in immature plant tissue, and that a 'toxic window' during which cattle tend to eat more larkspur extends from the flower stage into the pod stage. In the reported case, cattle were exposed to the toxic alkaloids during the flowering stage. The seasonal occurrence of poisonings matches the clinical picture typically described in larkspur toxicosis. In cattle, 30 g fresh larkspur  $\text{kg}^{-1}$  bodyweight produced recumbency (Marsh *et al.*, 1916). The estimated oral lethal dose 50 ( $\text{LD}_{50}$ ) for cattle given larkspur is 25–40 mg  $\text{kg}^{-1}$  bodyweight of total toxic alkaloids (Pfister *et al.*, 1994b), or 2.48 g  $\text{kg}^{-1}$  bodyweight of the dried plant (Olsen, 1978). Most of the toxicity of tall larkspur can be attributed to MLA and 14-deacetylnudicauline (Manners *et al.*, 1993). Purified MLA administered intravenously to cattle resulted in clinical signs of larkspur poisoning but not death when given at 2 mg  $\text{kg}^{-1}$  bodyweight, while 50 mg  $\text{kg}^{-1}$  bodyweight of deltaline had to be administered to cause the same clinical signs (Pfister *et al.*, 1999). MLA is the dominant alkaloid found in low larkspurs (Bai *et al.*, 1994), while deltaline is the dominant alkaloid found in most tall larkspurs. In general, the risk associated with ingestion of larkspurs may vary considerably. It is thought that larkspur alkaloids are absorbed rapidly and not hydrolysed in the rumen leading to a rapid onset of clinical signs after ingestion of the plants. Although detailed kinetic studies have not been published, larkspur alkaloids are diterpenoids that contain functional groups that are known substrates for phase-II biotransformation reactions (Parkinson, 1996).

*Aconitum napellus* (Echter Eisenhut, Monk's hood) and *Aconitum vulparia* (Gelber Eisenhut, Wolf's bane) are variable species in which numerous local variants have been recognized as species or subspecies. These perennials are commonly found in central Europe, especially in the Alps near creeks and meadows, and their flowering season extends from June through to September. Aconitine is extremely toxic. In horses, ingestion of 200–400 g of fresh *A. napellus* is likely to result in death (Gerbaud, 1980). Leaves can contain 0.2–1.2% aconitine, while the root can contain 0.3–2% aconitine (Bentz, 1969). In adult humans, the lethal dose of purified aconitine is 3–6 mg (Frohne and Pfänder, 1983). Thus, ingestion of less than 1 g of the root can be deadly to an adult. In humans, most cases are related to accidental ingestion of the wild plant. The root has been mistaken for horse-radish root (Martens and Vandeveld, 1993). Although cattle usually avoid *Aconitum* spp. exposure represents a risk, especially in alpine regions where Monk's hood is abundant. Reports of aconitine poisoning in cattle are not published. The finding of aconitine in the gastrointestinal contents however, is proof of ingestion of plants from the *Aconitum* genus.

*Delphinium* spp. and *Aconitum* spp. poisoning should be suspected when appropriate clinical signs are found along with evidence of consumption of plant material that contains diterpenoid alkaloids. Most animals will avoid larkspurs until the late flower and early pod stage. Toxicosis usually occurs during a 'toxic window' or when little else is available to eat. The presence of larkspurs in mountain rangeland has often dictated summer grazing management. A great variety of management options have been reviewed (Pfister *et al.*, 1999). In the reported case, the producer may avoid livestock losses by moving the cattle to a higher elevated pasture before the flowering stage, or by grazing the cattle on the larkspur infested pasture later in the season.

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## Chapter 5

# Toxicity of a Pyrrolizidine Alkaloid, Riddelliine, in Neonatal Pigs

B.L. Stegelmeier<sup>1</sup>, L.F. James<sup>1</sup>, K.E. Panter<sup>1</sup>, R.J. Molyneux<sup>2</sup>, D.R. Gardner<sup>1</sup>, T.K. Schoch<sup>2</sup>, S.T. Lee<sup>1</sup>, M.H. Ralphs<sup>1</sup> and J.A. Pfister<sup>1</sup>

<sup>1</sup>Poisonous Plant Research Laboratory USDA Agricultural Research Service, Logan UT 84341, USA; <sup>2</sup>Western Regional Research Center, USDA Agricultural Research Service, Albany CA 94710, USA

### Introduction

More than 350 pyrrolizidine alkaloids (PAs) have been identified in over 6000 plants in the *Boraginaceae*, *Asteraceae* and *Fabaceae* families. About half of the identified PAs are toxic and several are carcinogenic (Mattocks, 1986; Chan *et al.*, 1994). Many PA-containing plants are introduced species that are considered invasive, noxious weeds that infest open ranges and fields, replacing desirable plants. Most are not palatable and livestock avoid eating them if other forages are available. PA-containing plants often invade fields or crops and plant parts or seeds contaminate prepared feeds and grains. Human poisonings are most often a result of food contamination or when PA-containing plants are used for medicinal purposes. Regulations controlling PA exposure in feeds and food have been enacted in several countries and additional regulations are likely. The purpose of this work is to document the effects of animal age on PA toxicity, especially the effects of PAs on neonatal animals. These findings are supported by clinical reports of poisoning in neonatal animals and human infants while similarly exposed adults were unaffected.

### Materials and Methods

Five groups of 12 pigs at 0, 3, 6, 12 and 52 weeks of age were randomly divided into groups of three and dosed via gavage with purified riddelliine at doses of 0, 5, 10 and 20 mg kg<sup>-1</sup> for 14 days. Animal work and assessment of intoxication was done under the care of a licenced veterinarian following protocols reviewed and approved by the Utah State University Animal Care and Use Committee. All pigs were monitored daily and weighed weekly when blood samples were collected. After dosing the pigs for 14 days, they were euthanized, necropsied and tissues were collected for histological and chemical studies. Serum biochemistries were done on a Beckman CX5 analyser using Beckman reagents and following

the manufacturer's protocols (Beckman Coulter Inc., San Brea, CA). PA metabolites were extracted from pig livers and detected using gas chromatography and mass spectrometry as previously described (Schoch *et al.*, 2000). Animal weights, serum biochemical data and histological grades were compared using an analysis of variance (ANOVA) with both mixed and generalized linear models for a repeated measures design. Mean separations were done using Duncan's method (SAS Statistical Software 1986, SAS Institute Inc., Cary, NC). The level of significance was set at  $P < 0.05$ .

## Results and Discussion

All age groups of animals dosed with riddelliine gained less or lost weight ( $P < 0.001$ ). Weight loss correlated directly with dose for all age groups ( $r = 0.78$ ). When changes in weight were normalized for body size there were no significant age differences at different riddelliine doses. Clinically, all animals dosed with riddelliine at  $20 \text{ mg kg}^{-1}$  developed severe hepatic disease characterized by icterus, anorexia, scruffy-dry skin and reluctance to move. Riddelliine doses of 5 and  $10 \text{ mg kg}^{-1}$  caused similar but less severe clinical changes. Age groups of 3 and 6 weeks were most severely affected and several of the high dose animals from these groups had to be euthanized several days early. These animals also had the most severe gross lesions. Six-week-old pigs treated with  $20 \text{ mg kg}^{-1}$  had extensive ascites and extensive visceral oedema. The livers of these animals were swollen with prominent red lobular patterns and sub capsular oedema (Fig. 5.1).

Serum biochemical changes were similar to those previously reported with riddelliine toxicosis (Molyneux *et al.*, 1991). All dosed animals had elevated serum alkaline phosphatase (ALP), aspartate amino transferase (AST), gamma glutamyl transferase (GGT) and creatinine kinase (CK) activities. Pigs dosed with  $20 \text{ mg kg}^{-1}$  also had elevated serum bilirubin and bile acid concentrations. Treated pigs that were 0, 3 and 6 weeks old had reduced serum albumin and total proteins that were significantly different from the 12- and 52-week-old groups at doses of  $20 \text{ mg kg}^{-1}$  ( $P = 0.02$ ) and  $10 \text{ mg kg}^{-1}$  ( $P = 0.0003$ ). The older 6-, 12- and 52-week-old pigs tended ( $P < 0.10$ ) to have higher serum enzyme activities (ALP, AST and GGT), serum bilirubin and serum bile acid concentrations than the younger groups.

All treated animals had significant hepatocellular swelling, vacuolation and necrosis with associated collapse of hepatic cords, haemorrhage and inflammation. All treated pigs had similar lesions of varying degrees of severity. The lesions were subjectively scored for severity of necrosis, haemorrhage and inflammation. The most severe lesions were found in the 3- and 6-week-old pigs. These pigs had extensive hepatocellular necrosis with collapse of hepatic cords and haemorrhage (Fig. 5.2). Older 12- and 52-week-old pigs had more inflammation, but hepatocellular swelling and degeneration was similar to that seen in similarly treated newborn pigs (Fig. 5.2).



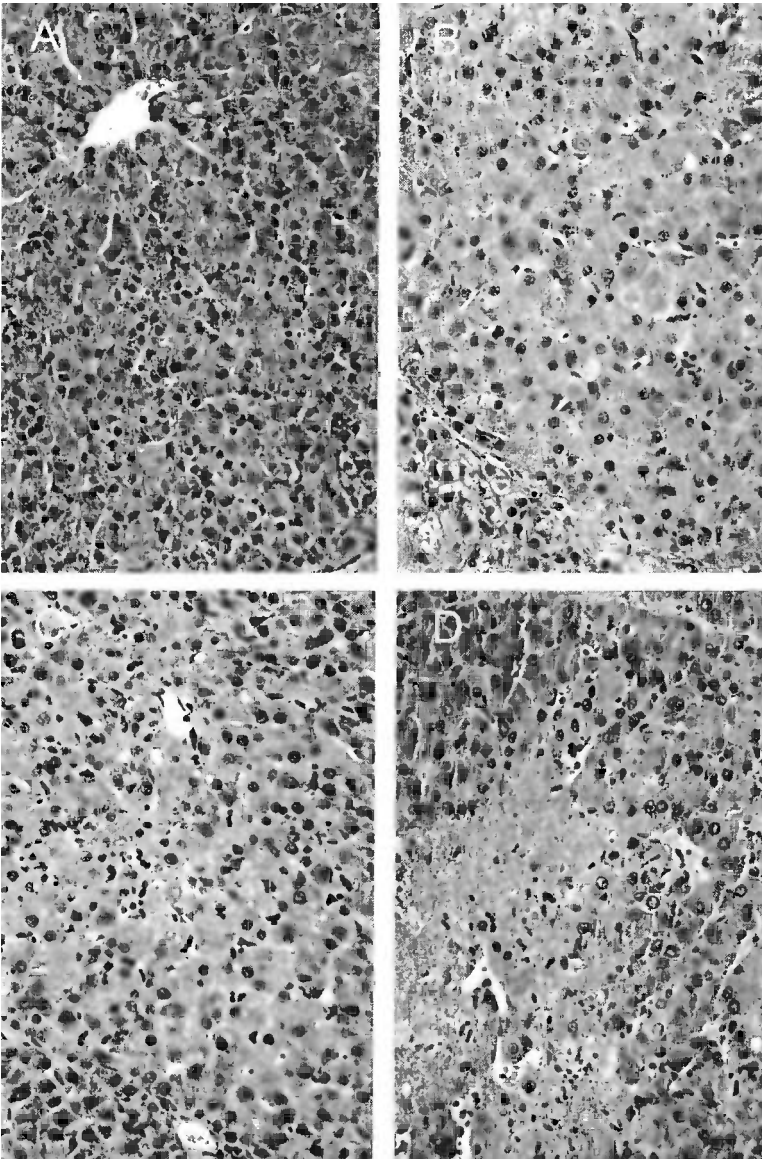
**Fig. 5.1.** Liver of 6-week-old pig treated with 20 mg kg<sup>-1</sup> riddelliine for 14 days. Note the prominent lobular pattern on the cut surface.

Previous PA studies report that pigs commonly develop extrahepatic lesions in the lungs and kidneys (Harding *et al.*, 1964; Hooper and Scanlan, 1977). All animals, except those aged 6 weeks had no detectable extrahepatic lesions.

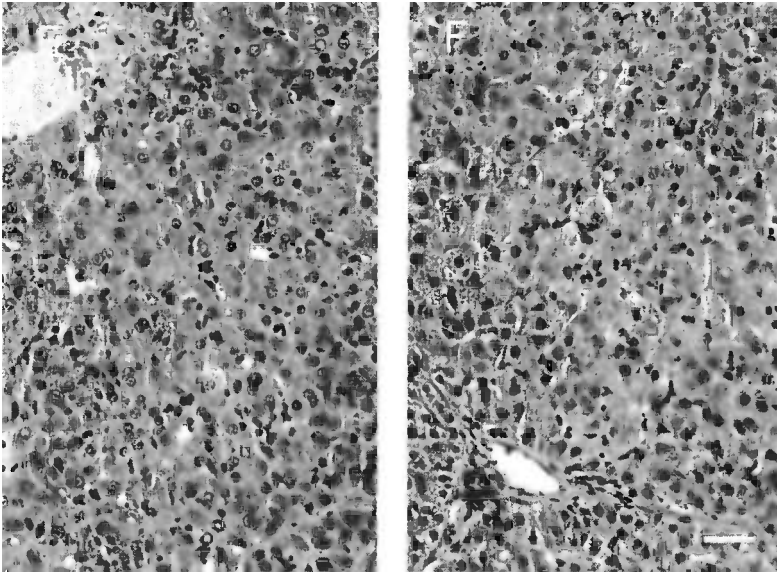
Several small arteries in 6-week-old pigs that were treated with 20 mg riddelliine kg<sup>-1</sup> had vascular media oedema and fibrosis with mild endothelial hyperplasia. No lesions were found in the kidneys of any of the pigs. Liver from treated pigs contained pyrroles but the amount of pyrrole detected was not consistent and it did not correlate with dose. The current methodology for pyrrole detection in tissues and serum is qualitative (Schoch *et al.*, 2000). Improvement of these techniques is needed to better identify intoxications and to better prognose the outcome of poisoned animals.

## **Conclusion**

Riddelliine is toxic in pigs at doses that are comparable to those of other species. Poisoning resulted in hepatic lesions and vascular lesions in the lungs of 6-week-old pigs. Poisoned 3- and 6-week-old pigs developed clinical signs that were more severe than the other age groups. These animals also developed hepatic disease that was more extensive than the younger and older pigs. These findings



**Fig. 5.2.** Photomicrographs of liver from: (A) A newborn control pig. Note the intact hepatic cords. (B) A newborn pig (0 weeks) treated with  $20 \text{ mg kg}^{-1}$  riddelliine. Note the cord disruption and intense hepatocyte swelling. (C) A 3-week-old pig also treated with riddelliine at  $20 \text{ mg kg}^{-1}$ . Note the hepatocyte swelling and necrosis. (D) A 6-week-old pig treated at  $20 \text{ mg kg}^{-1}$ . Notice the extensive necrosis with haemorrhage.



**Fig. 5.2 (cont).** Photomicrographs of liver from: (E) A 12-week-old pig treated at  $20 \text{ mg kg}^{-1}$ . Notice the hepatocyte necrosis with mild inflammation. (F) A 52-week-old pig treated at  $20 \text{ mg kg}^{-1}$ . Note the hepatocyte swelling with associated lymphocytic inflammation. Sections are  $5 \mu\text{m}$  thick and stained with haematoxylin and eosin. Bar is  $20 \mu\text{m}$ .

suggest that weanling pigs near 3-6 weeks of age are most susceptible to PA poisoning. Age susceptibility is likely to be an important consideration in determining exposure risk to both humans and livestock.

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## Chapter 6

# Bacteria and Corynetoxin-like Toxins in Nematode Seed-galls in *Festuca nigrescens* from North America and New Zealand

I.T. Riley<sup>1</sup>, A.R. Gregory<sup>2</sup>, J.G. Allen<sup>2</sup> and J.A. Edgar<sup>3</sup>

<sup>1</sup>Applied and Molecular Ecology, Adelaide University, Glen Osmond SA 5064, Australia; <sup>2</sup>Animal Health Laboratories, Department of Agriculture, South Perth WA 6151, Australia; <sup>3</sup>CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong Vic 3220, Australia

*Rathayibacter toxicus* produces a characteristic mixture of 16 tunicaminylluracil antibiotics, referred to collectively as corynetoxins, which are responsible for livestock poisoning in Australia and South Africa (Edgar *et al.*, 1982). Seed-gall nematodes (*Anguina* spp.) are vectors for *R. toxicus*, carrying it into grasses where it colonizes both nematode galls and seedheads. In Australia, *R. toxicus* is found in association with *Anguina funesta* in *Lolium rigidum* and *Vulpia myuros* and with an *Anguina* sp. in *Agrostis avenacea* and *Polypogon monspeliensis* (McKay and Ophel, 1993; Riley, 1995). In South Africa it is found in *L. rigidum* (Schneider, 1981), and the toxins produced are identical to those found in Australian bacterial galls (Cockrum and Edgar, 1985). It is likely that the bacterium was introduced to South Africa in contaminated ryegrass seed from Australia (Nicholas, 1981).

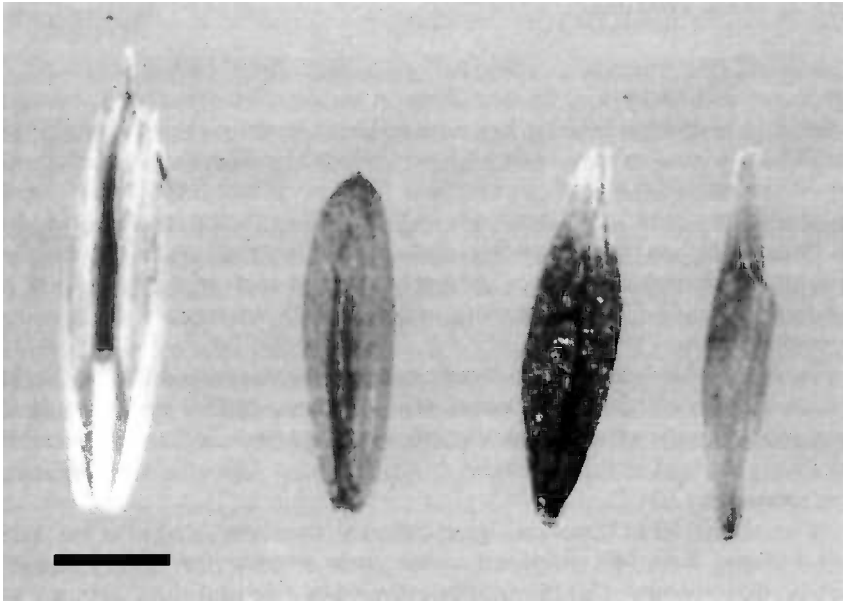
The widespread occurrence of *R. toxicus* in Australia is consistent with it being an Australian endemic. However, the main hosts (*viz.* *L. rigidum* and *P. monspeliensis*) are of Mediterranean origin and have become pandemic. So, it is possible that *R. toxicus* occurs elsewhere in the world, depending on vector availability. Equally, *R. toxicus* could occur in association with other grasses because experimentally it has been shown to be carried by other *Anguina* spp. (Riley and McKay, 1990; Riley, 1992; Riley *et al.*, 2001). Knowledge of the distribution of *R. toxicus* is important because corynetoxin poisoning of livestock may be misdiagnosed unless the possibility is recognized. Livestock deaths associated with infection of *P. monspeliensis* by *R. toxicus* occurred annually for nearly 20 years before the cause was determined (Finnie, 1991).

The cause of livestock deaths associated with nematode infestation of the turf grass selection Chewings fescue (*Festuca nigrescens*) in Oregon, USA from the 1940s to 1960s (Haag, 1945; Shaw and Muth, 1949; Jensen, 1961) was never determined. Since nematode galls colonized by a coryneform bacterium were found (Galloway, 1961) and descriptions of the clinical disease suggest a close similarity to corynetoxin poisoning (McKay and Ophel, 1993), it is reasonable to

suggest that corynetoxins produced by *R. toxicus* may have caused the deaths. Although original samples are unavailable, bacterially colonized nematode galls have recently been found in samples of the *F. nigrescens* selection Chewings fescue from New Zealand (NZ) and New Jersey, USA.

### Examination of Bacteria and Toxins in *Festuca* Galls

Seed-galls from *F. nigrescens* (Fig. 6.1) from NZ and USA were examined with a dissecting microscope. Those appearing by their translucent yellow colour to be bacterially colonized were separated for assay. The galls from NZ were from a sample of the *F. nigrescens* selection Chewings fescue seed collected in 1980. The sample contained seed, nematode galls and a small number of bacterially colonized galls. The galls from USA were supplied by the USDA Nematode Collection (accession 12020), and came from the same host species and selection from New Jersey lodged in 1944.



**Fig. 6.1.** *Festuca nigrescens* diaspore, seed, nematode gall (dark purple-black, *Anguina* sp.) and bacterially colonized nematode gall (yellow, translucent, *Rathayibacter toxicus* or variant). Scale bar = 1 mm.



### Bacterial content

Microscopic examination (x 1000) of stained suspensions from the galls revealed coryneform bacterial cells consistent with *Rathayibacter*. As the galls were old and the bacterium no longer viable, the identity of the bacterium was determined by an ELISA based on a monoclonal antibody for a specific polysaccharide antigen of *R. toxicus* (A.R. Gregory, unpublished data).

Initially bacterially colonized galls from *F. nigrescens* (two from NZ and three from USA) and controls of galls colonized by *R. toxicus* and seeds from *L. rigidum* collected in Australia were assayed. Positive results were obtained for both galls from NZ, albeit somewhat weaker than that obtained for control galls from *L. rigidum*. Negative results were recorded for the galls from USA. Further assays were conducted on retained material of galls assayed for toxins (described below). Again it was found that the galls from NZ gave a relatively weak positive response and the galls from the USA gave a negative response.

These results indicate that *R. toxicus* was present in the galls from NZ and is associated with *Anguina* sp. infestation of *F. nigrescens*.

### Toxicity of bacterial galls

Extracts of two bacterially colonized galls each from both sources of *F. nigrescens* and from *L. rigidum* from Australia, were analysed using a quantitative ELISA specific for corynetoxin-like substances (Than *et al.*, 1998). The extracts were also assayed by high performance liquid chromatography with UV detection (HPLC) (Cockrum and Edgar, 1983, 1985) and liquid chromatography-mass spectrometry (lc-ms). The gall extracts were also examined for their ability to inhibit uridine diphospho-N-acetylglucosamine: dolichyl phosphate N-acetylglucosamine-1-phosphate transferase, an enzyme that is specifically inhibited by corynetoxins and related tunicaminylluracil antibiotics (Stewart, 1998).

The toxin ELISA and HPLC readily detected corynetoxin-like toxins in the extracts. The levels of toxin measured using the toxin ELISA were 0.45 µg of toxin per gall in the material from the US, 1.1 µg per gall in the galls from NZ and 1.6 µg per gall in the galls from *L. rigidum* from Australia. Similar results were obtained by HPLC.

When 1-min HPLC fractions were collected from the extracts of the galls from NZ and USA and subjected to the toxin ELISA, the ELISA positive fractions corresponded with the putative corynetoxin-like peaks and there was no activity in other fractions.

The identity of individual components seen by HPLC in the galls from NZ and USA was confirmed by lc-ms. The toxin peaks displayed the expected molecular weights for tunicaminylluracil antibiotics at the relative retention times expected from previous work (Cockrum and Edgar, 1983) and relative to the corynetoxins in the positive control galls from *L. rigidum*.

The mixtures of toxins found in the galls from NZ and USA, although quite similar to each other, differed in relative amounts of some individual components from those typical in galls from other grasses colonized by *R. toxicus*. The mixture of toxins produced by *R. toxicus* has been shown to be consistent in gall extracts from widespread regions of Australia and from South Africa, even after long storage (Cockrum and Edgar, 1985) and regardless of the host grass species (Edgar *et al.*, 1994). The latter observation argues against the different toxin profiles seen with the galls from NZ and USA being simply due to growth of *R. toxicus* in *F. nigrescens* rather than *L. rigidum*. The similarity in the toxin profiles obtained with the galls from NZ and USA indicates that the bacterium from both sources was the same, but differed from *R. toxicus* producing the Australian and South African toxin profile.

The extracts of the galls from USA, NZ and Australia were all strongly positive in the *in vitro* N-acetylglucosamine-1-phosphotransferase inhibition assay, thereby confirming the expected biological activity of the toxins.

A fuller description of the chemistry of the toxins in these galls from *F. nigrescens* is given by Anderton *et al.* (Chapter 9 this volume).

### Interpretation

For the galls from NZ, the presence of yellow coryneform bacteria that were weakly positive in the ELISA for *R. toxicus*, and the detection of corynetoxin-like tunicaminyuracil antibiotics similar to corynetoxins, indicates that a variant of *R. toxicus*, or closely related species, had colonized the galls.

For the galls from USA, the similarity in tunicaminyuracil antibiotic composition with the NZ galls indicates that the same bacterium had colonized the galls and was involved in toxin production, despite not being detected by the ELISA for *R. toxicus*. It is most likely that the bacterial antigens had decayed as the galls were quite old. The nematode galls from this sample only contained remnants of nematodes indicative of decay during storage. The case for the galls from the two sources having the same bacterium is strengthened by the fact that the *F. nigrescens* selection known as Chewings fescue, widely grown throughout the world as turf grass, originates from NZ and is consistent with an Australasian origin of *R. toxicus*.

### Conclusions

The data presented cannot conclusively establish the cause of the livestock deaths in Oregon; this would require analysis of original samples. Nevertheless, the findings point to corynetoxin-like toxins produced by a variant of *R. toxicus* being the most likely cause.

This view is based on the following evidence.

1. The toxic *F. nigrescens* selection Chewings fescue was infested with a seed-gall *Anguina* and an unidentified coryneform bacterium (Jensen, 1961).

2. Toxins similar to those produced by *R. toxicus* were found in galls from Chewings fescue from NZ and USA.
3. The bacterium in the galls from NZ is *R. toxicus* or a variant.
4. The *F. nigrescens* selection Chewings fescue came from NZ (Aiken *et al.*, 1996) with considerable quantities having been exported to the USA (Whittet, 1969).

Although this supports an Australasian origin for *R. toxicus*, it also indicates a possible global distribution of the bacterium. Therefore, even though significant risk to livestock only occurs where conditions favour high field populations of a suitable vector or toxic materials are concentrated in screenings, the possibility of corynetoxin poisoning should be considered whenever investigating otherwise unexplained deaths or convulsive poisoning of livestock (Edgar, 1994).

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## Chapter 7

# Preliminary *In Vitro* Studies of *Cistus salvifolius* Leaves in Relation to Metabolic Disorders in Sheep

A.M. Bruno-Soares<sup>1</sup>, J.M. Abreu<sup>1</sup> and F. Soler<sup>2</sup>

<sup>1</sup>*Instituto Superior de Agronomia, DPAA, Tapada da Ajuda 1349-017 Lisboa, Portugal;* <sup>2</sup>*Facultad de Veterinaria, Unidad de Toxicología, Depto. Medicina y Sanidad Animal, Av. De la Universidad s/n (Campus Universitario) 10071 Cáceres, Spain*

### Introduction

In natural Mediterranean pastures, during periods when forages are scarce, the abundance of *Cistus* spp. induces animals to consume these shrubs. Symptoms of intoxication and photosensitivity were detected in sheep from several flocks, in Spain, Turkey and Portugal.

Sheep flocks after ingestion of *Cistus ladanifer* and *Cistus salvifolius*, showed different intoxication symptoms and signs, such as polyuria, oedema of the head, thinning until death and muscular twitches (Soler *et al.*, 1997). Identical symptoms were observed in sheep in the Alentejo Region, together with others, such as hypersensitivity to sound or movement, and animals falling and pedalling when *C. salvifolius* leaves were ingested (Louzã *et al.*, 1999). However, studies using Merino rams nourished with *C. salvifolius* leaves (at no more than 30% of the diet) did not show remarkable alterations in their behaviour, or in the following anatomopathological exams (Bruno-Soares *et al.*, 1999). The quantitative and qualitative nature of the substances responsible in *Cistus* spp. for this poisoning is not yet clear. Flavonoid compounds were described in *Cistus laurifolius* as responsible for the convulsive syndrome (Vogt and Gulz, 1994). Some of them are certainly related to the condensed tannins, and can have anti-nutritive properties and also oestrogenic activity (e.g. formononetin).

The consumption of saponins by animals may induce hepatogenous photosensitization diseases (Miles *et al.*, 1994). Alternatively the simultaneous presence of the saponin and flavonoid compounds in the plant may lead to synergic effects, thus increasing the toxic effects of each substance.

The aim of the work presented here was: (i) to evaluate the nutritive value of *C. salvifolius* leaves during the vegetative development of the shrubs, so as to better understand their nutritive contribution, and (ii) to evaluate its anti-nutritional factors (phenolic and saponin compounds) in order to understand the relationship between consumption of *C. salvifolius* leaves and sheep intoxication.

## Materials and Methods

Leaves of *C. salvifolius* were randomly collected in farms (Alentejo Region) where sheep intoxication had occurred. The leaf samples were collected in all parts of the shrubs, thus comprising both young and old leaves. Leaves obtained were mixed in five composite samples (06/26, 07/09, 09/26, 10/30 and 12/03). After lyophilization and milling (1 mm), samples were assessed for dry matter (DM) – drying at 100-105°C, ash – complete incineration at 550°C, crude protein (CP) – Kjeldhal method, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) according to Robertson and Van Soest (1981), gross energy (GE) in a calorimeter bomb (Parr 1261), total phenol (TP) content according to Julkumen-Titto (1985), condensed tannins (CT) according to Broadhurst and Jones (1978) and crude saponins (CS) according to Tava *et al.* (1993).

The gas volume produced was evaluated according to Menke and Steingass (1988) in samples of 200 mg DM after 2, 4, 6, 8, 12, 24, 30, 48 and 72 h of incubation.

Polyethylene glycol (PEG) was used to evaluate the relationship between anti-nutritional compounds and gas production. PEG, previously wetted with distilled water, was mixed with *C. salvifolius* leaves (in a 1:2 ratio) and the mixture was subsequently lyophilized.

DM and CP losses in the rumen were also evaluated using the nylon bag technique (Ørskov *et al.*, 1980). Losses in the rumen at every incubation time were corrected by deduction of the particle losses, which were determined separately.

Data on chemical composition and on gas volume produced in 24, 30 and 48 h incubation were subject to regression analysis to study how they varied with the vegetative development of the shrubs. A stepwise analysis was used to determine which of the variables studied might best explain the variations in gas production.

## Results

### Chemical composition and gross energy

Some points are worth stressing about the chemical composition of *C. salvifolius* leaves (Table 7.1): the high levels of CP, ADL and CS (ca. 13%, 11% and 10% respectively) and the low levels of fibre (NDF) and CT (ca. 32% and 0.51 g 100 g<sup>-1</sup> DM). The saponin level of *C. salvifolius* leaves is about four times the level quoted by Tava *et al.* (1993) for lucerne.

### Gas production

Table 7.2 shows gas production at different periods of incubation for the different dates of collection of *C. salvifolius* leaves. The volume of gas at 72 h incubation

**Table 7.1.** Chemical composition of *Cistus salvifolius* leaves (% dry matter).

Date	DM	Ash	CP	NDF	ADF	ADL	TP <sup>a</sup>	CT <sup>a</sup>	CS	GE <sup>b</sup>
06/26	28.8	5.6	11.0	37.4	27.0	9.7	2.7	0.265	11.6	18.4
07/09	35.8	5.8	11.5	31.7	23.2	8.7	3.3	0.498	10.4	18.2
09/26	37.8	6.7	13.0	32.4	24.3	15.1	3.1	0.416	8.8	18.5
10/30	24.0	6.5	14.2	28.5	22.5	13.2	4.1	0.977	10.8	18.8
12/03	30.9	5.8	13.9	29.4	20.2	10.6	3.1	0.371	9.5	18.9

<sup>a</sup> (g 100 g<sup>-1</sup> DM); <sup>b</sup> (MJ kg<sup>-1</sup> DM)

DM = dry matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; TP = total phenol; CT = condensed tannins; CS = crude saponins; and GE = gross energy.

**Table 7.2.** Gas production of *Cistus salvifolius* leaves (ml 200 mg<sup>-1</sup> dry matter).

Date	Incubation time (h)							
	2	4	6	8	24	32	48	72
06/26	0.3	1.0	0.6	1.6	5.2	5.7	6.5	7.9
07/09	0.8	1.4	1.2	2.3	6.0	6.7	7.2	9.3
09/26	0.6	1.4	1.6	2.7	6.8	7.7	8.8	12.4
10/30	3.6	4.4	6.0	7.8	13.3	14.7	19.1	23.6
12/03	1.5	1.9	2.1	3.4	8.4	9.1	10.7	13.3

**Table 7.3.** Gas production of *Cistus salvifolius* leaves with addition of PEG (ml 200 mg<sup>-1</sup> dry matter).

Date	Incubation time (h)							
	2	4	6	8	24	32	48	72
06/26	1.1	1.7	2.1	3.0	7.7	9.8	12.7	15.0
07/09	1.8	3.5	4.3	6.0	13.6	14.6	15.6	16.4
09/26	1.8	3.5	4.6	6.3	14.0	15.5	15.9	16.4
10/30	5.6	7.4	9.9	12.4	20.5	22.5	24.0	25.5
12/03	2.0	4.2	5.4	7.4	15.2	16.9	17.3	18.0

was low, varying between about 8 and 24 ml 200 mg<sup>-1</sup> DM. However, the addition of PEG (Table 7.3) to the leaves increased the gas production, which about doubled at every incubation time (7.9 vs. 15.0 ml 200 mg<sup>-1</sup> DM at 72 h).

#### DM and CP losses in the rumen

DM and CP losses at different incubation times in the rumen are presented in Table 7.4. Both losses were low, reaching about 47% and 51% respectively, in 48 h.

**Table 7.4.** Dry matter (DM) and crude protein (CP) rumen losses of *Cistus salvifolius* leaves (% DM).

Rumen losses	Sample date	Incubation time (h)				
		3	6	16	24	48
DM	07/09	13.9	24.0	27.5	30.5	45.1
	09/26	23.8	24.1	25.9	33.2	47.9
	10/30	23.2	23.7	31.6	39.8	47.9
	12/03	23.9	24.4	29.3	31.6	47.9
CP	07/09	7.0	16.8	18.3	19.0	46.3
	09/26	17.7	19.7	17.7	25.8	53.2
	10/30	22.7	21.7	22.6	33.0	51.6
	12/03	16.9	17.5	17.5	18.8	53.7

During the initial incubation times, lag times of about 6 h were seen in DM disappearance, while in CP disappearance the lag times reached 24 h.

## Discussion

All composition values, except ash, CP and NDF, were time-independent ( $P < 0.05$ ). CP increased ( $r^2 = 92.5\%$ ,  $P < 0.05$ ) and NDF decreased ( $r^2 = 76.0\%$ ,  $P < 0.05$ ) with time. The mixing of new and old leaves may have hidden other time effects.

Gas volumes were low in every sample and incubation time. Values obtained by Bruno-Soares *et al.* (1999), in similar studies with legume forages, were about twofold higher at 48 h than the ones reported here (36 vs 18 ml 200 mg<sup>-1</sup> DM).

The results obtained indicate a direct correlation between TP levels, namely CT, and gas production (*c.* G24 h;  $r = 0.91$ ;  $P \leq 0.05$ ), and a lack of any correlation ( $P > 0.05$ ) between crude saponin (CS) and gas production. Consequently, the TP levels (namely CT) in this study could explain ( $P < 0.05$ ) more than 82% of the variation in gas production in each incubation time. This fact contrasts with the known inhibition action of the condensed tannins in gas production, as observed in other feeds.

The TP level of the *C. salvifolius* leaves might not be the true limiting factor in gas production. The generally low gas production may be explained by the presence of tannins – as suggested by the marked PEG effect.

On the other hand, *Cistus* have high saponin levels, some of these may be toxic and may also have synergetic effects with the tannins, these effects leading to a strong decrease in rumen liquor activity.

The significant increase ( $P < 0.01$ ) of gas production caused by addition of PEG to the leaves was greater in the first date samples and in the initial hours of incubation. This fact is probably due to the presence of tannins or other secondary metabolites present in *C. salvifolius* leaves.



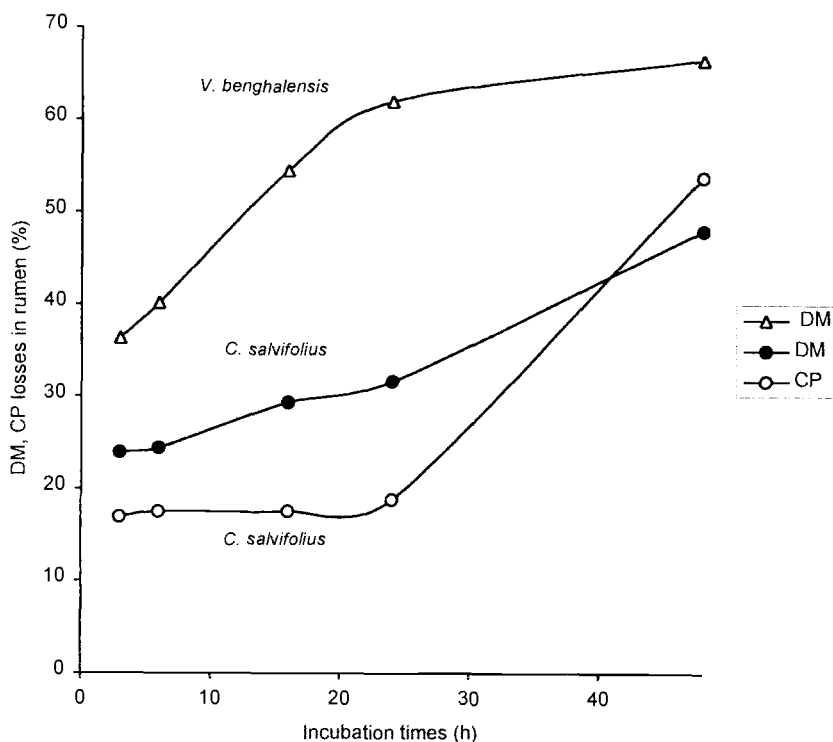


Fig. 7.1. Losses in rumen of *Vicia benghalensis* and *Cistus salvifolius* leaves.

DM and CP losses in the rumen were also low for all of the samples. Working with vetch leaves, Bruno-Soares (1996) obtained approximately twice the losses in rumen obtained here with *C. salvifolius* leaves (Fig. 7.1).

The degradation kinetics of vetch leaves showed a typical pattern, with a good fit to a non-linear model (RSD < 1.9). With *C. salvifolius* leaves the degradation kinetics of DM and CP follow an untypical pattern, which makes it impossible to fit one of the commonly used non-linear models to the data.

## Conclusion

CP, NDF and GE contents assessed in *C. salvifolius* leaves are similar to those observed in some common ruminant feeds, namely in legume forages. However, the very low gas production and very low DM losses in the rumen suggest that these leaves have a very low nutritive value.

Epidemiological data strongly suggest that the long-term ingestion of *C. salvifolius* leaves as the sole feed available to sheep, during periods of forage scarcity in extensive production systems, may cause metabolic disorders in sheep. We are still unable to confirm and explain this cause-effect relationship. We might hypothesize that the rumen microbes do not detoxify this feed.

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## Chapter 8

# Fusarochromanone and Wortmannin: Novel *Fusarium* Toxins

W.L. Bryden<sup>1,3</sup>, M. Lowe<sup>1</sup>, T.M. Amba<sup>1</sup> and H.K. Abbas<sup>2</sup>

<sup>1</sup>Faculty of Veterinary Science, University of Sydney, Camden, NSW 2570, Australia; <sup>2</sup>USDA, ARS, Crop Genetics and Production Research Unit, Stoneville MS 38776, USA; <sup>3</sup>Present address for correspondence: School of Animal Studies, University of Queensland, Gatton Qld 4343, Australia

The fungal genus *Fusarium* produces a number of agriculturally important mycotoxins, namely zearalenone (Hagler *et al.*, 2001), fumonisins (Marasas *et al.*, 2001) and the tricothecene, deoxynivalenol (Miller *et al.*, 2001). In addition to these economically significant toxins the fungi produce many other toxins that are not widespread in nature but have unique biological effects. Fusarochromanone and wortmannin are novel *Fusarium* mycotoxins which perturb distinct physiological systems (Bryden *et al.*, 2001).

### Fusarochromanone

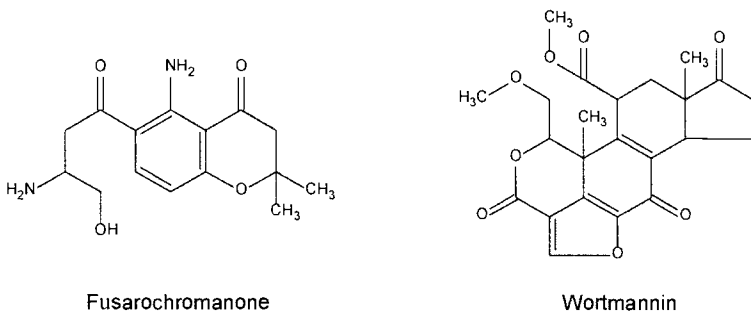
Fusarochromanone (Fig. 8.1) is named to reflect its *Fusarium* origin and chromanone ring structure. This toxin is unique as it is the first reported naturally occurring chromanone derivative (Pathre *et al.*, 1986) and is produced by *Fusarium equiseti*. Fusarochromanone synthesis by *F. equiseti* is rare. Wu *et al.* (1990) detected its production by only three of 62 *F. equiseti* isolates taken from a wide range of geographic locations. These include *F. equiseti* isolates from Germany, Alaska and Denmark but not from other *Fusarium* species nor from other *F. equiseti* isolates taken from different geographic areas (Wing *et al.*, 1993). Fifteen strains of *F. equiseti* capable of producing fusarochromanone are listed in the International Toxic *Fusarium* Reference Collection (Marasas *et al.*, 1984) and these include the 'Alaska 2-2' strain from which fusarochromanone was isolated (Walser *et al.*, 1980). It is unusual for so few isolates of a species to produce a given toxin. Some of the other toxins produced by *F. equiseti* include butenolide, moniliformin, tricothecenes, equisetin and zearalenone (Thrane, 1989).

There have been no systematic surveys to determine the natural occurrence of fusarochromanone in feeds and feedstuffs. It has been reported in Danish poultry feed associated with field cases of tibial dyschondroplasia (TD) in broiler chickens. The main clinical feature of fusarochromanone intoxication is its

capacity to increase the incidence of avian TD, which is an abnormality of bone growth plates in which there is an accumulation of uncalcified and avascular cartilage in the proximal head of the tibiotarsal bone. This toxin has also been shown to induce chicken embryonic mortality and thereby decrease hatchability of fertile chicken eggs (Mirocha *et al.*, 1985) without effect on egg production or egg weight (Lee *et al.*, 1985a). It has been suggested that fusarochromanone might be involved in the aetiology of the human bone disorder, Kashin-Beck disease (Lee *et al.*, 1985b), however, this is unlikely as the human disorder has fundamental pathological differences from TD (Wright *et al.*, 1987; Sokoloff, 1989).

Several studies indicate that fusarochromanone has some influence on immune function, particularly the suppression of humoral immunity. However, immunosuppression does not always occur with the induction of TD (Wu *et al.*, 1993). Immunosuppression by fusarochromanone has been demonstrated to occur at levels below which there were no changes in growth and feed intake. No adverse effect on bodyweight or feed consumption were observed when the concentration of the toxin in the feed caused severe reduction in antibody titres (Chu *et al.*, 1988). Fusarochromanone has also been demonstrated to have immunostimulatory activities on bovine lymphocytes at low doses (Minervini *et al.*, 1992).

Despite many years of study and the delineation of the factors that influence the incidence of TD, the cause of TD remains unknown. A reproducible model of TD has been developed with fusarochromanone (Walser *et al.*, 1982; Bryden and Lowe, 2000). Using this experimental model it was unequivocally demonstrated that genetic predisposition is the major factor in determining the incidence of the disease. Some strains of broiler chickens are more susceptible to toxic insult by the mycotoxin and exhibit a higher incidence of the bone abnormality (Amba and Bryden, 1997). Of the nutrients examined (sulphur-containing amino acids, calcium, phosphorus) in the fusarochromanone model, only dietary vitamin D (Lowe *et al.*, 1998; Bryden and Lowe, 2000) varied the incidence of the disease. The mechanism of the interaction remains to be elucidated.



**Fig. 8.1.** Chemical structure of fusarochromanone and wortmannin.

## Wortmannin

Wortmannin (Fig. 8.1) was originally isolated in 1955 by Brian *et al.* (1957) from *Penicillium wortmanni* and subsequently from *Myrothecium roridum* (Petcher *et al.*, 1972). It was found to be an antifungal antibiotic, inhibiting a variety of fungi including *Fusarium graminearum* (Brian *et al.*, 1957) but it is not antibacterial. Some three decades later isolates of *Fusarium oxysporum* and *Fusarium sambucinum* collected from the Arctic regions of Norway were found to cause haemorrhage in rat feeding tests (Abbas *et al.*, 1987, 1989) and the active compound was isolated and called haemorrhagic factor or H-1. Later, the toxin was found in *Fusarium* cultures obtained from New Zealand and Alaska (Bosch *et al.*, 1989; Abbas *et al.*, 1991) and shown to be wortmannin (Abbas and Mirocha, 1988). Recent taxonomic studies of *F. sambucinum* have shown that this complex contains two other species, *Fusarium torulosum* and *Fusarium venenatum* (for review see Thrane, 2001). Secondary metabolite profiling of these species and *Fusarium avenaceum* and *F. oxysporum*, also reported to produce wortmannin, has allowed Thrane and Hansen (1995) and Thrane (2001) to conclude that all wortmannin producers in *Fusarium* should be identified as *F. torulosum*. Changes in taxonomy make it difficult to reliably correlate earlier literature and can cause considerable confusion in *Fusarium* mycotoxicology (Marasas, 1986; Desjardins and Proctor, 2001).

Pathological studies of wortmannin in various laboratory animals showed that rats and mice were more sensitive to the toxin than guinea pigs (Gunther *et al.*, 1989a). Wortmannin caused haemorrhage in the urinary bladder, gastrointestinal tract, thymus and myocardium of rats (Gunther *et al.*, 1989a; Abbas *et al.*, 1992). Haemorrhage of various organs has also been observed in poultry (Wu and Mirocha, 1992). There are a number of unresolved field reports of haemorrhagic mycotoxicoses and it has been postulated that wortmannin was involved in the aetiology of a human mycotoxicosis, alimentary toxic aleukia, in the USSR in the 1940s and also contributed to haemorrhagic syndromes in farm animals (Joffe, 1986; Mirocha and Abbas, 1989).

Wortmannin induces immune alterations in parameters such as thymus weight, serum IgG and *in vitro* mitogen stimulation (Gunther *et al.*, 1989b) and 11-desacetoxy-wortmannin is a highly active anti-inflammatory agent in rats (Wiesinger *et al.*, 1974). However, use of wortmannin and its analogues in this regard has been limited by their mammalian toxicity (Closse *et al.*, 1981; Baggiolini *et al.*, 1987; Abbas and Mirocha, 1988). Mammalian cytotoxicity was high when four cultured mammalian cell lines were tested for wortmannin cytotoxicity (H.K. Abbas, unpublished results). Wortmannin can induce apoptosis and does so by disrupting signalling pathways mediated by activation of phosphatidylinositol 3-kinase (Riley, 1998). The ability of the toxin to inhibit this signalling pathway has been used as an approach to studying insulin action and glucose homeostasis.

In plants, wortmannin is very active and causes complete inhibition of POOH-terminal pro-peptide-mediated transport to the vacuoles in tobacco cells

(Matsuoka *et al.*, 1995). Recently, wortmannin was tested for its phytotoxicity on duckweed (*Lemna pausicostata* L.), a small aquatic plant, as described by Tanaka *et al.* (1993). Wortmannin caused dramatic phytotoxic effects in this bioassay, including conductivity increase, growth inhibition and chlorophyll loss (H.K. Abbas, unpublished results). Mammalian toxicity limits wortmannin's use as a herbicide, although it is possible that suitable analogues could be useful in weed control.

## Conclusion

Fusarochromanone is produced by only a few isolates of *F. equiseti* and while there is some change in the identification of the *Fusarium* species that produce wortmannin, species of at least two other fungal genera produce this secondary metabolite. These observations suggest very different roles for both compounds in fungal ecology and pathology but little is known of their respective mycological importance.

The association of these mycotoxins with overt human and animal disease has not been clearly defined. The novel biological effects of both toxins suggest, however, that they could exert considerable subtle effects if consumed in low doses in foods or feeds. Their possible interaction with other mycotoxins produced by *Fusarium* awaits investigation.

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## Chapter 9

# Improved Methods for the Extraction and Purification of Corynetoxins for Preparative or Analytical Purposes using Water Solubilizing Additives

N. Anderton, Y. Cao, S.M. Colegate\*, J.A. Edgar, A.M. Knill, A. Michalewicz, V. Olsen and K.A. Than

*Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Victoria 3220, Australia*

\* Author to whom correspondence should be addressed.

Corynetoxins (CTs) are a family of tunicaminyuracil-glycolipids (Fig. 9.1) produced by a bacterium (*Rathayibacter toxicus*) that colonizes nematode (*Anguina* spp.) galls in the seed-heads of various grasses (Edgar *et al.*, 1982). The CTs cause Annual Ryegrass Toxicity, Stewart's Range Syndrome and Floodplain staggers when the bacterium/nematode complex is associated with *Lolium rigidum* (annual ryegrass), *Polypogon monspeliensis* (annual beardgrass) and *Agrostis avenacea* (blowngrass) respectively (Bryden *et al.*, 1994).

The amipathic properties of the CTs, resulting from the hydrophilic tunicaminyuracil moiety and the lipophilic fatty acid side chain, and the limited solvent solubility were first noted in early studies on the cause of Annual Ryegrass Toxicity (ARGT) (Vogel *et al.*, 1981).

In view of the very poisonous nature of these natural toxins and their potential to contaminate animal and human food supplies (Colegate *et al.*, 1998), efficient extraction and purification protocols to provide CTs for toxicological studies and analytical purposes are essential. The CSIRO Plant Toxins Research Group has developed methods for the isolation, identification and sensitive quantitation of these compounds and these HPLC, LCMS and ELISA procedures are constantly being improved. The use of aqueous methanol solutions (20-40% water in methanol, v/v) has proved useful in the extraction of CTs for laboratory purposes but is undesirable (cost and safety concerns) for wide-scale screening of samples for CTs and is not appropriate for the concurrent ELISA measurement of the presence of the CT-producing bacterium.

The application of a quaternary ammonium phase transfer reagent, with similar solubility properties to the CTs, and cyclodextrins has improved the protocols for the isolation and analysis of corynetoxins respectively by rendering the corynetoxins soluble in water. Thus, safety issues associated with methanol are avoided and the 100% aqueous solution is suitable for simultaneous extraction

of the CT-producing bacterium antigen, thereby allowing analysis of the same extracts for bacterium and CT.

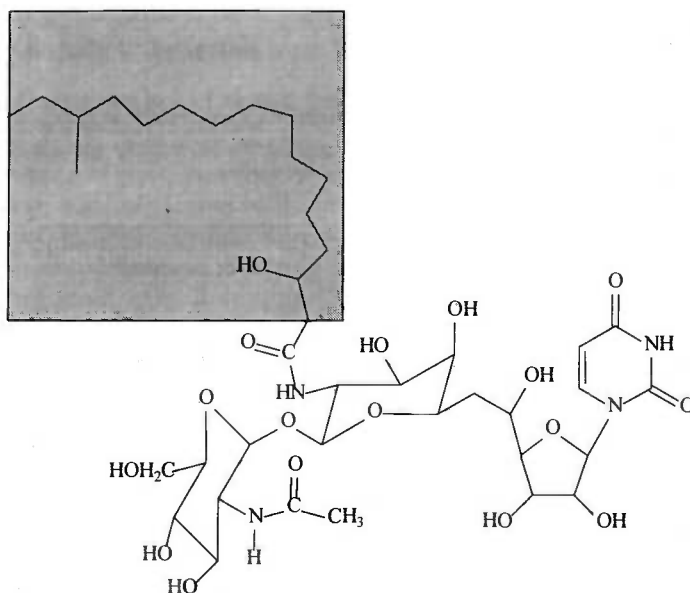
## Analytical Scale Extraction of Corynetoxins

Previous research (Stewart *et al.*, 1998) on developing therapeutic or prophylactic treatments for ARGV, identified cyclodextrins with strong affinities for the CTs. The affinity arises from an interaction of the lipophilic discriminator of the CTs with the hydrophobic, annular centre of the cyclodextrin cone (Fig. 9.2).

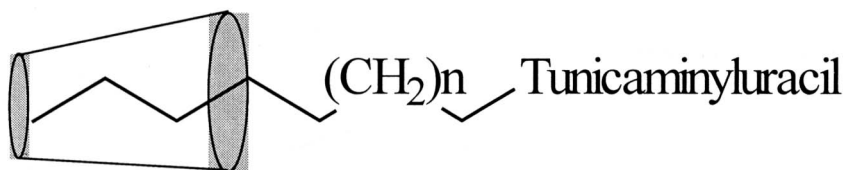
Aqueous solutions of various cyclodextrins were compared with the aqueous methanol (80% of methanol in water, v/v) benchmark for efficiency in extracting CTs from bacterial galls collected from annual ryegrass seed heads.

The results (Table 9.1) clearly show that, for a single extraction of 12 uncrushed galls in 1 ml of solvent, 2% methyl- $\beta$ -cyclodextrin in water (w/v) and 80% methanol in water (v/v) have similar efficacy in extracting CTs from bacterial galls (Fig. 9.3).

The efficiency of extraction was measured using HPLC, estimating CT content of extracts as previously described (Cockrum and Edgar, 1985).



**Fig. 9.1.** Structure of corynetoxin H17a, a major component in the series of approximately 16 corynetoxins that differ only in the aliphatic side chain (shaded area) (Cockrum and Edgar, 1985).



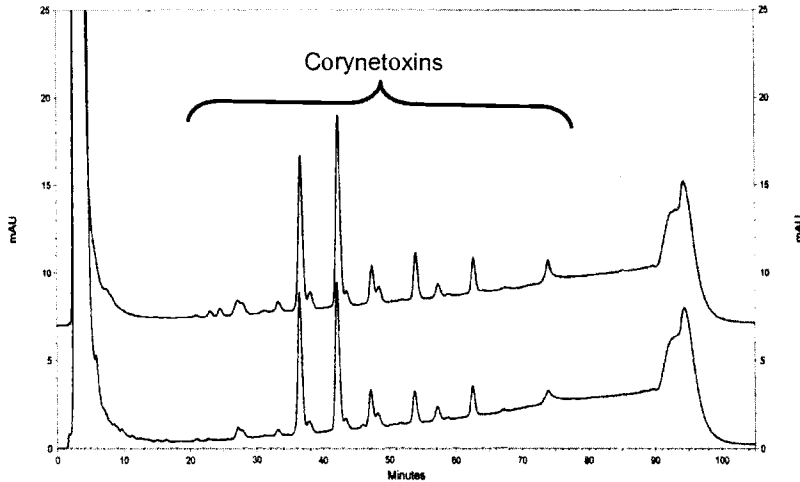
**Fig. 9.2.** Schematic diagram showing association of corynetoxins with cyclodextrins.

Not only has the use of aqueous cyclodextrins proved useful for the HPLC analysis of CTs, the ELISA method of detecting and quantitating CTs has also been optimized for use with the cyclodextrin extracts (Than *et al.*, Chapter 59 this volume and unpublished, 2001). For application with the CT ELISA, a 1% (w/v) solution of methyl- $\beta$ -cyclodextrin in water was found to be almost as effective as the 2% solution in extraction efficiency but was more compatible with the ELISA and less expensive for wide-scale screening of samples.

In addition, the use of aqueous cyclodextrin as a sample solvent has also been shown to be amenable to the detection of the bacterium-derived antigen in the ELISA screening method for presence of the bacterium that produces the CTs in grain and fodder samples (Andrew Gregory, Department of Agriculture, Western Australia, personal communication).

**Table 9.1.** Corynetoxin extraction (12 uncrushed galls in 1 ml of solvent) efficiency of various solvent systems as measured by HPLC. Maximum recovery estimated by exhaustive extraction of residual CTs with 80% methanol in water after crushing the galls.

Solvent	Single extraction efficiency (% of maximum)
Methanol	5
Water	0
0.05M sodium hydroxide	8
80% methanol:water (v/v)	70
0.5% hydroxypropyl- $\beta$ -cyclodextrin	27
1.0% hydroxypropyl- $\beta$ -cyclodextrin	39
2.0% hydroxypropyl- $\beta$ -cyclodextrin	48
5.0% hydroxypropyl- $\beta$ -cyclodextrin	66
0.5% methyl- $\beta$ -cyclodextrin	45
1% methyl- $\beta$ -cyclodextrin	59
2% methyl- $\beta$ -cyclodextrin	73
2% $\beta$ -cyclodextrin	40
2% $\alpha$ -cyclodextrin	32



**Fig. 9.3.** HPLC trace of extract of 12 bacterium-colonized galls with: (Top) 80% methanol in water (v/v), and (Bottom) 2% methyl- $\beta$ -cyclodextrin in water (w/v) showing the similarities in qualitative and quantitative extraction.

## Large-scale Extraction and Purification

A toxicological study of CTs and the chemically- and toxicologically-related tunicamycins, recently commenced by the Plant Toxins Research Group, requires the isolation of gram quantities of pure CTs as their naturally occurring mixture.

Large-scale extractions of the infected ryegrass (15 kg) using methanol/water mixtures (80% methanol in water, 400 l) followed by evaporation of the solvent under reduced pressure, routinely deliver the desired CTs as a mixture with a purity of about 0.1%. This crude CT extract is then treated at room temperature with aqueous sodium hydroxide (0.5 M) to hydrolyse the co-extracting and interfering glycerides. Acid-induced precipitation of the CTs provided a crude CT mixture, which previously was then subjected to large scale, reverse phase silica column chromatography and subsequent preparative HPLC. This procedure was time consuming, inefficient and expensive.

The improved protocol involves solvent partitioning of the base-treated, acid-precipitated crude CT extract with ethyl acetate. The solid that consequently formed at the water/ethyl acetate interface was removed and shown by HPLC to comprise 10-15% CTs. The enriched CT mixture was then extracted with an aqueous solution of cetyltrimethylammonium bromide, a phase-transfer catalyst having similar solubility properties to those of the CTs. A very selective extraction was observed, improving the CT content of the extract to 65-70% with minimal effort. Following lyophilization of the phase transfer reagent/CT solution, the phase transfer reagent was removed by washing the dried residue

with dichloromethane. The CT-rich residue, cleaned of the cetyltrimethylammonium bromide, was then easily purified using either reverse phase HPLC or dry column chromatography to yield CTs with a purity > 95%.

## Summary

- One per cent and 2% (w/v) solutions of methyl- $\beta$ -cyclodextrin in water have proven to be useful solvents, comparable to the 80% methanol in water benchmark, for extracting CTs from bacterium-infected nematode galls in annual ryegrass seed heads.
- The aqueous cyclodextrin CT extracts are suitable for analysis by HPLC and ELISA for the presence of CTs.
- The aqueous cyclodextrin CT extracts are also suitable for determining the presence of the CT-producing bacterium since the bacterium-specific antigen recognized by the ELISA is water soluble (Andrew Gregory, Department of Agriculture, Western Australia, personal communication).
- The use of an ampipathic quaternary ammonium bromide provides a simple, efficient, safe and relatively inexpensive method to rapidly enhance the purity of crude CT extracts.
- Toxic and flammable methanol has been replaced, in assays and extraction protocols for CTs, by non-flammable, aqueous solutions of cyclodextrin and cetyltrimethylammonium bromide, both registered for human use.
- The use of the commercially available and inexpensive cyclodextrins and/or quaternary ammonium phase transfer reagents could have value in the extraction and purification of other lipophilic or ampipathic natural toxins.

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## Chapter 10

# Nephrotic Syndrome in Cattle Caused by the Shrub *Nolletia gariepina* in the Kalahari Sandveld of Southern Africa

E.C. du Plessis<sup>1</sup>, J.P.J. Joubert<sup>2</sup>, L. Prozesky<sup>1</sup>, T.W. Naudé<sup>3</sup>, P. Herman<sup>4</sup> and G.B.M. van der Westhuizen<sup>5</sup>

<sup>1</sup>Pathology Section, Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110, South Africa; <sup>2</sup>Toxicology Section, Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110, South Africa; <sup>3</sup>University of Pretoria, Pretoria, 0001, South Africa; <sup>4</sup>Botanical Research Institute, Pretoria, South Africa; <sup>5</sup>Private Practitioner, Kuruman, South Africa

### Introduction

A sudden 18% mortality occurred in a herd of range beef cattle in excellent condition at the beginning of the winter in May/June 2000 on two adjoining farms in the Kalahari sandveld of southern Africa. More than 200 animals died with signs of extensive renal failure from 3 days to 3 weeks after suspected exposure. Investigations indicated a nephrotoxic aetiology, but none of the known nephrotoxic plants or agents could be incriminated (Kellerman *et al.*, 1988). A small perennial shrub, identified as *Nolletia gariepina* (Asteraceae) grew extensively in the camps where the affected animals had grazed and was one of the dominant shrubs that had been eaten. According to the stockman on the second farm, the cattle immediately started to graze on *N. gariepina* shrubs as soon as they were released on to the grazing. Feeding of the shrubs to a steer under experimental conditions caused the same nephrotic syndrome clinically, as was observed in the field cases. Post-mortem and histopathological lesions were identical to those found in the field cases. Dosing of the shrubs via rumen fistule to two sheep caused severe depression, abdominal pain and anorexia, and on post-mortem histopathological examination, the lesions were similar to the field cases. Guinea pigs were also dosed with material from the shrub, and histological lesions found in the kidneys of affected guinea pigs resembled those from the field cases as well as the other experimental animals. This is the first confirmed report of toxicity due to the ingestion of this shrub.

## **Plant Description**

*Nolletia gariepina* is a much-branched perennial dwarf shrub, densely covered with fine hairs. The leaves are alternate and thin, and the flowers appear to consist only of yellow disc florets, the ray florets being severely reduced. Flowers can be present all year round. It grows in Namibia and the Northern Cape Province, which are the sandy, dryer parts of southern Africa.

## **Clinical Signs**

Almost 200 grazing animals from weaner calves to adult cattle were affected. Anorexia, depression, weakness and a variable amount of subcutaneous oedema mainly in the perineal region were noted. The duration of illness was a few days in most animals, and the mortality rate was high.

## **Necropsy lesions**

Severe ascites, sometimes accompanied by hydrothorax, were seen in association with severe perirenal oedema. The renal cortices were pale, indicating nephrosis.

## **Histopathological lesions**

Severe tubular degeneration and necrosis occurred in the renal cortices with formation of numerous protein casts in the tubular lumens. Calcification of scattered tubular structures was also noted, especially in the cortico-medullary region. In more chronic cases tubular epithelium regeneration could be found.

## **Discussion**

Although *N. gariepina* plants were distributed very widely in the Northern Cape Province during the period of the outbreaks, confirmed deaths only occurred on the two adjacent farms. According to the epidemiological data gathered, the syndrome only appeared after the cattle had been mustered for pregnancy examination and held in kraals (pens) for 24-48 hours with water, but without food. This suggests that, upon release on to the grazing, the starved animals must have ingested large amounts of the shrub before toxicity occurred, as no adverse effects occurred in animals grazing the same camps under normal conditions.

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## Chapter 11

# The Concentration of Steroidal Saponins in and the Degree of Fungal Infection on *Narthecium ossifragum* Plants in Møre and Romsdal County, Norway

A. Flåøyen<sup>1,2</sup>, A.L. Wilkins<sup>3</sup>, M.E. di Menna<sup>4</sup> and M. Sandvik<sup>1</sup>

<sup>1</sup>National Veterinary Institute, PO Box 8156 Dep., 0033 Oslo, Norway;

<sup>2</sup>Department of Large Animal Clinical Sciences, Norwegian School of Veterinary Science, PO Box 8146 Dep., 0033 Oslo, Norway; <sup>3</sup>Chemistry Department, University of Waikato, Private Bag 3105, Hamilton, New Zealand; <sup>4</sup>New Zealand Pastoral Agriculture Research Institute Ltd, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand

### Introduction

*Narthecium ossifragum* (Eng.: Bog asphodel), a member of the lily family, is known to cause alveld, a hepatogenous photosensitization of lambs in Norway, the British Isles and the Faroe Islands (Flåøyen, 1999). Steroidal saponins of the plant have been suggested to cause the liver lesions resulting in retention of the photosensitizing agent phylloerythrin (Flåøyen, 1999).

Steroidal saponins are spirostanol or furostanol glycosides bearing one or more sugar chains, usually one at C-3 and one at C-26 (Osbourn, 1996). The classical definition of saponins is based on their surface activity, for many saponins have detergent properties and give stable foams in water (Hostettmann and Marston, 1995).

Alveld prevalence varies significantly between years and not all *N. ossifragum* pastures seem to be toxic. It has therefore been suggested that the concentration of saponins in the plants may vary between years and sites.

Alveld is more prevalent in cold and rainy summers than in hot and dry summers, and it has been suggested that fungi growing on *N. ossifragum* plants may be involved in the aetiology of alveld because fungal infections are thought to be more common in years with high humidity and much rain. Saponins are part of the defence system of the plant and some saponins are known to have antifungal activity (Osbourn, 1996). It can be hypothesized that there is a relationship between saponin concentration in and the degree of fungal infection on *N. ossifragum* plants and that fungal infections may stimulate the production of saponins or *vice versa*.

The present work was undertaken to determine the variation in saponin concentration in *N. ossifragum* plants growing in an area where alveld is a common disease, and to explore the possible relationship between saponin concentration in, and the degree of fungal infection on, *N. ossifragum* plants.

## Materials and Methods

### Materials collected

Between 21 June and 16 August 1998, five collections of, optimally, 100 g *N. ossifragum* leaves cut at ground level were made at intervals of 2 weeks at five sites in Møre and Romsdal County, Norway (Table 11.1). There were two sub-sites A and B at each site. The A and B site was 50-100 m apart. The plant materials were collected by local sheep farmers, put in paper bags and kept refrigerated for 1 night. One day after collection, the paper bags were enclosed in waterproof envelopes and sent by an overnight service to the National Veterinary Institute, Oslo. The material was deep frozen until wash counts could be made or until extraction for chemical analysis.

### Chemical analysis

#### *Sapogenin extracts*

Portions of the plant material were frozen and freeze-dried for 24 h. Plant material was sequentially extracted in a Soxhlet apparatus for 12 h with dichloromethane and methanol (150 ml of each solvent). The methanol extracts, which contained the plant saponins, were concentrated to dryness using a rotary evaporator and the resulting residue was taken up in 0.5 M HCl (10 ml) and transferred, with repetitive washing (3 x 3 ml of 0.5 M HCl), to a boiling tube which was heated in a water bath for 90 min at 85-90°C. After cooling, the hydrolysed solution was extracted with dichloromethane (4 x 4 ml), the combined extracts were dried over MgSO<sub>4</sub>, filtered through a short silica column (*c.* 3 cm) packed in a Pasteur pipette, evaporated to dryness under a stream of warm nitrogen, and acetylated at room temperature for *c.* 16 h using pyridine-acetic anhydride (1:1) (0.5 ml).

#### *GC-MS analyses*

GC-MS analyses of the acetylated extracts were performed as reported previously by Wilkins *et al.* (1994) except that the pyridine solutions were diluted 1:1 with chloroform and the analyses were performed using a 25 m x 0.2 mm id DB-1 column installed in a Fisons GC8000 gas chromatograph interfaced to a VG Trio 1000 mass spectrometer. Quantification was achieved using *m/z* 139 ion profiles. Sarsasapogenin propionate was utilized as the internal standard. The *m/z* 139 ion

response factor for sarsasapogenin acetate relative to sarsasapogenin propionate was determined to be 1.03. Other sapogenins (smilagenone, sarsasapogenone, smilagenin acetate, epismilagenin acetate, sarsasapogenin acetate and episarsasapogenin acetate) were quantified using a unit response factor, relative to sarsasapogenin propionate.

### Mycological studies

Leaves were shaken with ten times their weight of water in a stoppered container for 2-3 min. Usually 10 g of leaves were used but as little as 6 g when the sample was scant. Fungal elements, conidia and hyphae, in 2 mm<sup>3</sup> of wash water were counted using a haemocytometer slide.

### Results

The results from the saponin analysis of *N. ossifragum* are shown in Table 11.1. The concentrations reported in Table 11.1 are those of steroidal sapogenins found after hydrolysis of parent saponins.

*Cladosporium magnusianum* was the most common fungus infecting the *N. ossifragum* plants. Numbers of fungal elements in leaf washings tended to increase with time (Table 11.2).

Regression analysis revealed that the sapogenin concentration alone explained 43% of the variation in number of conidia found in wash counts of samples collected at the same site 14 days later.

**Table 11.1.** Sites and times of collection of *Nartheceium ossifragum* leaves and the concentrations (mg kg<sup>-1</sup> DM) of steroidal sapogenins (derived from hydrolysis of parent saponins) in the plant materials.

Day	Site <sup>a</sup>									
	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
21.06	1320	1187	* <sup>b</sup>	*	*	*	2129	662	*	*
05.07	1912	2405	1155	*	1505	1144	531	1526	806	1345
19.07	6085	1279	775	1853	2402	*	3865	3044	2004	1248
02.08	2969	2605	2190	4266	4208	3039	4043	3394	3495	3113
16.08	*	3928	3714	4089	1607	2501	7115	2599	5279	3307
Mean	3072	2281	1959	3403	2431	2228	3537	2245	2896	2253
N	4	5	4	3	4	3	5	5	4	4
SD	2122	1122	1314	1345	1251	997	2459	1130	1932	1108

<sup>a</sup>Sites: 1 Valsøybotn, Halså; 2 Vindøldalen, Surnadal; 3 Gurskøy; 4 Frøysadal, Hellesylt; 5 Skinvikdalen, Volda.

<sup>b</sup>\*Sample not available.

**Table 11.2.** Sites and times of collection of *Nartheccium ossifragum* and number of conidia and hyphae found per g leaves in wash counts. The results are given in thousands g<sup>-1</sup>. Samples collected on 21 June were not analysed for fungal elements.

Day		Site <sup>a</sup>									
		1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
05.07	Hyphae	0	15	15	* <sup>b</sup>	0	0	0	0	10	15
	Conidia	0	0	0	*	0	0	0	0	0	0
19.07	Hyphae	45	35	10	45	20	20	15	10	25	10
	Conidia	5	10	10	0	5	15	10	15	0	0
02.08	Hyphae	10	10	20	30	15	0	5	20	0	0
	Conidia	55	5	5	15	5	0	15	5	0	0
16.08	Hyphae	2	2	0	4	10	14	6	6	12	10
	Conidia	12	10	22	6	18	16	14	10	46	34

<sup>a</sup> and <sup>b</sup> as for Table 11.1.

## Discussion

There was a large variation in the saponin concentrations found after hydrolyses of parent saponins. The leaf sample which afforded the highest saponin concentration contained 13 times more saponins than the leaf sample with the lowest saponin concentration.

Our results do not indicate any systematic difference in saponin concentrations between plants harvested at different sites, however, saponin concentrations seemed to increase throughout the season. Sampling of more sites at the same time, in combination with more frequent sampling at each site and sampling of the same sites for several consecutive years may reveal significant differences. The great variation in saponin concentrations between sub-sites show that the local variation in saponin concentrations, and possibly also variations between plants, is significant. Our results are consistent with the proposal that saponins stimulate sporulation in the fungi infecting *N. ossifragum* leaves. We suggest that sporulation may occur in response to increased saponin concentration in order to protect the fungus from the antifungal properties of the saponins.

Assuming that saponins are implicated in the development of alveld in lambs, the large differences in saponin concentration between plants and location may, at least in part, explain the sporadic occurrence of the disease.

Our results indicate that saponin concentrations influence fungal growth rather than the opposite. The reasons why saponin concentrations fluctuate in the plants cannot be elucidated from the results obtained in this study.

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## Chapter 12

# Calcinosis in Ruminants due to Plant Poisoning: Contributions on the Pathogenesis

E.J. Gimeno<sup>1</sup>, E.L. Portiansky<sup>1</sup>, M.S. Gomar<sup>1</sup>, E.F. Costa<sup>1</sup>, A.R. Massone<sup>1</sup>, C.R. Alonso<sup>2</sup>, M.E. Dallorso<sup>3</sup> and S.S. Barros<sup>4</sup>

<sup>1</sup>Institute of Pathology and <sup>2</sup>Department of Anatomy, Faculty of Veterinary Sciences, National University of La Plata, PO Box 296, (1900) La Plata;

<sup>3</sup>Zootecnic Pathology and Hygiene, School of Agricultural Sciences, National University of Lomas de Zamora, Buenos Aires, Argentina; <sup>4</sup>Department of Pathology, Veterinary Faculty, Federal University of Pelotas, PO Box 354, CEP 96010-900 Pelotas, Brazil

### Introduction

Enzootic calcinosis (EC) are chronic plant intoxications of grazing livestock characterized by the calcification of soft tissues and loss of body condition. Different calcinogenic plants have been identified in many countries (Worker and Carrillo, 1967; Morris, 1982; Puche and Bingley, 1995). *Solanum glaucophyllum* (Sg) (synonym: *S. malacoxylon*) and *Nierembergia veitchii* (Nv) cause considerable economic losses from EC in Argentina, Brazil and Uruguay (Worker and Carrillo, 1967; Puche and Bingley, 1995). Calcinogenic plants contain high levels of 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) as glycoside derivatives (Corradino and Wasserman, 1974). In fact, they contain not only 1,25(OH)<sub>2</sub>D<sub>3</sub> but also other metabolites, such as vitamin D<sub>3</sub> and 25(OH)<sub>2</sub>D<sub>3</sub> (Esparza *et al.*, 1982).

The earliest signs in intoxicated animals are stiffening and loss of weight leading to emaciation (Worker and Carrillo, 1967). EC affected animals show metastatic calcification of the vascular system, lungs and other tissues (Puche *et al.*, 1980). Soft tissue mineralization in EC is preceded by fragmentation of elastic fibres, extracellular accumulation of proteoglycans and mesenchymal metaplasia (Barros *et al.*, 1981). As far as the skin is concerned, we have found only occasional reference to the rough and discoloured appearance of the hair coat (Puche and Bingley, 1995).

The effects of the vitamin D involve not only mineral metabolism but also essential and varied processes such as cell differentiation and cell proliferation (Dusso and Brown, 1998). Here, we study the effects of plant-induced hypervitaminosis D on cell differentiation in the skin, aorta and lung.

## Materials and Methods

Samples of skin of experimentally *Sg*-intoxicated and control heifers were studied histologically. Cellular differentiation and proliferation were analysed by immunohistochemical expression of cytokeratins 10 and 11 (clone 8.60, Sigma Chemical Co., USA), involucrin (Novocastra Laboratories Ltd, UK) and proliferating cell nuclear antigen (PCNA)(clone PC10, Sigma Chemical Co.). The results were obtained by image processing and analysis and statistically evaluated. The procedures have been previously described (Gimeno *et al.*, 2000).

The aortas and lungs of sheep with spontaneous EC induced by *Nv* and the same organs of rabbits experimentally poisoned by *Sg* were analysed by using electron microscopy and immunohistochemical techniques. Primary monoclonal antibodies were directed to chondroitin sulphate (clone #CS-56, Sigma Chemical Co.) and to non-collagenous bone matrix proteins: osteocalcin (clone #OC1, Biodesign International, Kennebunk, ME, USA), osteonectin (clone #N50, Biodesign International, Kennebunk, ME, USA) and osteopontin (clone MPIIB10, Developmental Studies Hybridoma Bank, Iowa, USA). Details of materials and methods have been previously published (Barros and Gimeno, 2000; Gomar *et al.*, 2000).

Collagenous and elastic systems present in the aorta of normal cows as well as in bovines experimentally intoxicated with *Sg* were quantitatively analysed. Collagen and reticulin fibres were detected in sections stained with the Picrosirius red technique and viewed with polarized light. For this purpose the sections were deparaffinized, hydrated through graded ethanol and stained for 1 h in a 0.1% solution of Sirius Red (Direct Red 80, Aldrich, Milwaukee, WI, USA) dissolved in aqueous saturated picric acid (Montes, 1996). For the identification of the elastic system fibres, three adjacent sections were stained by one of the following methods: Verhoeff's iodine iron haematoxylin or by Weigert's resorcin-fuchsin, the latter either without any previous treatment or after oxidation with oxone. While Verhoeff's method demonstrates fully mature elastic fibres selectively, elaunin and oxytalan fibres are not stained by this staining procedure; this can be explained by the well known fact that not all biochemically detectable elastin is Verhoeff-positive and, thus, only mature fibres (which contain large amounts of elastin) are demonstrated by Verhoeff's technique. On the other hand, Weigert's resorcin-fuchsin is more sensitive and, thus, also stains elaunin fibres (which contain less elastin). Oxytalan fibres remain unstained unless they are oxidized prior to staining by Weigert's resorcin-fuchsin (oxona method) (Montes, 1996). The procedures are published elsewhere (Portiansky *et al.*, 2002).

## Results

The histological analysis of the skin demonstrated conspicuous differences between *Sg*-poisoned and control animals. The affected heifers showed a highly statistically significant decrease of the epidermal thickness due to a reduction of

cell layers. A qualitative and quantitative atrophy of sebaceous and sweat glands was observed. The hair follicles changed from anagen to telogen phase. The intensity and distribution of the analysed markers of differentiation changed in relation to Sg-poisoning. Involucrin was expressed at the epidermal suprabasal layers in control animals. On the other hand, in Sg-intoxicated heifers, involucrin was heavily stained in all layers of the epithelium. A quantitative evaluation showed an increase in the percentage of epidermal staining in intoxicated cattle in comparison with control animals. A similar change was observed for the expression of cytokeratins 10 and 11. Sg-intoxication also affected the proliferation of keratinocytes. Immunoreactivity to PCNA was profuse in different cells of normal skin, including the epidermal basal cell layer, hair follicles, sebaceous and sweat glands. The skin sections of experimentally intoxicated heifers showed a reduction in the expression of PCNA.

The main ultra-structural alterations observed in aorta and lung of EC affected sheep and rabbits were a modification of smooth muscle cells (SMCs) and the activation of fibroblasts in the interstitium. The modified SMCs showed conspicuous rough endoplasmic reticulum, increased number of mitochondria, free ribosomes and decreased number of myofibres. There was an increase of extracellular matrix and precipitation of calcium in a laminated appearance or in amorphous aggregates. Some macrophages and multinucleated giant cells, with calcium crystals in the cytoplasm, were found in areas of increased matrix. Thickening and reduplication of the basal lamina of capillaries was very prominent. The bone proteins osteocalcin, osteopontin and osteonectin were detected by immunohistochemistry in the cytoplasm of activated fibroblasts, in modified SMCs and in the extracellular matrix. Immunoreactivity to chondroitin sulphate was observed only in the hyaline cartilage of the bronchi.

In aortas, the Picosirius red-polarization method showed the presence of two different collagenous populations: weakly birefringent greenish and thin masses characteristic of reticulin type fibres, contrasting with the strongly birefringent thick yellow to red fascicles, characteristic of collagen fibres. The red appearance indicative of collagen fibres decreased from about 20% present in normal aortas to less than 4% after 8 weeks of intoxication. On the contrary, the green appearance typical of reticulin fibres fluctuated between 2.38% in the control aortas and 1.41% after 8 weeks of intoxication.

We found a decrease in the amount of elastic fibres as a function of the intoxication time. This reduction was significant after 15 days of intoxication when the aortas were stained with the Verhoeff technique. No significant differences with the controls were observed when using the other two staining methods.

## Discussion

The ingestion of calcinogenic plants greatly elevated the levels of plasma  $1,25(\text{OH})_2\text{D}_3$  and consequently an elevation in plasma calcium and phosphate

(Corradino and Wasserman, 1974). Previous works occasionally refer to the rough and discoloured aspect of the hair coat in EC affected animals (Puche and Bingley, 1995). Apparently, histology of the skin has not been previously studied in hypervitaminosis D or in EC. We have found that the intoxication with Sg induced atrophy of the epidermis, hair follicles, sebaceous and sweat glands. The expression of involucrin and cytokeratins 10 and 11 in all the epidermal layers, including the basal cell layer, was expected according to the well known effects of  $1,25(\text{OH})_2\text{D}_3$  (Eckert *et al.*, 1997). Cell division appears to be reduced, although the differences were non-significant. The results seem to indicate that the reduced thickness in the epidermis is not a consequence of blocking of the basal cells, but the result of increased differentiation of suprabasal keratinocytes.

Mesenchymal metaplasia, i.e. the development of cartilage and bone tissue, has been repeatedly reported in EC (Morris, 1982). These changes are preceded by proliferation of mesenchymal cells (Barros *et al.*, 1981). The differentiation of SMCs in the arteries has been reported ultrastructurally and attributed to the action of  $1,25(\text{OH})_2\text{D}_3$ . These cells increased the rough endoplasmic reticulum, and went on to synthesize collagen fibres and extracellular matrix (Barros *et al.*, 1981). We have found that osteocalcin, osteopontin and osteonectin were synthesized in the mesenchymal cells and secreted to the extracellular environment. The expression of these proteins is induced by interaction of  $1,25(\text{OH})_2\text{D}_3$  with specific genes that respond to vitamin D (Dusso and Brown, 1998). Non-collagenous bone matrix proteins play a pivotal role in cell differentiation, cell activation and normal tissue mineralization (Sommer *et al.*, 1996). There are, however, very few references on the participation of these proteins in pathological calcification (Mohler *et al.*, 1997). The expression of non-collagenous matrix proteins by activated fibroblasts or SMCs is partially coincident with membranous ossification (Furusawa *et al.*, 1996). Therefore, the mineralization of soft tissues in EC is a complex process. The observed morphological and biochemical modifications could be attributed to specific genomic effects of  $1,25(\text{OH})_2\text{D}_3$ .

Mineralization in EC is preceded by fragmentation of elastic fibres, accumulation of proteoglycans and mesenchymal metaplasia (Barros *et al.*, 1981). We provide evidence of a decrease in collagen relative to reticulin fibres and a decrease of elastic fibres in relation to the length of the intoxication. Previous studies have shown that  $1,25(\text{OH})_2\text{D}_3$  down regulates type I collagen synthesis (Norman, 1998) and tropoelastin levels decreased after treatment with  $1,25(\text{OH})_2\text{D}_3$  (Hinek *et al.*, 1991). It could be speculated that changes described in EC are induced by high levels of  $1,25(\text{OH})_2\text{D}_3$ . The decrease of collagen and elastin could also be due to the increase of bone matrix proteins and proteoglycans. The present studies demonstrated that cell differentiation plays a pivotal role in the pathogenesis of dermal and mesenchymal lesions in plant-induced EC.

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## Chapter 13

# Evaluation of *Ipomoea carnea* Toxicity to Growing Goats: Clinical, Biochemical, Haematological and Pathological Alterations

B. Schumacher-Henrique, S.L. Górnaiak, M.L.Z. Dagli and H.S. Spinosa

*Research Center for Veterinary Toxicology (CEPTOX), Department of Pathology, School of Veterinary Medicine, University of São Paulo, Brazil*

### Introduction

*Ipomoea carnea*, a shrub from the Convolvulaceae family, has been incriminated as responsible for several outbreaks of livestock poisoning, mainly in goats (Tartour *et al.*, 1974; De Balogh *et al.*, 1999). Swainsonine, an indolizidinic alkaloid, is the most important active principle displayed by *I. carnea*. It promotes cellular accumulation of non-metabolized oligosaccharides due to the inhibition of acid or lysosomal enzyme  $\alpha$ -mannosidase, causing cellular vacuolation. This alkaloid also inhibits Golgi mannosidase II, affecting glycoprotein synthesis, processing and transport, resulting in cellular adhesion dysfunction, in addition to hormonal changes and alterations in some membrane receptors. *Ipomoea carnea* also presents two other alkaloids, calistegeins C1 and B2, which inhibit  $\beta$ -glycosidase (Asano *et al.*, 1995) and because of this may aggravate swainsonine effects.

Animals that consume *I. carnea* exhibit a variety of clinical signs, such as depression, staggering gait, muscle tremor, ataxia and nervousness, mainly when stressed. Haematological parameters are also altered in animals intoxicated by *I. carnea*. Alterations range from subtle changes in the values of haemoglobin and packed cell volume (PCV) (De Balogh *et al.*, 1999) to anaemia and progressive leucopaenia (Tartour *et al.*, 1974; Amir *et al.*, 1987). Biochemical alterations in some renal and hepatic parameters have also been reported: goats intoxicated by *I. carnea* presented increased  $\gamma$ -glutamyltransferase (GGT), creatinine kinase (CK) and blood urea nitrogen (BUN) (Tartour *et al.*, 1973; De Balogh *et al.*, 1999).

Histologically, *I. carnea* toxicosis is mainly characterized by cytoplasmatic vacuolation in central nervous system (CNS), liver and kidney cells, consistent

with lysosomal storage disorders (Damir *et al.*, 1987; Srilatha *et al.*, 1997; De Balogh *et al.*, 1999).

Many trials conducted in different animal species have demonstrated the toxic effects of *I. carnea*. However, no studies have been found in the literature showing the dose-dependent effects of *I. carnea* (or swainsonine) in animals. Therefore, the objective of the present study was to evaluate the degree of *I. carnea* toxicosis when three different doses of fresh leaves of this plant were fed to growing goats.

## Materials and Methods

### Plant material

*Ipomoea carnea* leaves and stems were collected from a culture at the Research Center for Veterinary Toxicology (CEPTOX), School of Veterinary Medicine, University of São Paulo, Brazil. Plant material was collected and shredded immediately before feeding the animals.

### General procedures

Twenty mixed-breed male goats, 4 to 6-months-old, were randomly separated into four equal groups: three experimental and one control. Goats from experimental groups were fed 5, 10, or 30 g of *I. carnea* kg<sup>-1</sup> bodyweight day<sup>-1</sup>, for 4 months. The leaves and stems of *I. carnea* were given between 07:00 and 08:00. Animals were used in accordance with the guidelines of the Committee on Care and Use of Animal Resources of the School of Veterinary Medicine, University of São Paulo; these guidelines are similar to those of the National Research Council, USA. For the whole experimental period, control and *I. carnea* treated animals were fed 100 g concentrate and sugar cane (*Saccharum officinarum*) *ad libitum*, and had free access to drinking water.

Every week, the animals were clinically evaluated, and bodyweights were recorded. Before beginning the trial and then every other week, blood samples were taken from the jugular vein for haemogram evaluation (red and white cell counts, PCV, haemoglobin concentration, haematological indices and differential leukocytes counts) and for determination of serum levels of glucose, cholesterol, alanine transaminase (ALT), aspartate transaminase (AST), GGT, BUN and creatinine serum levels, using commercial kits (Merck®).

At the end of the experimental period, all goats were killed and fragments of brain, spinal cord, a portion of the sciatic nerve, thyroid, myocardium, liver, kidney, spleen, lung, pancreas and intestine were collected. Specimens were fixed in 10% formalin and embedded in paraffin, and 5 µm sections were stained by the haematoxylin and eosin (HE) technique. Brains were sectioned serially, and blocks were prepared from level cuts at the olfactory tubercle and cortex, cerebral



cortex, basal nuclei, thalamus, mesencephalon, pons, cerebellum and medulla oblongata.

Data are reported as mean  $\pm$  SEM and were statistically analysed by one-way analysis of variance, followed by Dunnett's test. Significance level was set at  $P < 0.05$ .

## Results

Clinical signs observed in goats from all experimental groups included nystagmus, muscular tremors, weakness of hind limbs, incoordination, and ataxia. Four animals treated with the highest dose of *I. carnea* and three animals treated with  $10 \text{ g kg}^{-1}$  died or were scarified 'in extremis' during the third month of the experiment. Goats treated with  $30 \text{ g kg}^{-1}$  of *I. carnea* leaves presented significant decrease ( $P < 0.05$ ) in bodyweight gains when compared to controls from the first month and the treated animals from all groups presented significant decrease ( $P < 0.05$ ) in bodyweight gains from the third month (Fig. 13.1).

Goats treated with the different doses of *I. carnea* leaves presented, in general, increased levels of ALT, when compared to the control group (Fig. 13.2). AST levels fluctuated during the trial and creatinine levels were similar in the control and treated groups. The results of haematological parameters (Fig. 13.3) revealed that only the haemoglobin concentration was significantly reduced ( $P < 0.05$ ) in those goats from group  $5 \text{ g kg}^{-1}$  during the last 3 months of the experiment.

Histopathological evaluation revealed degenerative vacuolar alterations of liver, pancreas, thyroid and kidney cells, and in CNS neurons, mainly in the cerebellum. In all experimental goats, however, the severity of these alterations was more prominent in those animals treated with the highest dose of *I. carnea*.

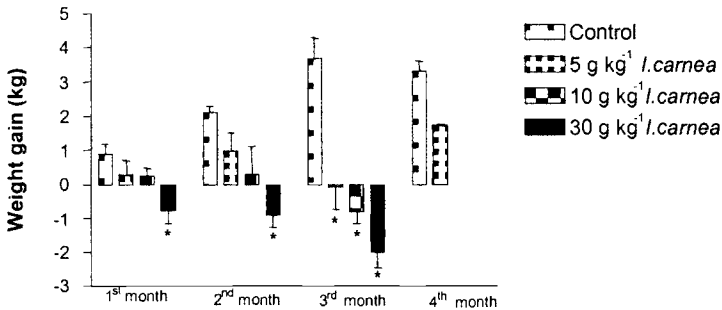


Fig. 13.1. Mensal weight gain (kg) in growing goats treated for 4 months with different doses of leaves of *Ipomoea carnea*. ( $P < 0.05$ , ANOVA followed by the Dunnett's test.)

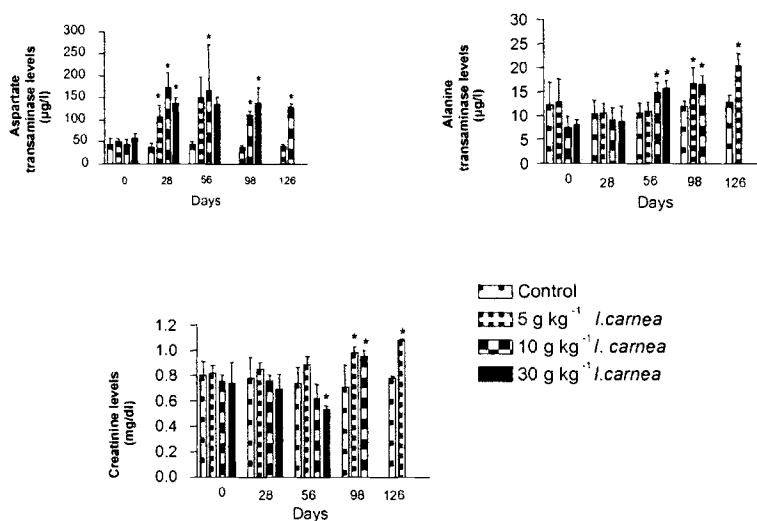


Fig. 13.2. Biochemical parameters (aspartate transaminase, alanine transaminase and creatinine) in growing goats treated for 4 months with different doses of *Ipomoea carnea* leaves. ( $P < 0.05$ , ANOVA followed by the Dunnett's test.)

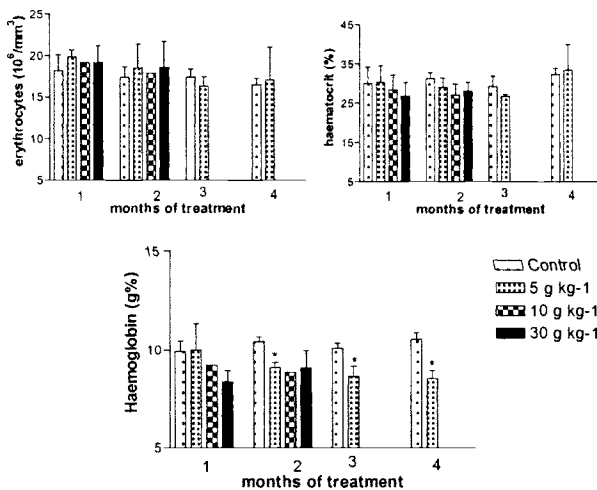


Fig. 13.3. Haematological parameters (erythrocytes, haematocrit, haemoglobin) in growing goats treated for 4 months with different doses of *Ipomoea carnea* leaves. ( $P < 0.05$ , ANOVA followed by the Dunnett's test.)

## Discussion

In a previous study conducted in ruminant species Tokarnia *et al.* (1960) verified that these animals showed addiction to *I. carnea*. In our experiment, this effect was clearly evident and all experimental animals showed avidity to eat the plant during the entire experimental period. On the other hand, De Balogh (1999) reported exactly the opposite behaviour, that is in that study, all experimental goats showed great aversion to *I. carnea*. Little can be hypothesized about this controversy at this moment. However, it should be considered that both Tokarnia's and the present study were performed using Brazilian *I. carnea*, unlike the trial conducted by De Balogh using *I. carnea* from Mozambique. Thus, it is possible to suppose that *I. carnea* from Africa and Brazil may have some differences in relation to the presence of secondary metabolites, which can lead to feed avidity or feed aversion in the goats.

Bodyweight is one of the most important parameters for toxicological evaluations in order to determine the often precocious onset of toxicological effects of a given substance in animals (Stevens and Gallo, 1989). In fact, earlier trials have shown that one of the earliest signs of *I. carnea* toxicity is weight loss (Tokarnia *et al.*, 1960; De Balogh *et al.*, 1999). In the present study this effect was also well demonstrated in all experimental animals. As the *I. carnea*-fed goats did not show any deviation from their nutritional habits, it may be assumed that weight gain impairment was not linked to the possible anorexic effect of the plant, but probably to changes in oligosaccharide metabolism.

Among the clinical signs and symptoms displayed by goats, neurological ones are the most prominent (Tokarnia *et al.*, 1960; Tartour *et al.*, 1974; Tirkey *et al.*, 1987; Damir *et al.*, 1987; Srilatha *et al.*, 1997; De Balogh *et al.*, 1999). The results of the present trial are in agreement with those of previous reports; all experimental goats presented headshakes, nystagmus, opisthotonus, fasciculations and muscular tremors, weakness of the hind limbs, incoordination and ataxia. These alterations reflect CNS lesions. In fact, the histopathological study revealed typical alterations related to neuronal storage disease.

Toxic effects of *I. carnea* have been reported to occur in kidney and liver (Damir *et al.*, 1987; Srilatha *et al.*, 1997). Again, the results of the present trial were in agreement with these previous studies showing degenerative process in those organs. In addition, the biochemical evaluation corroborates the histological findings.

According to Damir *et al.* (1987) one of the most conspicuous effects of *I. carnea* toxicity in goats is the development of the normocytic normochromic anaemia. In our study, this alteration was also observed. Thus, all experimental goats showed haematological changes from the third month of plant intake and this pathological alteration occurred in a dose-dependent manner. This clinical finding may be important in the diagnosis of *I. carnea* intoxication.

In conclusion, prolonged ingestion of *I. carnea* produces intoxication in goats and this toxicity is mainly manifested by neurological disorders. Signs of intoxication occur in a dose-dependent manner.

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## Chapter 14

# Chemical and Pharmacological Investigation of Macrocyclic Diterpenoids Isolated from *Euphorbia* Species

J. Hohmann<sup>1</sup>, D. Rédei<sup>1</sup>, I. Máthé<sup>1</sup>, P. Forgó<sup>2</sup>, G. Blazsó<sup>3</sup>, G. Falkay<sup>3</sup>, J. Molnár<sup>4</sup>, K. Wolfard<sup>4</sup>, A. Molnár<sup>4</sup> and T. Thalhammer<sup>5</sup>

<sup>1</sup>Department of Pharmacognosy, <sup>2</sup>Department of Organic Chemistry,

<sup>3</sup>Department of Pharmacodynamics and Biopharmacy, <sup>4</sup>Department of Medical Microbiology, University of Szeged, H-6720, Szeged, Hungary; <sup>5</sup>Department of Pathophysiology, University of Vienna, A-1090 Vienna, Austria

### Introduction

The genus *Euphorbia* is characterized by the occurrence of highly irritant and frequently tumour-promoting latex causing many toxicological problems to humans and animals. Various diterpene type toxins based on a tiglane and ingenane skeleton, referred to as 'phorboids', were isolated from *Euphorbia* species and identified as responsible for these effects (Evans and Taylor, 1983). Besides the presence of the well-known ingenanes and tiglianes, the genus also produces a high diversity of macrocyclic diterpenoids, which are regarded as precursors of polycyclic derivatives and occur in plants generally as complex mixtures in low concentration (Jakupovic *et al.*, 1998; Appendino *et al.*, 1998, 1999; Abbas *et al.*, 2000). Among these jatrophanes and lathyranes are the pre-eminent in terms of distribution, biogenetic relevance and structural complexity (Fig. 14.1).

Because of the presence of the toxic phorboids, the use of *Euphorbia* species in traditional medicine was very restricted, and phytochemical, pharmacological studies on this genus focused mainly on the irritant phorboids. Thus, the chemical behaviour and biological potential of the macrocyclic compounds remained for a long time to be unknown.

The aim of our work was the isolation, chemical characterization and pharmacological evaluation of the macrocyclic types of diterpenoids. Our investigations were performed on *Euphorbia esula* L., *E. salicifolia* Host. and *E. serrulata* Thuill., which are found in Europe mostly as weeds.

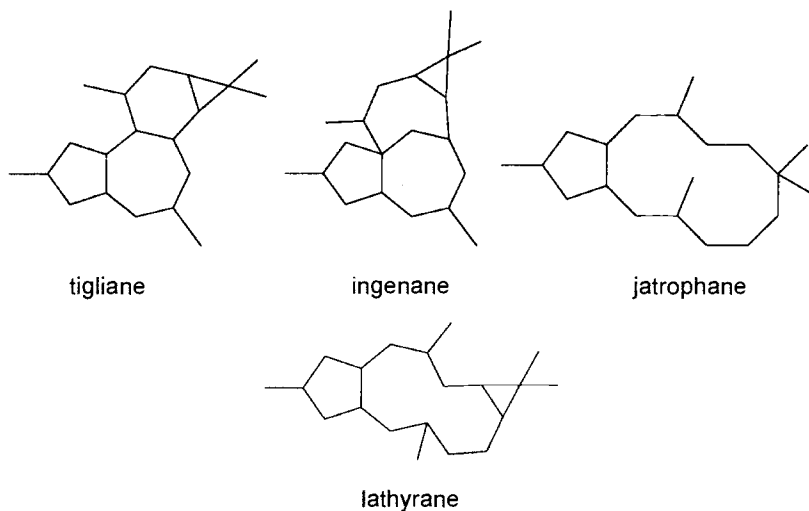


Fig. 14.1. Structural types of diterpenes from Euphorbiaceae.

## Results and Discussion

The whole undried plants of *E. esula*, *E. salicifolia* and *E. serrulata* were percolated with MeOH at room temperature. The extracts were subjected to solvent partitioning to furnish dichloromethane- and water-soluble fractions. The dichloromethane extracts were chromatographed on a polyamide open column with various MeOH–H<sub>2</sub>O mixtures as eluents. The fractions obtained with MeOH–H<sub>2</sub>O (3:2) were separated by vacuum liquid chromatography on Si gel and by NP- and RP-HPLC to afford compounds 1-4 from *E. esula*, 1 and 5 from *E. salicifolia* and 6-8 from *E. serrulata* as detailed in references Hohmann *et al.* (1997, 2000, 2001).

Structure determination was carried out using mass spectroscopy, high field NMR techniques including <sup>1</sup>H, <sup>13</sup>C, <sup>1</sup>H-<sup>1</sup>H COSY, JMOD, HSQC, HMBC and NOESY experiments and X-ray analysis. The elucidation of the structure of the compounds is detailed in references Hohmann *et al.* (1997, 2000, 2001). Spectral analyses revealed that compounds 1-8 are new members of the group of jatrophane diterpenes with exception of 5, which is based on a modified 5-13 fused jatrophane skeleton. All compounds are highly functionalized with acetyl, benzoyl, isobutanoyl and nicotinoyl groups (Fig. 14.2).

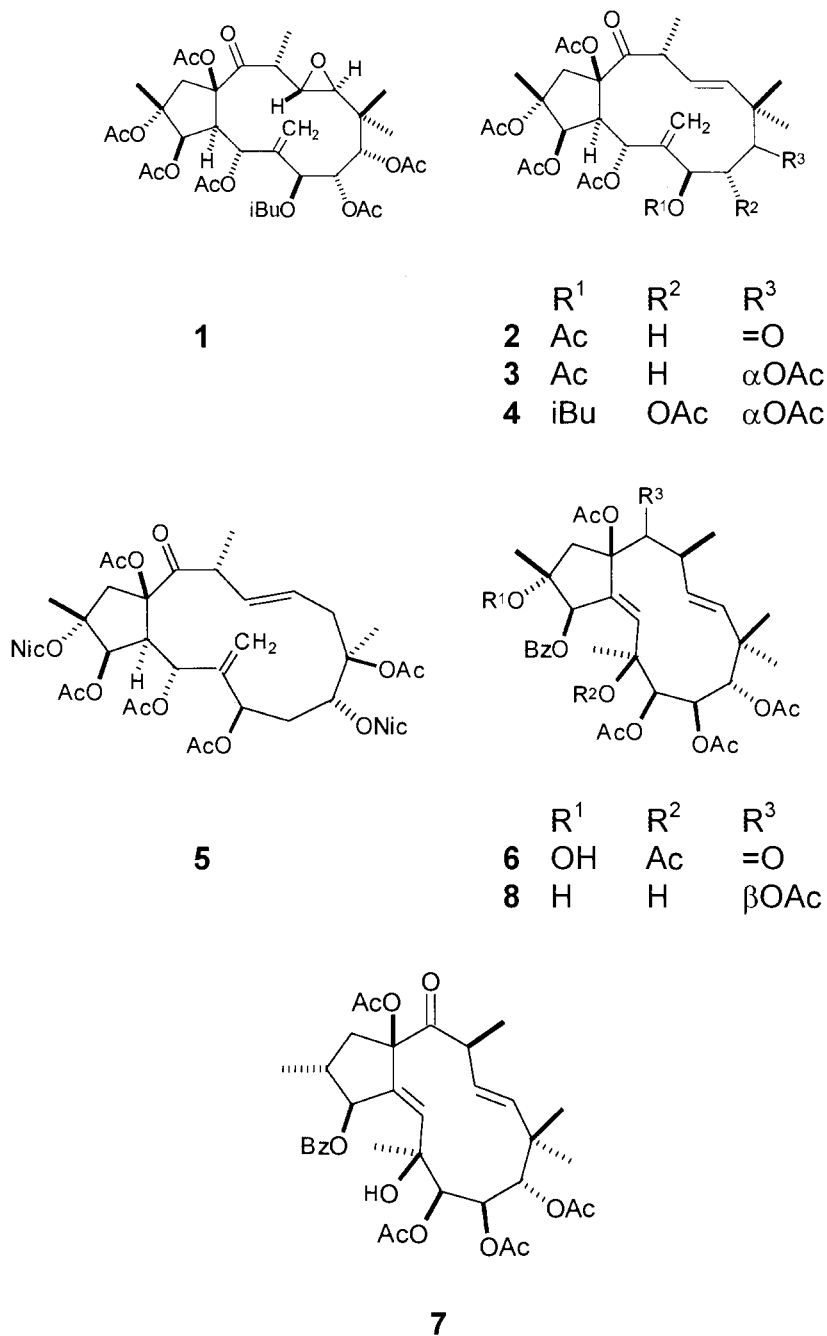


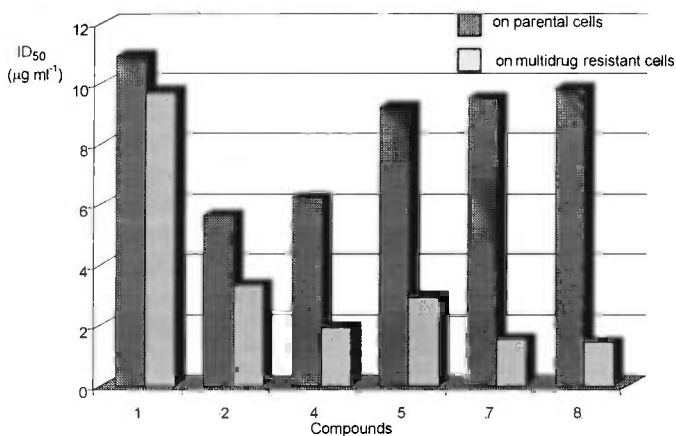
Fig. 14.2. Chemical structure of compounds 1–8.

The crude dichloromethane extracts and the pure compounds were subjected to pro-inflammatory assay (Table 14.1) (Hecker *et al.*, 1966). All of the tested extracts exhibited mouse ear irritant activity with  $ID_{50}^{4h}$  values of 56–325  $\mu\text{g}$  per ear. In order to establish whether the macrocyclic diterpenes contribute to the irritant activity of the plant extracts, compounds 1-4 were also tested. It was found that 1-4 are inactive up to a dose of 200  $\mu\text{g}$  per ear, indicating that the jatrophone type compounds do not play a significant role in the skin irritant activity of *Euphorbia* species.

With the aim to inform about the pharmacological potential of the macrocyclic diterpenes, the antiproliferative and multidrug resistance reversing (mdr) activities of 1-8 were examined on mouse lymphoma cells. The antiproliferative potency was investigated on both drug sensitive and drug resistant cell lines (Cory *et al.*, 1991). The results presented in Fig. 14.3 revealed a cell growth inhibitory effect of all tested compounds. Interestingly, 2-5, 7 and 8 proved to be more potent inhibitors of the growth of human mdr gene-transfected cells ( $IC_{50}$  values 1.4 – 3.3  $\mu\text{g ml}^{-1}$ ) than those of parental cells ( $IC_{50}$  values 5.6 – 9.8  $\mu\text{g ml}^{-1}$ ). Only compound 1 displayed a marginal antiproliferative effect in both tests.

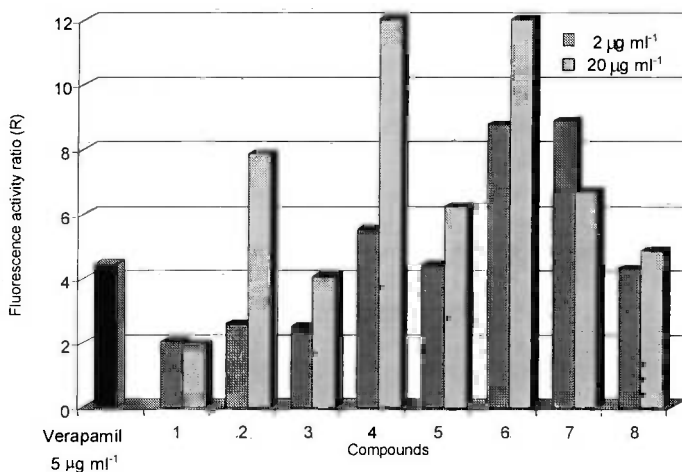
**Table 14.1.** Mouse ear irritant activity of the dichloromethane extracts of *Euphorbia* species.

Species	$ID_{50}$ ( $\mu\text{g ml}^{-1}$ ) (SD)	
	After 4 h	After 24 h
<i>Euphorbia esula</i>	284 (1.18)	317 (1.21)
<i>E. salicifolia</i>	56 (1.21)	63 (1.27)
<i>E. serrulata</i>	325 (1.27)	> 800 (-)



**Fig.14.3.** Antiproliferative effect of diterpenes (1, 2, 4, 5, 7 and 8) on mouse lymphoma cells.





**Fig.14.4.** Multidrug resistance reversing activity of diterpenes 1-8 on mouse lymphoma cells.

The assay for *mdr* activity demonstrated a pronounced effect of compounds 4 and 6-8 in inhibiting the efflux pump activity of tumour cells (Fig. 14.4). The anti-*mdr* effects of compounds 2, 4, 6 and 7, which are expressed in fluorescence activity ratio (R) (Hohmann *et al.*, 2001), were higher than that of the positive control verapamil.

From the above results it could be concluded that the jatrophone diterpenes do not play a significant role in the irritant effect of *Euphorbia* species, rather they are considered therapeutically relevant natural products.

## Acknowledgements

This work was supported by grants OTKA T035200, FKFP 0598/1999 and FKFP 0024/2001 and ETT 115 03/2001. The authors thank Tamás Rédei (Department of Taxonomy and Ecology, Eötvös Lóránd University, Budapest, Hungary) for the identification and collection of plant materials, and Dr Pál Szabó (Institute of Chemistry, Chemical Research Center, Hungarian Academy of Sciences, Budapest, Hungary) for the mass spectroscopic investigations.

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## Chapter 15

# Disposition of Swainsonine in Sheep Following Acute Oral Exposure

J.B. Taylor<sup>1</sup>, J.R. Strickland<sup>2</sup>, C.R. Krehbiel<sup>3</sup>, A.K. Clayshulte<sup>2</sup>, M.A. Siepel<sup>2</sup> and R.L. Ashley<sup>2</sup>

<sup>1</sup>*Department of Animal and Range Sciences, North Dakota State University, Fargo, North Dakota 58105, USA;* <sup>2</sup>*Department of Animal and Range Sciences, New Mexico State University, Las Cruces, New Mexico 88003, USA;*

<sup>3</sup>*Department of Animal Science, Oklahoma State University, Stillwater, Oklahoma 74078, USA*

### Introduction

Swainsonine, an indolizidine alkaloid, has been identified as the primary toxicant in locoweed (*Oxytropis* and *Astragalus* spp.; Molyneux and James, 1982) and currently costs livestock producers approximately \$330 million in annual production losses (Avant, 1998). To date, the majority of research concerning locoweed poisoning has addressed swainsonine exposure rates and frequencies (late subacute and early subchronic) resulting in clinical expression of toxicity (e.g. depression, ataxia, tremors, emaciation, etc.; James *et al.*, 1970). Surprisingly, few investigations have addressed the effects of swainsonine within the first 24 h after initial ingestion. Only three studies (Smith *et al.*, 1992; Stegelmeier *et al.*, 1995, 1998) have attempted to determine the elimination rates of swainsonine, and one (Smith *et al.*, 1992) the absorption rates in cattle or sheep. Although data in these studies support multiple compartment elimination kinetics, no clear distinction between rates of distribution and elimination were defined. A clear understanding of the toxicokinetics of swainsonine following both acute and subacute/subchronic exposures is needed in order to predict serum swainsonine levels following locoweed consumption on the range. These data coupled with clinical dose response data could potentially allow the prediction of how long an animal could graze a locoweed infested pasture without adverse effects on health and (or) production. Therefore, the following study was conducted to provide a preliminary model of swainsonine disposition in sheep by characterizing the rates of absorption, distribution and elimination of swainsonine following acute oral exposure.

## Materials and Methods

### Animal, treatment and sampling protocol

Animal use was approved and followed the guidelines of the Institutional Animal Care and Use Committee, New Mexico State University, Las Cruces. Ten wethers (BW =  $74.7 \pm 7.6$  kg) were housed outdoors in individual pens (4 x 1.5 m). Sheep were stratified by bodyweight (BW) and assigned to either 0.4 (0.4E2, n = 5) or 1.6 (1.6E2; n = 5) mg swainsonine  $\text{kg}^{-1}$  bodyweight treatments. Swainsonine was administered orally as a locoweed (*Oxytropis sericea*) extract immediately following h 0 sampling. Blood samples were collected via jugular venepuncture at 1 h intervals from 0 to 12 h, 3 h intervals from 15 to 24 h, 6 h intervals from 30 to 48 h, and 12 h intervals from 60 to 168 h. The swainsonine extract was obtained by boiling ground (1 mm) locoweed in methanol for 12 h. Subsequently, the liquid fraction was separated from plant matter and the methanol was removed by evaporation. The remaining extract was diluted with distilled water (1:9), vigorously mixed and centrifuged at  $3000 \times g$  for 30 min to separate lipid- and water-soluble fractions. The water-soluble fraction was collected and analysed for swainsonine activity. Basal diet (blue gramma grass and lucerne hay) was provided in metal feeders after treatment delivery and water was available *ad libitum* via automatic watering systems attached to the crates. The basal diet was formulated to meet protein, energy and mineral requirements (NRC, 1985) for maintenance.

### Sample preparation and analysis

Blood samples were allowed to clot for 30 min following collection and then centrifuged ( $1500 \times g$ , 25 min). Serum was decanted and stored ( $< 20^\circ\text{C}$ ) until analysis. Swainsonine in serum or plant was determined using an  $\alpha$ -mannosidase inhibition assay as described by Taylor (2000; lower detection limit =  $0.025 \mu\text{g ml}^{-1}$ ; intra- and interassay CV  $< 12\%$ ). Briefly, serum samples were thawed and boiled in a water bath for 30 min and centrifuged for 30 min at  $11,500 \times g$ . In triplicate, 20  $\mu\text{l}$  of the supernatant was transferred to a 96 well plate (well volume = 320  $\mu\text{l}$ ) with 100  $\mu\text{l}$  of citrate buffer (79.2 mM, pH = 4.5) and 20  $\mu\text{l}$  of  $\alpha$ -mannosidase enzyme ( $0.025 \text{ U ml}^{-1}$ ; Sigma Chemical Co., St Louis, MO), then incubated for 15 min at  $37^\circ\text{C}$ . Following incubation, 20  $\mu\text{l}$  of  $p$ -nitrophenyl  $\alpha$ -D-mannopyranoside (20 mM; Sigma) was added to each well and then incubated for an additional 90 min. The reaction was stopped and colour was developed with 80  $\mu\text{l}$  of borate buffer (200 mM, pH = 9.8) added to each well. Optical density was determined at 405 nm (MRX Microtiter Plate Reader; Dynatech Laboratories Inc., Chantilly, VA).

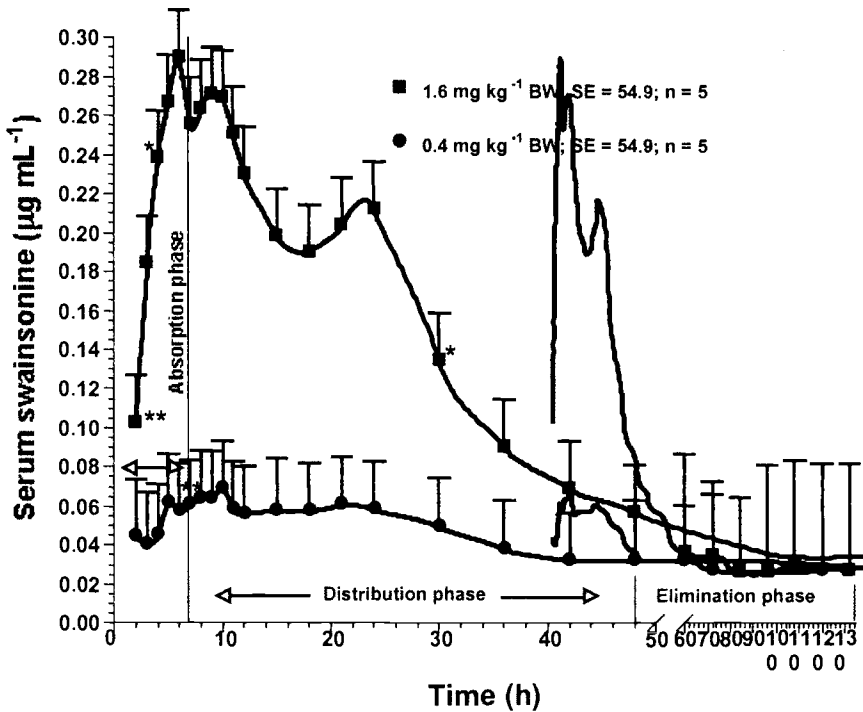
## Statistics

Serum swainsonine activity was analysed using mixed models procedure of SAS 6.12 (SAS, 1996). Data were analysed as unequally spaced repeated measures using the 'spatial power law' structure (SP[POW]; SAS, 1996). The effects of treatment, sampling time and the corresponding interaction were included in the model. Preplanned pairwise comparisons were made within time to test for treatment differences and within treatment to estimate difference from h 0 sampling. A  $P$  value of  $< 0.05$  was considered significant. No statistical analysis of treatment differences in rates of absorption, distribution or elimination are included due to a lack of swainsonine assay sensitivity causing a paucity of data at sampling times greater than 60 to 72 h in all animals. However, rates of absorption, elimination and distribution were calculated on overall treatment least squared means using the curve stripping program, PK Solutions v2.0 (Summit Research Services, Montrose, CO).

## Results and Discussion

Figure 15.1 illustrates the serum swainsonine profiles of wethers receiving either the 1.6E2 or 0.4E2 swainsonine treatments. A treatment by time interaction was detected at h 3, with 1.6E2 serum swainsonine concentrations being higher ( $P < 0.05$ ) than 0.4E2 values and remained so through h 30. At h 36, 1.6E2 and 0.4E2 were again similar ( $P > 0.05$ ). Serum swainsonine was detected in all wethers at h 2 for the 1.6E2 treatment, and at h 7 for the 0.4E2 treatment. The apparent delay in detecting serum swainsonine in all wethers on the 0.4E2 treatment may be a consequence of the limited sensitivity of our current swainsonine assay. Maximum serum swainsonine levels were reached at h 6 for the 1.6E2 ( $0.290 \mu\text{g ml}^{-1}$ ), and at h 10 for the 0.4E2 ( $0.069 \mu\text{g ml}^{-1}$ ) treatment. These agree with the time for maximal serum swainsonine concentration reported by Taylor and Strickland (2001; 6 to 12 h) in lactating ewes and cows receiving an acute dose of swainsonine delivered via a methanol extract of locoweed (0.2 or 0.8 mg swainsonine  $\text{kg}^{-1}$  bodyweight). In contrast, Smith *et al.* (1992) reported that oral dosing of two cows with 3300 or 1950 mg swainsonine (delivered as locoweed) resulted in maximal serum levels at 12 to 24 h after dosing. Additionally, Taylor and Strickland (2001) reported that in lactating cows allowed free access to locoweed forage for 2 h resulted in maximal serum concentrations between 12 and 18 h. The longer time to maximal serum levels, demonstrated by Smith *et al.* (1992) and Taylor and Strickland (2001), is likely due to the time needed to digest the locoweed in order to release swainsonine from the plant matrix.

For the 1.6E2 treatment, absorption (h 0-6) occurred at a rate of  $0.514 \text{ h}^{-1}$  ( $t_{1/2} = 1.4 \text{ h}$ ), distribution (h 7-48) at  $0.055 \text{ h}^{-1}$  ( $t_{1/2} = 12.6 \text{ h}$ ) and elimination (h 60-132) at  $0.004 \text{ h}^{-1}$  ( $t_{1/2} = 184.2 \text{ h}$ ). Rates for the 0.4E2 treatment were  $0.002 \text{ h}^{-1}$ ;  $0.228 \text{ h}^{-1}$ ; and  $0.113 \text{ h}^{-1}$  for elimination (h 48-120;  $t_{1/2} = 289.4 \text{ h}$ ), absorption (h 0-10;  $t_{1/2} = 3.0 \text{ h}$ ) and distribution (h 11-42;  $t_{1/2} = 6.2 \text{ h}$ ), respectively. The



**Fig. 15.1.** Acute appearance of swainsonine in the serum (jugular) of sheep gavaged with swainsonine (locoweed extract) providing either 0.4 or 1.6 mg swainsonine kg<sup>-1</sup> bodyweight immediately following sampling of the blood at h 0. \*Swainsonine concentrations are greater ( $P < 0.05$ ) for 1.6 than 0.4 mg treatment from h 3 to 30. \*\*First time point at which all wethers had detectable serum swainsonine activity.

absorption data presented here are the first of their kind for swainsonine in the refereed literature. The rapid rate of absorption would support a non-saturable concentration dependent method of transport into the systemic circulation.

Assuming first order kinetics for all rates, the kinetic data in our study would indicate that swainsonine elimination from the body could be modelled as a two compartment open model. Two reports (Stegelmeier *et al.*, 1995, 1998) studying the elimination kinetics of swainsonine in livestock would support this type of model for swainsonine elimination. Stegelmeier *et al.* (1995) reported the serum half-life of swainsonine in cattle and lactating ewes to be approximately 16-20 h following 12 or 30 d (respectively) of locoweed consumption. These values are somewhat equivalent to the data we report for distribution  $t_{1/2}$  above. However, comparison of our data to that reported by Stegelmeier *et al.* (1995) is difficult and should be viewed with caution, given that they calculated their elimination

kinetics after subacute/subchronic oral exposure. Smith *et al.* (1992) reported elimination half-life values of 6 to 12 h (in cattle) on samples collected through 48 h following last exposure. Their sampling times and half-life data correlate well with our distribution values of 6 to 12 h.

Stegelmeier *et al.* (1998) demonstrate that tissue clearance half-life in sheep was approximately 50-60 h for liver, kidney and spleen, considerably longer than the approximately 20 h they report for serum. The discrepancy between tissue and serum half-lives definitely argues for a multi-compartment model of elimination. In fact, Bowen *et al.* (1993) utilized a three compartment open model to describe the results of a preliminary experiment in which the terminal half-life of swainsonine in mice was found to be 31.6 min. No one to date has reported the exceedingly long elimination half-lives (184 h plus) we report here for wethers. As such, we are currently pursuing alternative assay procedures to improve our swainsonine detection limit.

## Conclusion

In conclusion, our findings tend to agree with those of Bowen *et al.* (1993), suggesting a multi-compartment model for swainsonine toxicokinetics, which is further supported by Stegelmeier *et al.* (1995, 1998). Additionally, the data support a non-saturable, concentration driven mode of absorption for swainsonine. Swainsonine was measured indirectly by an  $\alpha$ -mannosidase inhibition assay with a detection limit of only  $0.025 \mu\text{g ml}^{-1}$  (CV < 12%). This lack of sensitivity currently restricts our ability to fully define the toxicokinetics of swainsonine. As such, further research, using more sensitive detection methods (e.g. gas chromatography), is necessary to fully characterize the toxicokinetics of swainsonine. Using the data presented here it would take approximately 80 to 120 days (i.e. 10 elimination half-lives) to completely clear swainsonine from the body.

## Acknowledgements

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## Chapter 16

# Stimulating Effect of Aflatoxin B1 on Lipid Peroxidation in the *In Vitro* Model Systems

J.E. Dvorska<sup>1</sup> and P.F. Surai<sup>2</sup>

<sup>1</sup>*Sumy State Agrarian University, Sumy, Ukraine;* <sup>2</sup>*Avian Science Research Centre, SAC, Ayr, KA6 5HW, Scotland, UK*

### Introduction

The global occurrence of mycotoxins is considered as a major risk factor affecting human and animal health and about 25% of the world's crop production is contaminated to some extent with mycotoxins (Fink-Gremmels, 1999). Furthermore mycotoxicoses are considered to be a common cause of decreased poultry production in many countries. The mechanisms of mycotoxin action are complex and have not been fully elucidated.

Aflatoxins are highly oxygenated secondary metabolites produced by certain toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Jayashree and Subramanyam, 2000). There are four naturally occurring aflatoxins B1, B2, G1 and G2 and a range of their active metabolites with aflatoxin B1 (AFB1) being the most toxic and carcinogenic compound of this group of mycotoxins (Smith, 1997). This group of mycotoxins possesses high carcinogenic, teratogenic, mutagenic and immunosuppressive activities (Ellis *et al.*, 1991). However, molecular mechanisms of cellular damage caused by AFB1 have not been fully elucidated.

Nevertheless, lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many different compounds, including mycotoxins. Indeed, recent studies have shown that AFB1 enhances reactive oxygen species (ROS) formation and causes oxidative damage to various molecules including DNA, which could contribute to the cytotoxicity and carcinogenic effect of AFB1 (Yang *et al.*, 2000). For example, lipid peroxidation assessed as malondialdehyde (MDA) and diene conjugate accumulation was increased in rat liver 1 day after AFB1 administration (Shen *et al.*, 1994), after 6 weeks of AFB1 consumption (Rastogi *et al.*, 2001) or 72 hours after a single intraperitoneal dose of AFB1 (Souza *et al.*, 1999). This process was dose dependent and the highest concentration of MDA was detected in microsomes, followed by nuclear fraction and mitochondria. Lipid peroxidation was also enhanced in cultured primary rat hepatocytes (Shen *et al.*, 1995; Liu *et al.*, 1999; Yang *et al.*, 2000). Increased activities of blood

alanine aminotransferase and aspartate aminotransferase in rats fed on a diet containing AFB1 (Choi *et al.*, 1995; Souza *et al.*, 1999) probably reflect damage to the liver. However, in an experiment with laying hens there was no alteration in activity of those enzymes as a result of AFB1 consumption (Fukal *et al.*, 1988), indicating that the toxic effect of AFB1 is probably dose- and species-specific. In general, AFB1 has a high affinity to lipid domains of the cell membrane and liposomes composed of unsaturated fatty acids bound twice as much AFB1 as those composed of saturated ones (Müller and Petzinger, 1988). Furthermore, AFB1 consumption by broiler chickens (Merkley *et al.*, 1987) or rats (Kendall, 1978) was associated with a significant increase in total liver lipids. In general, the membrane-active character of AFB1 (Amstad *et al.*, 1984) could be an important point in consideration of its pro-oxidant action.

The aim of the present work was to evaluate the pro-oxidant properties of AFB1 using *in vitro* model systems.

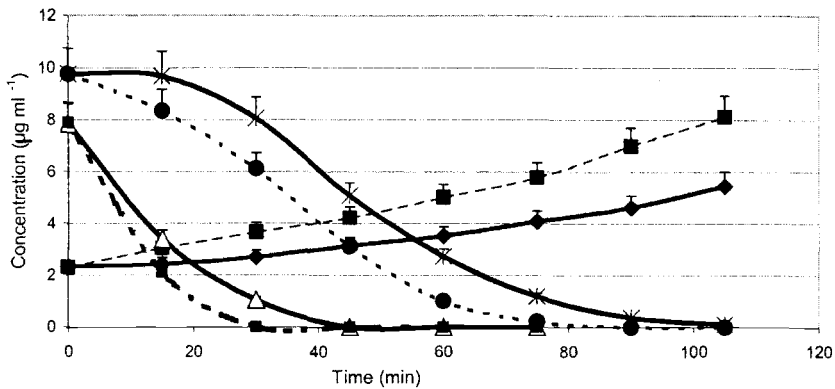
## Materials and Methods

Two model systems were used. The model system A was based on the peroxidation of phosphatidylcholine (PC) liposomes in the absence (spontaneous) or presence (stimulated) of inducers of peroxidation. Liposomes were formed with or without AFB1 and incubated at 37°C. PC hydroperoxide (PC-OOH) and thiobarbituric acid reactive substances (TBARS) were determined by HPLC. The model system B was based on the oxidation of linoleic acid. In this system, AFB1 and/or vitamin E were added and samples were incubated at 37°C. In some cases, peroxidation inducers were used. Linoleic acid hydroperoxides and vitamin E were monitored by HPLC.

## Results and Discussion

Incubation of the PC liposomes at 37°C caused peroxidation and PC-OOH formation as indicated by HPLC. Inclusion in the system of the inducers significantly increased PC-OOH and TBARS formation and this process was time-dependent. Incorporation of AFB1 in the PC liposomes stimulated lipid peroxidation when the liposomes were incubated *in vitro*. When the lipid peroxidation inducers were included in the system the pro-oxidant effect of AFB1 was more pronounced compared to spontaneous lipid peroxidation.

Inclusion of AFB1 in the second model system was also associated with increased linoleic acid hydroperoxide formation (Fig. 16.1). Similarly to the first system, AFB1 stimulated lipid peroxidation more effectively in the presence of lipid peroxidation inducers, indicating that the physical interaction of aflatoxin with other pro-oxidants is an important mechanism of its pro-oxidant properties. Inclusion in the system of vitamin E significantly reduced lipid peroxidation but this vitamin was oxidized itself in the presence of AFB1 showing that the



**Fig. 16.1.** Effect of AFB1 on  $\alpha$ - and  $\gamma$ -tocopherol oxidation and linoleic acid hydroperoxide accumulation in the model system B. Linoleic acid hydroperoxide accumulation without AFB1 ( $\blacklozenge$ ); linoleic acid hydroperoxide accumulation with AFB1 inclusion into the medium (-■-);  $\alpha$ -tocopherol oxidation without AFB1 ( $\bullet$ );  $\alpha$ -tocopherol oxidation with AFB1 inclusion into the medium (-■-);  $\gamma$ -tocopherol oxidation without AFB1 (\*);  $\gamma$ -tocopherol oxidation with AFB1 inclusion into the medium ( $\bullet$ ).

afatoxin interaction with antioxidants is also an important mechanism of their pro-oxidant action. Once vitamin E was consumed completely, the rate of lipid peroxidation increased significantly. Therefore these results confirmed the pro-oxidant effect of AFB1, suggesting that more attention should be paid to aflatoxin interactions with antioxidants.

Similarly, AFB1 in the feed interfered with the accumulation of carotenoids in chicken tissues (Schaeffer *et al.*, 1988a) inducing pale bird syndrome in chickens. In fact, AFB1 caused a significant depression of lutein in the toe web, liver, serum and mucosa (Schaeffer *et al.*, 1988b). Pigment restoration was accomplished by feeding the same diet supplemented with lutein ( $70 \text{ mg kg}^{-1}$ ). In young chickens, AFB1 reduced up to 35% the lutein content of the jejunal mucosa and the serum lutein was reduced up to 70% (Tyczkowski and Hamilton, 1987a) suggesting that AFB1 interfered with the absorption, transport and deposition of carotenoids. Therefore, AFB1 impaired lutein absorption in chickens (Tyczkowski and Hamilton, 1987b). In similar fashion, ochratoxin A was shown to affect carotenoid assimilation in chickens. Again, depression of the uptake of carotenoids by the intestinal mucosa and depressed transport in serum were considered as important mechanisms of action of AFB1 on carotenoid metabolism (Schaeffer *et al.*, 1987). In general, malabsorption syndrome is considered to be a common result of mycotoxicoses. A reduced glutathione (GSH) depletion occurred in cultured rat hepatocytes as a result of AFB1 toxicosis (Liu *et al.*, 1999).

Intraperitoneal administration of AFB1 to rats ( $2 \text{ mg kg}^{-1}$ ) was also associated with a decreased level of GSH in the liver. In contrast, in 3-week-old male chickens having daily aflatoxin gavage ( $2 \text{ mg kg}^{-1}$  bodyweight, in corn oil) for 5 and 10 days, hepatic and renal GSH was elevated after 10 days of aflatoxin treatment (Beers *et al.*, 1992a). Similarly, hepatic GSH increased 2 and 8 h, respectively, following a single AFB1 dose and continued to increase through 5 daily doses of AFB1 (Beers *et al.*, 1992b). There was a GSH depletion in cultured rat hepatocytes as a result of AFB1 toxicosis (Liu *et al.*, 1999).

Since AFB1 stimulates peroxidation, a protective effect of antioxidants would be expected. Indeed, protective effects of vitamin E were shown in aflatoxicosis (Shen *et al.*, 1994; Choi *et al.*, 1995; Souza *et al.*, 1999). Dietary carotenoids inhibited AFB1-induced liver DNA damage in rats (Gradelet *et al.*, 1997, 1998) and xanthophylls inhibited the mutagenicity of AFB1 in a dose-dependent manner (de Mejia *et al.*, 1997). The authors suggested that the inhibitory mechanism of lutein against AFB1 mutagenicity is the result of formation of a complex between lutein and AFB1, direct interaction between lutein and AFB1 metabolites and modulation of the metabolic activation of AFB1 by lutein. Selenium has also a protective effect against aflatoxicosis (Burguera *et al.*, 1983) enhancing the aflatoxin detoxification process (Gregory and Edds, 1984). Therefore, dietary antioxidant additives such as Se are considered to be effective in the reduction of aflatoxicosis in poultry (Dalvi, 1986).

Thus, AFB1 is involved in stimulation of lipid peroxidation, however, the molecular mechanisms of this effect need further investigation.

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## Chapter 17

# An Indirect Competitive ELISA for Pyrrolizidine Alkaloids of *Heliotropium europaeum*

V. Cavallaro, K.A. Than, S.M. Colegate and J.A. Edgar

CSIRO Livestock Industries, Australian Animal Health Laboratory, Portarlington Rd, Geelong, Victoria 3220, Australia

### Introduction

Common heliotrope (*Heliotropium europaeum*) is a pyrrolizidine alkaloid (PA)-containing annual herb that is native to southern and central Europe, western Asia and northern Africa. It was introduced to Australia accidentally in the 19th century and is now spread through the south and east of Australia, especially in wheat-growing areas. *Heliotropium europaeum* growth is favoured by higher than average rainfall in the summer months.

Heliotrope has been the cause of poisoning of livestock, such as pigs, poultry and cattle, in Australia (Gaul *et al.*, 1994; Hill *et al.*, 1997). Heliotrope poisoning occurs as a progressive liver disease of livestock grazing on heliotrope-infected wheat stubble and pasture or through contamination of wheat and commercial stockfeeds. Humans can be exposed to PAs through the consumption of plants for medicinal or dietary needs (Prakash *et al.*, 1999). There is a risk of contamination of staple foods (cereals/flour), by *Heliotropium* spp. seeds, for human consumption. Cases of PA poisoning have occurred in Afghanistan (Tandon *et al.*, 1978), Tadjikistan (Mayer and Luthy, 1993) and India (Tandon *et al.*, 1976). The alkaloids are particularly harmful to the liver and lungs, with the major human clinical symptom being hepatic veno-occlusive disease (Prakash *et al.*, 1999).

Indications are that, based on the genotoxic, carcinogenic properties of some PAs, low Tolerable Daily Intakes will be allowed for humans. For example, in Germany regulations restrict oral exposure to PAs and their N-oxides in herbal preparations to  $0.1 \mu\text{g day}^{-1}$  (German Federal Health Bureau, 1992). No food standards have been established for PAs although an extrapolation to food of the German herbal regulations may result in equally low allowable levels of PAs in food.

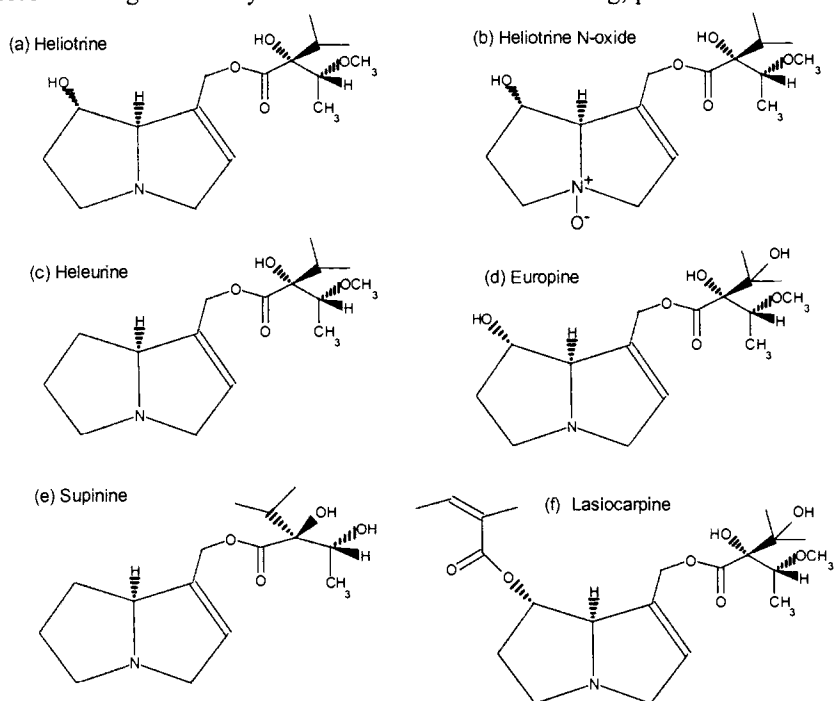
The screening of food or plant material for PA contamination to these low levels requires sensitive methods of detection. A number of ELISA methods have been published for PAs, for example retronecine-based PAs (Bober *et al.*, 1989), retrorsine (Roeder and Pflueger, 1995) and senecionine (Langer *et al.*, 1996).

Here, we describe an indirect competitive ELISA for the detection of PAs from *H. europaeum* using primary ovine antibodies generated against the major PA of heliotrope, heliotrine. Cross-reactivity studies were performed with free bases and N-oxides derived from heliotrope. The methodology was also investigated for its applicability for the detection of PAs in foodstuffs, namely wheat and honey.

## Competition ELISA and Cross-reactivity Studies

An indirect competitive ELISA was developed for the detection of heliotrine and other heliotrope PAs. The anti-serum used in this study displayed a high sensitivity to free heliotrine at  $\sim 15 \text{ pg well}^{-1}$ . The major PAs (and their N-oxides) found in *H. europaeum* are heliotrine and lasiocarpine, followed by europine, with small quantities of heleurine and supinine. Acetyl-lasiocarpine is found in the plant in trace amounts and was not tested in these studies. The structures of some of these PAs are shown in Fig. 17.1.

To analyse the cross-reactivity of the anti-heliotrine anti-serum, microtitre plates were coated with a bovine serum albumin-heliotrine N-oxide conjugate (Erlanger, 1980) at  $25 \text{ ng well}^{-1}$ . Plates were washed (with saline/Tween-20) before blocking with assay buffer for 1 hour. After blocking, plates were washed



**Fig. 17.1.** Structures of pyrrolizidine alkaloids that were used to determine the cross-reactivity of the heliotrope ELISA.



and standards of heliotrine or other PAs (prepared in assay buffer) were added to wells along with the optimum dilution of anti-heliotrine anti-serum (1 in 2000).

After 2 hours of competition for antibody binding between solid and liquid phase toxins, the unbound reagents were washed out. The antibodies bound to the wells were subsequently detected by the addition of anti-sheep IgG conjugated to horse-radish peroxidase. After a further 1 hour, plates were washed and 3, 3', 5, 5' tetramethylbenzidine substrate was added and incubated for 15 min before stopping the reaction with sulphuric acid (0.05 M). A comparison of the optical densities for the different amounts of PA (free bases and N-oxides) is shown in Fig. 17.2. Cross-reactivities were quantified at the 80% and 50% of maximum anti-serum binding level (Table 17.1).

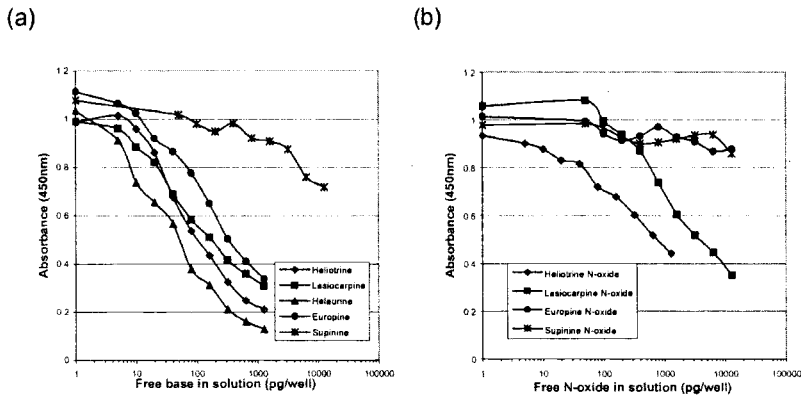
The order of recognition by the anti-serum for free bases was heleurine > heliotrine  $\equiv$  lasiocarpine > europine > supinine. Cross-reactivity of the N-oxides was different to that seen for the free bases, with lasiocarpine N-oxide recognized much less than heliotrine N-oxide by the anti-serum and little recognition towards supinine N-oxide and europine N-oxide. The overall sensitivity of the anti-serum for the N-oxides was much lower than their respective free bases. This lack of recognition for the N-oxides is important as in plant-derived material PAs are found predominantly as the N-oxide, thus requiring an additional reduction step to convert N-oxides to free bases prior to the ELISA.

## Quantitation of Heliotrope PAs in Foodstuffs

Because of the potential for interfering co-extractives in real samples, the ELISA needs to be individually assessed for adverse effects with different sample matrices. Two types of food matrices were investigated, wheat and honey.

**Table 17.1.** Cross-reactivity determined using the competition heliotrope ELISA. The mean concentrations of PAs (in solution) to achieve a decrease in binding of 20% ( $I_{80}$ ) and 50% ( $I_{50}$ ) of antibody to the plate-bound toxin are shown as pg or ng well<sup>-1</sup>.

Pyrrolizidine alkaloid	Mean		% Cross-reactivity	
	$I_{80}$	$I_{50}$	$I_{80}$	$I_{50}$
Heliotrine	23 pg	112 pg	100	100
Lasiocarpine	23 pg	150 pg	100	75
Europine	50 pg	400 pg	45	28
Supinine	2 ng	15 ng	0.01	< 0.01
Heleurine	8 pg	50 pg	250	220
Heliotrine N-oxide	60 pg	1000 pg	33	11
Lasiocarpine N-oxide	300 pg	2500 pg	8	5
Europine N-oxide	15 ng	500 ng	< 0.01	< 0.01
Supinine N-oxide	15 ng	250 ng	< 0.01	< 0.01
Heleurine N-oxide	Not determined		Not determined	



**Fig. 17.2.** Standard absorbance curve for anti-heliotrine anti-serum binding to BHNOx conjugate, coated at  $25 \text{ ng well}^{-1}$  with doubling dilutions of standard PAs, showing (a) the free bases and (b) the N-oxides.

## Wheat

Clean wheat, spiked with heliotrope seeds at 0, 1, 2, 5 or 10 seeds  $100 \text{ g}^{-1}$ , was extracted for  $\sim 18$  hours by continuous shaking in sulphuric acid (0.05 M). Samples were diluted with methanol (1:1) and mixed with indigo-carmin redox resin for 0.5–2.0 hours to reduce the N-oxides to parent bases.

Post-reduction aliquots were diluted in assay buffer at 1/10, 1/50 or 1/100 for use in the ELISA. These samples acted as the competitor (similar to standard solutions of PAs in the cross-reactivity studies) for antibodies, against the plate-coated conjugate. The amount of competition detected was proportional to the amount of PAs in the extract and was quantified against a heliotrine standard curve (prepared in 10% reduced clean wheat extract/assay buffer).

The collated data for three sampling replicates is shown in Table 17.2 as mean content of PAs (ppb). Values were corrected for all dilutions, including the ELISA (1 in 10), the reduction (1 in 2), the extraction (1 in 2.5) and an empirically determined reduction efficiency of 54% for conversion of N-oxide to free base. The PA content per heliotrope seed was determined at  $14\text{--}22 \mu\text{g seed}^{-1}$ .

To gauge the presence of free bases in the natural matrix and/or the low level of cross-reactivity of the N-oxides to the anti-serum, PAs in the wheat extracts were quantified as previously, in the absence of resin reduction. As expected, PA quantities were much lower, although an increase was noted proportional to the number of seeds spiked (Table 17.2). This result supports the presence of free bases at low levels in the seed and/or the low cross-reactivity of the more abundant N-oxides.

**Table 17.2.** The amount of PAs (free base) quantified in wheat extracts spiked with heliotrope seeds by the ELISA against a heliotrine standard curve.

Heliotrope seeds in clean grain (100 g <sup>-1</sup> )	Heliotrine equivalents (ppb)					Adj. mean	Mean ( $\mu\text{g seed}^{-1}$ )
	0.5 h	1.0 h	2.0 h	Mean			
1 seed	reduction (1/10)	76	72	70	73	135	14
	no reduction				8		
2 seeds	reduction (1/10)	172	172	164	169	313	16
	no reduction				12		
5 seeds	reduction (1/50)	–	680	720	700	1296	22
	reduction (1/100)	–	547	380	464		
	no reduction				27		
10 seeds	reduction (1/50)	–	1288	1346	1317	2439	21
	reduction (1/100)	–	912	1007	959		
	no reduction				62		

### Honey

The aims of these preliminary investigations were: (i) to ascertain whether heliotrope PAs, in the honey matrix, could be detected by the ELISA; and (ii) to validate ELISA results by comparison with levels estimated by liquid chromatography-mass spectrometry (LCMS).

Two types of honey samples were investigated by the ELISA and LCMS methods. The first involved the detection of PAs, extracted from honey by solid phase extraction techniques, reconstituted in aqueous solution (methanolic). The other, ELISA analysis of neat honey, diluted in ELISA buffer (1 in 10). The results from this comparison work showed the ELISA detected 43–70% of PAs to that estimated by LCMS in the first instance and 45–118% of PAs when using neat honey samples. This work, being preliminary in nature, requires further optimization of the ELISA for use with honey samples. Nevertheless, the ability to use neat honey samples in this ELISA was extremely promising.

### Conclusion

An indirect competition ELISA for detecting heliotrope PAs has been optimized. Initial investigations have supported its applicability to wheat and honey samples.

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in the production of anti-serum and protein conjugates and Kerrie Beales for preparation of honey samples and their analysis by LCMS.

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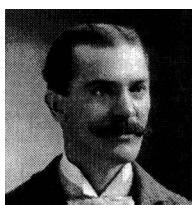
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## Chapter 18

# Victor King Chesnut (1867–1938), a Poisonous Plant Pioneer

D.J. Wagstaff

276 East 100 South, Pleasant Grove, Utah 84062, USA



Victor King Chesnut was born on 28 June 1867 to John Andrew Chesnut and Henrietta Sarah King at Nevada City in the gold mining region of California shortly after the gold rush. The family moved to Oakland, California where he attended public schools. He was trained as a botanist and as a chemist at the University of California at Berkeley where he got a BS degree in 1890. Later he acquired some training at

the University of Chicago and Columbian University which is now George Washington University. He taught chemistry at the University of California and at Montana State College (MSC) which later became Montana State University (MSU).

In 1894 he was the first person employed full time to research plant poisonings by the United States Department of Agriculture (USDA) in the Bureau of Plant Industry under the supervision of the botanist Frederick V. Coville. One project was to interview American Indians of Mendocino County, California regarding their use of plants in preparation for displays at the World Fair in Chicago (Chesnut, 1902).

He married Olive Branch Spohr on 18 July 1899 in Oakland, California. She was trained as a mathematician. The family tell of spirited discussions of whether botany or mathematics is the more basic science. About the time of Chesnut's marriage, Edmund V. Wilcox, a veterinarian at the Montana Experiment Station transferred to the USDA in Washington, DC. Before his transfer, he had written some brief reports on plant poisonings. The poisonous plant situation in Montana probably was discussed in USDA, because both Chesnut and Wilcox spent the summer months of 1900 in Montana investigating plant poisonings. They had the cooperation of livestock owners, veterinarians, the experiment station, state officials, chemists and laboratory animal researchers. In addition, they had the support of P.A. Rydberg of the New York Botanical Garden to verify plant identifications.

They travelled over 7000 miles via railroads, stage-coaches and on horseback investigating outbreaks of plant poisoning in every county of that large state. Their voluminous report (Chesnut and Wilcox, 1901) documented several types of plant poisoning especially those caused by *Delphinium*, *Zigadenus* and *Lupinus*. Some of the poisonous plant problems resulted from overgrazing by the huge number of livestock on the open ranges. For example, one sheep rancher, Charles Bair, had

transport to market (Anonymous, 1910). Many outbreaks occurred in hungry livestock that were unloaded from railroad cars into patches of poisonous plants. Another problem was driving animals so hastily that they grazed indiscriminately and thus consumed plants they would ordinarily avoid.

Chesnut returned to Bozeman, Montana for a few years where he further investigated poisonous plants and taught chemistry at MSC. Charles Russell, the Montana Cowboy artist, romanticized the lives of the cowboys. Victor Chesnut documented some of the problems behind the romance. Conditions have changed since 1900. The number of livestock has decreased, the open ranges have been fenced, and railroads no longer transport livestock. However, the basic principles have not changed. Now, as then, animals that are hungry or thirsty graze poisonous plants. In addition, animals that are hastily driven graze plants they ordinarily would avoid. His extensive writings about poisonous plants, not only of Montana but of the entire USA, were widely circulated and were translated into various languages (Anonymous, 1906).

An interesting mystery regarding Chesnut in Montana has recently become known (Scott, 1999). During a 1903 poisonous plant investigation, he visited the sheep ranch of Charles W. Cook near Unity, Meagher County, Montana. Apparently Cook was impressed with his academic ties and told him that he and two friends, David E. Folsom and William Peterson, in 1869 had made the first definitive exploration of the region which was shortly to become Yellowstone National Park. He further explained that through a series of unfortunate circumstances the account of their exploration had never been fully published. Cook turned over to Chesnut his only copy of the manuscript taken from the diary the trio had made during their trip. Chesnut made a transcript of the manuscript and took it to David E. Folsom in Helena, Montana who made some corrections to it. However, Chesnut never got around to publishing the manuscript before moving to Washington, DC in 1907. The original Cook manuscript was destroyed in a fire at the Chemistry building at MSU in 1916. However, Chesnut kept the transcript. Finally in 1922, Oscar O. Mueller, a son-in-law of Charles Cook, contacted Chesnut who rummaged through his files and found some original material. Then in 1979, a small collection of papers from Chesnut's files appeared on a desk in the Special Collections Department of MSU. Among other things, it contained a few pages of a typewritten manuscript of the Cook-Folsom-Peterson expedition that had not been previously published. The staff of Special Collections told me on 21 June 2001 that the identity of the donor of the small collection of Chesnut's papers was unknown and that for all they knew the collection could have been in the Special Collections Department or elsewhere in the MSU library for some time.

Chesnut moved from Bozeman to Washington, DC in 1907 to accept a position in the Bureau of Chemistry that later became the US Food and Drug Administration. He published a few articles after that but not on the subject of poisonous plants. Although he lived in Hyattsville, Maryland where he died on 29 August 1938, he was buried in the cemetery at St John's Episcopal Church in Beltsville, Maryland.

Victor King Chesnut laid the foundation upon which others, including C. Dwight Marsh, built a solid body of poisonous plant knowledge. The Montana legacy of

Victor King Chesnut laid the foundation upon which others, including C. Dwight Marsh, built a solid body of poisonous plant knowledge. The Montana legacy of Chesnut lived on in the work of those who followed him. One of the first field stations used in the poisonous plant research of Marsh was not far from Bozeman at Greycliffe, Montana. Later his son, Hadleigh Marsh, wrote a definitive textbook of sheep diseases that included much material about poisonous plants (Marsh, 1965). Hadleigh Marsh became head of the MSU Department of Veterinary Science and Veterinary Research Laboratory. The laboratory was subsequently named Marsh Laboratory in his honour.

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## Chapter 19

# Phomopsin Intoxication of the Rat Causes Prodigious Loss of Bodyweight and Liver Weight

J.G. Allen

*Department of Agriculture, 3 Baron-Hay Court, South Perth, WA 6151, Australia*

Phomopsins are the toxic metabolites of the fungus *Diaporthe toxica* (anamorph *Phomopsis* sp.) that are responsible for causing the disease lupinosis (Culvenor *et al.*, 1977). These compounds are predominantly hepatotoxic (Peterson, 1986) and in sheep cause bodyweight loss in subacute and chronic intoxications, and increased and decreased liver size in acute and chronic intoxications, respectively (Allen, 1989). An experiment was conducted to examine the effect of a single intraperitoneal injection of phomopsin A on the bodyweight and liver weight of adult female rats.

### Materials and Methods

Fifty-two adult female rats (160-288 g, mean 229 g) of a Wistar derived, outbred strain that was not specific pathogen free, were used. They were kept in standard boxes in an air-conditioned room with controlled artificial lighting, and provided commercial rat pellets and water *ad libitum*. The phomopsins extract used was the same as that used by Allen and Hancock (1989), and it was shown by high performance liquid chromatography to contain predominantly phomopsin A. The rats were divided into two groups of 28 and 24, by random allocation from within liveweight strata. On day 0 the group of 28 (intoxicated) were each given 5 mg phomopsin A kg<sup>-1</sup> by intraperitoneal injection in a volume of approximately 2.5 ml, and the group of 24 (control) were each given an intraperitoneal injection of 2.5 ml of normal saline. The dose rate of phomopsin A was approximately the LD<sub>50</sub> for phomopsin A in adult rats by the intraperitoneal (Allen, 1992) and subcutaneous (Peterson, 1986) routes. All surviving rats were weighed daily until day 18, and then again on day 21. Four, 4, 4, 4, 2, 2 and 8 of the intoxicated rats were euthanized on days 2, 4, 5, 6, 7, 10 and 21 respectively, and all 24 control rats were euthanized on day 21, at which time liver weights were recorded and liver samples collected for microscopic examination. Intoxicated rats that had lost the most weight or appeared most severely affected were selected for euthanasia at each of the designated times during the experiment. This ensured that no rats actually died from intoxication.



## Results

### Body and liver weights

The mean bodyweights of the intoxicated rats were significantly less than those for the control rats from day 2 through to day 21 ( $P < 0.005$  to  $0.001$ ) (Fig. 19.1). All the surviving intoxicated rats were gaining weight by day 14.

In the intoxicated rats, mean liver weights were unchanged on days 2 to 5, but on day 6 they decreased markedly (Fig. 19.1). Mean liver weights on days 6 and 10 were significantly less than on day 2 ( $P < 0.05$ ), but the mean liver weight on day 21 was not. The mean liver weights of the intoxicated and control rats on day 21 were not significantly different. The pattern of changes in the mean liver weight: bodyweight ratio was similar (Fig. 19.1), with the mean ratios on days 6, 10 and 21 being significantly less than on day 2 ( $P < 0.05$ ). However, the mean ratio in the intoxicated rats was not significantly different to that in the control rats on day 21. In addition, if it is assumed that the ratio in the control rats remained the same throughout the experiment, then the mean ratios in the intoxicated rats were significantly increased on days 4 and 5 ( $P < 0.01$ ), and significantly decreased on days 6 and 10 ( $P < 0.01$ ).

### Gross pathology – intoxicated rats

The livers were swollen and creamy pink to creamy brown in colour up to day 5. From days 6 to day 10 they were reduced in size and were yellowy brown in colour. On day 21 they were normal colour and approximately normal size, but were usually misshapen, with some lobes shrunken and others enlarged relative to the sizes of lobes in the control rats.

### Microscopic pathology – intoxicated rats

Through to day 7 there was extensive vacuolation of predominantly periportal and mid-zonal hepatocytes. This was reduced by day 10, and only present in scattered hepatocytes by day 21. Oil red O stains of frozen sections revealed that the vacuolation was predominantly due to fat accumulation.

Hepatocytes and their nuclei generally increased in size as the experiment progressed, but the extent of the increase was very variable. On day 21 occasional hepatocytes and their nuclei were estimated to be 10 times the size of those in control rats. Regenerating nodules of normal sized hepatocytes were present in all the livers of intoxicated rats on day 21. In addition, as the experiment progressed there was substantial proliferation of Kupffer cells, cholangiolar cells and fibroblasts.

Arrested and abnormal mitotic profiles in metaphase were occasionally evident in periportal hepatocytes on day 2, and were very prevalent in hepatocytes throughout the lobules on days 6, 7 and 10. Only a few arrested

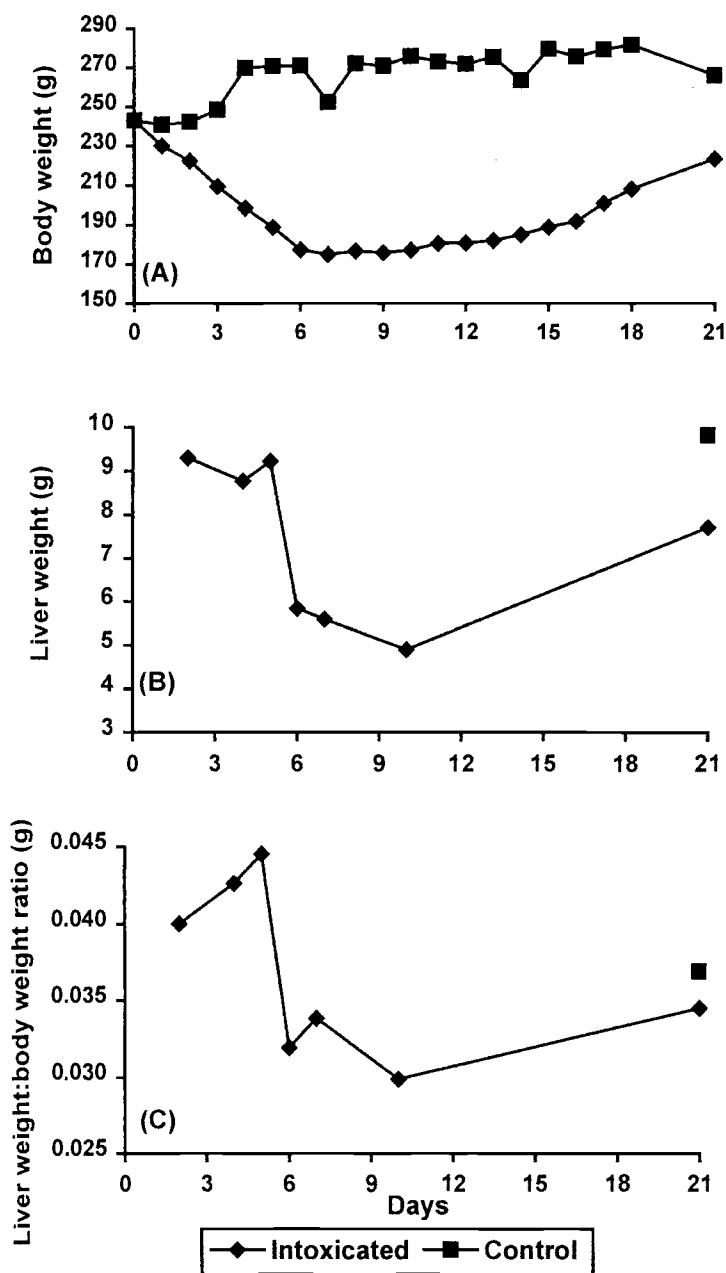


Fig. 19.1. Mean bodyweights (A), liver weights (B) and liver weight:bodyweight ratios (C) in rats given a single intraperitoneal injection of phomopsin A (intoxicated) or normal saline (control).

metaphase profiles were present on day 21, when there were also some anaphase profiles.

Two forms of hepatocyte death were observed. Focal midzonal and paracentral coagulative and lytic necrosis was occasionally seen in livers collected on days 4 to 10, but greatest cell loss was considered to be by apoptosis. This was apparent on day 2, most prevalent on days 4 to 10, and still occurring to a much lesser extent on day 21. Many of the hepatocytes affected by arrested mitosis were contracted, with strongly eosinophilic cytoplasm. These are early changes of apoptosis so it is presumed that hepatocytes affected by arrested mitosis were lost from the liver by apoptosis.

## Conclusions

Intoxication by phomopsin A profoundly affected bodyweight and liver weight of rats. The average bodyweight loss was 28% of the initial weight, but individual rats lost up to 31%. Bodyweight losses of up to 16% in adult mice (Gardiner and Petterson, 1972), 40-42% in sheep (Gardiner, 1965; Allen, 1989) and 56% in pigs (van Rensburg *et al.*, 1975) have been reported following phomopsins intoxication, but in each of these reports the animals were given multiple doses of the phomopsins.

Liver weights apparently increased, at least relative to bodyweight, during the first 5 days following intoxication, then over the next 5 days the livers lost 47% of their mass. Liver weight losses of 8% in adult mice following a single dose of phomopsins (Peterson and Lanigan, 1976) and 50% in sheep following multiple doses of phomopsins (Gardiner, 1965) have been reported.

The initial apparent increase in liver weight coincided with the period of intense fat accumulation in hepatocytes, and when cell loss was just beginning. The dramatic loss of liver mass during the sixth day coincided with the rapid increase in cell loss, predominantly by apoptosis, and occurred in spite of continued fat accumulation, increase in hepatocyte size, and proliferation of Kupffer cells, cholangiolar cells and fibroblasts. The rapidity with which it occurred is a reflection of apoptosis itself, which is a very rapid process of active, energy dependent, cellular self-destruction (Kerr, 1971). The half-life of histologically obvious apoptotic bodies is about 4 hours (Potten *et al.*, 1978), so even extensive apoptosis may be a relatively inconspicuous event (Searle *et al.*, 1982). The extent to which phomopsin A caused mitotic arrest and apoptosis suggests that it may have potential as an anti-cancer drug.

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## Chapter 20

# The Role of Condensed Tannins on the Rumen Degradation of *Lotus pedunculatus*

M.H.L. Bento<sup>1,2</sup>, T. Acamovic<sup>1</sup> and J.M.F. Abreu<sup>2</sup>

<sup>1</sup>Avian Science Research Centre, SAC, Ayr, KA6 5HW, Scotland, UK; <sup>2</sup>DPAA, Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisbon, Portugal

The presence of tannins in plants and in animal diets is frequently regarded as undesirable because of the antinutritional and toxic properties of tannins. It has been demonstrated that tannins bind to proteins and other nutrients, chelate metal ions and increase endogenous losses from animals. However, numerous authors have reported decreased ruminal degradation of protein in the presence of tannins, due to their ability to increase rumen bypass protein. Thus, more protein may be available for digestion in the small intestine. Condensed tannins (CT) strongly and selectively bind to proteins and form relatively stable complexes at rumen pH. These complexes are reported to become less stable at low and high pH where dissociation has been observed to occur with radiolabelled bovine serum albumin (D.M. McNeill, Sydney, 2001, personal communication).

The aim of this study was to assess the effect of CT present in *Lotus pedunculatus* (cv. Maku), grown in Portugal, on dry matter (DM) and nitrogen (N) degradation in the rumen, and to estimate the effects of these tannins on the post-rumen digestion using poultry as a model.

## Materials and Methods

### Rumen degradation

*Lotus pedunculatus* was harvested, dried and ground to a particle size of 2 mm. The composition of lotus (DM = 929.0 g kg<sup>-1</sup>) was as follows: N = 31.4, neutral detergent fibre (NDF) = 411.3, and CT = 96.8 g kg<sup>-1</sup> DM. Polyethylene glycol (PEG) was added to the ground lotus (1 g PEG:2 g lotus).

Lotus, treated with PEG (5 g) and control (PEG-free lotus) were weighed in duplicate in nylon bags (pore size of 40 µm), and incubated in the rumen of each of three sheep for 3, 6, 9, 12, 16, 24, 48, 72, 96 and 120 h. The nylon bags were removed, washed with cold water and dried in a forced-air oven at 45°C. DM disappearance from the nylon bags was determined. Concentration of N in the

residues was measured to determine protein losses. Condensed tannins (CT) were measured in lotus by the butanol-HCl assay.

The disappearance of DM and N ( $\text{g } 100 \text{ g}^{-1} \text{ DM}$ ) were fitted to the exponential equation described by Ørskov and McDonald (1979) [ $d = a + b(1 - e^{-(ct)})$ ], where  $d$  represents the disappearance from the bag after  $t$  hours,  $a$  the rapidly degradable fraction,  $b$  the slowly degradable fraction and  $c$  the rate of degradation of fraction  $b$ . Fraction  $b$  was re-estimated as  $B = (a + b) - A$ , where  $A$  = actual soluble fraction. Lag time ( $L$ ) is the time between incubation and the start of degradation, and it was calculated according to the equation  $L = 1/c [\ln(b/B)]$ . Effective degradability (ED) was estimated by using the pre-defined parameters and  $k$ , the outflow rate of digesta from the rumen ( $0.03 \text{ h}^{-1}$ ):

$$\text{ED} = A + [bce^{-(c+k)L}]/(c+k)$$

Disappearance of DM and N measured *in situ* were analysed in a repeated measure arrangement (GLM). Differences between treatments of *in situ* kinetic characteristics were subjected to the Mann-Whitney test.

### Post-rumen digestion

Post-rumen effects of tannins were assessed using the modified tube feeding technique in poultry (Ferraz de Oliveira *et al.*, 1994). Three-week old broilers were starved for 24 h before receiving 50 ml of an aqueous glucose solution by tube. After a further 24 h, each bird received about 10 g of PEG-treated lotus and control (PEG-free lotus) and the control birds received 50 ml of glucose solution by tube. Eight replicate birds were used per treatment. All excreta voided during the 48 h following feeding was quantitatively and individually collected, and was then frozen ( $-20^\circ\text{C}$ ), freeze dried, and finely ground (1 mm). The excreta voided by the control group birds were considered as an estimate of bird endogenous losses. True dry matter digestibility (TDMD) for PEG-treated lotus and the control was calculated according to the equation:

$$\text{TDMD} = \frac{[\text{DMin} - (\text{DMexc} - \text{DMexc}_{(G)})]}{\text{DMin}}$$

Where, DMin = dry matter intake (g); DMexc = dry matter excreted (g); DMexc<sub>(G)</sub> = dry matter excreted by the glucose fed birds (g).

Similarly, the coefficients of N retention (CNR) were calculated when N was substituted for DM in the above equation. Differences between treatments were analysed using the Mann-Whitney test.

All data were analysed using Statistical Package for Social Sciences (SPSS). All animal studies were conducted with the approval of the welfare committees of SAC and DPAA.

## Results

All animals survived the treatments in good health.

### Effects of lotus tannins on rumen degradation

Dry matter (DM) and N disappearances from the bags incubated in the rumen for the control and PEG-treated lotus are presented in Figs 20.1 and 20.2. PEG treatment significantly ( $P < 0.05$ ) increased DM and N disappearances of lotus from the bags at all times of incubation.

Ruminal degradation of the two treatments was reasonably described ( $R^2 = 0.98 \pm 0.004$ ) by the exponential equation defined by Ørskov and McDonald (1979).

The degradability characteristics of DM and N are found in Table 20.1. Lotus treated with PEG had significantly ( $P < 0.05$ ) higher values in all the DM degradability characteristics, except for the lag time (L), slowly degradable fraction (B) and effective degradability (ED) ( $P > 0.05$ ). The use of PEG resulted in higher ( $P < 0.05$ ) N degradability characteristics, except for the lag time (L) and effective degradability (ED) ( $P > 0.05$ ). However, the slowly degradable fraction (B) was significantly ( $P < 0.05$ ) lower for the PEG-treated lotus than the untreated lotus.

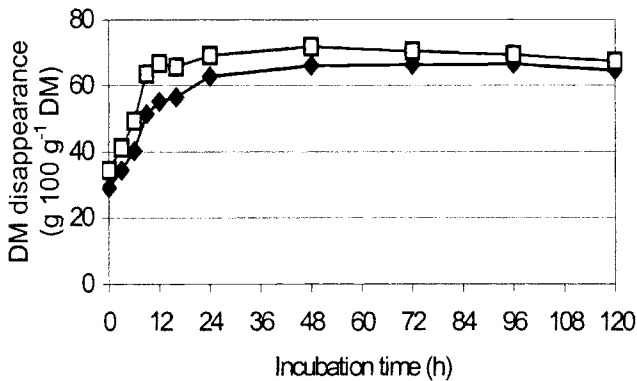


Fig. 20.1. Rumen degradability of *Lotus pedunculatus*: *in situ* dry matter (DM) disappearance curves of the control and PEG-treated lotus ( $n = 6$ ).

Lotus (control) ◆; Lotus/PEG □.

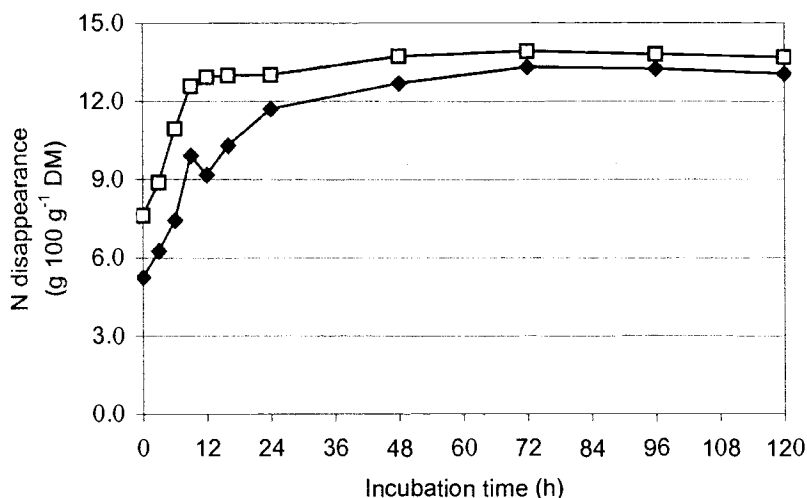


Fig. 20.2. Rumen degradability of *Lotus pedunculatus*: *in situ* nitrogen (N) disappearance curves of the control and PEG-treated lotus (n = 6).

Lotus (control) ◆; Lotus/PEG □.

Table 20.1. Ruminal kinetic characteristics and effective degradability of dry matter (DM) and nitrogen (N) of PEG-treated *Lotus pedunculatus* and control.

	Control	PEG-treated lotus
Dry matter		
A (g 100 g <sup>-1</sup> of DM)	28.8 <sup>a</sup>	34.4 <sup>b</sup>
B (g 100 g <sup>-1</sup> of DM)	36.9	35.4
A + B (g 100 g <sup>-1</sup> of DM)	65.7 <sup>a</sup>	69.8 <sup>b</sup>
c (% h <sup>-1</sup> )	0.110 <sup>a</sup>	0.196 <sup>b</sup>
Lag time (h)	1.8	2.1
ED (g 100 g <sup>-1</sup> of DM)	51.4	53.5
Nitrogen		
A (g 100 g <sup>-1</sup> of DM)	5.2 <sup>a</sup>	7.6 <sup>b</sup>
B (g 100 g <sup>-1</sup> of DM)	8.0 <sup>a</sup>	6.0 <sup>b</sup>
A + B (g 100 g <sup>-1</sup> of DM)	13.2 <sup>a</sup>	13.6 <sup>b</sup>
c (% h <sup>-1</sup> )	0.073 <sup>a</sup>	0.208 <sup>b</sup>
Lag time (h)	0.5	1.8
ED (g 100 g <sup>-1</sup> of DM)	10.6	11.0

<sup>ab</sup> Data in each row with different superscripts are significantly different ( $P < 0.05$ ).



### Effects of lotus tannins on post-rumen digestion

The data obtained from poultry demonstrated that TDMD and CNR were significantly ( $P < 0.05$ ) higher in PEG-treated lotus compared with control. The TDMD for treated and untreated lotus were  $0.725 \pm 0.16$  and  $-0.048 \pm 0.091$ , respectively.

The CNR values of the treated and untreated lotus were  $-0.13 \pm 0.55$  and  $-2.240 \pm 0.476$ , respectively. Total N excretion was considerably higher for the control ( $2.1 \pm 0.11$  g) than for PEG-treated lotus ( $1.5 \pm 0.13$  g).

As can be clearly seen, the addition of PEG to lotus forage improved the TDMD from  $-0.048$  to  $0.725$  suggesting that tannins have a substantial negative effect in post-rumen digestion of nutrients and/or on endogenous losses. Treatment of lotus with PEG improved the CNR from  $-2.24$  to  $-0.13$ , indicating that lotus increases endogenous losses in the lower gut of animals due to the presence of tannins.

### Conclusions

The reduction in the disappearance of DM and N in the rumen caused by lotus tannins may be due to the formation of tannin-protein complexes that are unavailable for microbial degradation at rumen pH. A larger quantity of dietary N will be available for post-rumen digestion and absorption in the small intestine when the lotus tannins are present.

The results shown above suggest that tannins in the duodenum increase the endogenous protein loss of the animal. This is of extremely high nutritional cost to the animal and supports the hypothesis that tannins can still adversely affect the lower gastrointestinal tract (GIT) of animals. The use of tannins as protein protection agents for ruminants and for their anthelmintic properties (Athanasidou *et al.*, 2001) needs to be considered with care to avoid adverse effects. Thus the type, form (either bound or free) and concentration of tannins used, require judicious selection. Lotus tannins exerted a negative effect on post-ruminal digestibility of DM and N (TDMD and CNR) when using poultry as a model. Others have reported the impairment of N absorption from the small intestine in tannin-fed animals. Barry *et al.* (1986) reported an increased faecal N excretion in sheep fed on *L. pedunculatus*. Similarly, Reed *et al.* (1990) and Wiegand *et al.* (1995) found that feeding high tannin fodders increased the proportion of faecal excretion of N and also the proportion of N attached to the NDF. These results are supported by the findings reported in this chapter. Furthermore, the increased excretion of N caused by tannins is due to increased endogenous losses from the gut of broilers used in our study. This may also occur in the duodenum of ruminants that have consumed tannins (Reed *et al.*, 1990; Wiegand *et al.*, 1995). The binding of the released (free) tannins to structural proteins within the GIT, along with the increased secretion of endogenous proteins from the intestine and the reduced activity of digestive enzymes, could be

factors in reducing the apparent digestibility of rumen undegradable protein in ruminants. Lesions of the gut mucosa have been reported in tannin-fed animals (Dawson *et al.*, 1999), and these may influence absorption of nutrients in the intestine. The negative CNR values observed in PEG-treated lotus indicate the presence of other factors that may have increased the N excretion.

Lotus tannins have effects not only in the rumen but also in the lower gut and may induce negative effects on post-rumen digestion of nutrients.

## Acknowledgements

Dr Miguel Bugalho (Centre for Applied Ecology, Lisbon, Portugal) for his advice on statistical analysis. This work is supported by a Praxis XXI studentship (Ministry of Science and Technology, Fundação para a Ciência e Tecnologia, Portugal). SAC is financially supported by Scottish Executive Environment and Rural Affairs Department.

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## Chapter 21

# Cyclopiazonic Acid: Food Chain Contaminant?

W.L. Bryden<sup>1,3</sup>, S. Suksupath<sup>1</sup>, D.J. Taylor<sup>1</sup> and B.C. Prasongsidh<sup>2</sup>

<sup>1</sup>Faculty of Veterinary Science, University of Sydney, Camden NSW 2570, Australia; <sup>2</sup>School of Food Science, University of Western Sydney Hawkesbury, Richmond NSW 2753, Australia; <sup>3</sup>Present address for correspondence: School of Animal Studies, University of Queensland, Gatton Qld. 4343, Australia

Cyclopiazonic acid (CPA) (Fig. 21.1) was discovered during toxicity screening of fungi isolated from groundnuts (Holzapfel, 1968). Although originally isolated from *Penicillium cyclopium* (now *P. griseofulvum*), CPA is produced by several species of the genera *Penicillium* and *Aspergillus*, including *Aspergillus flavus*. Significantly, CPA is produced by aflatoxigenic strains of *A. flavus* but not *Aspergillus parasiticus* and is likely to occur with aflatoxin (Bryden, 1991). Aflatoxin is a frequent contaminant of major agricultural commodities (Pittet, 2001) but surveys of the occurrence of CPA in agricultural commodities are limited.

### Occurrence and Association with Disease

The widespread occurrence of fungi capable of producing CPA signals the likelihood of contamination of agricultural commodities and field intoxications with this toxin. The natural occurrence of CPA was first reported in 1978, when maize contaminated by *A. flavus* was found to contain CPA (Gallagher *et al.*, 1978). Later, Lansden and Davidson (1983) reported natural contamination of peanuts with CPA and Le Bars (1979) showed contamination of cheese, Rao and

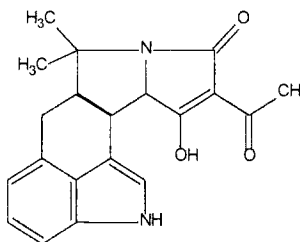


Fig. 21.1. Chemical structure of cyclopiazonic acid (CPA).

Husain (1985) demonstrated contamination of kodo millet and Stoltz *et al.* (1988) described contamination of maize.

Naturally occurring CPA toxicosis has not been unequivocally confirmed but there are a number of disease episodes (Table 21.1) where there is good circumstantial evidence for CPA involvement. The outbreak of mouldy maize toxicosis in cattle described by Albright *et al.* (1964) was attributed to the toxic effects of three fungal species isolated from the maize. One species, *P. cyclopium*, has since been shown to produce CPA. Of the syndromes listed in Table 21.1, the episode of reproductive failure and feed refusal in sows is the least convincing as a possible CPA intoxication due to a lack of information on effects of CPA in sows. However, in this case 10 mg CPA kg<sup>-1</sup> was detected in the mouldy sunflower screenings responsible for the field case (Ross *et al.*, 1991). The feedstuffs associated with Kodua poisoning (Rao and Husain, 1985) and death of quails (Stoltz *et al.*, 1988) contained CPA. In these latter cases and the other cases in Table 21.1, affected people, animals and birds exhibited neurological signs during the course of the disease. Neurological signs especially of a neuromuscular nature are characteristic of CPA intoxication (Bryden, 1991). Stoltz *et al.* (1988) based their diagnosis of CPA toxicosis of quail on clinical signs of opisthotonus at death, histopathology and a level of 6 mg CPA kg<sup>-1</sup> feed. The feed also contained aflatoxin (0.45 mg kg<sup>-1</sup>) and ochratoxin A (0.5 mg kg<sup>-1</sup>) but the neuromuscular signs displayed by the quail and the relative levels of toxins found would implicate CPA as the major cause of death.

It is accepted that aflatoxin is the causative agent of Turkey 'X' disease and it was from this disease outbreak in 1961 that aflatoxin was discovered. Cole (1986) presented a persuasive case for the involvement of CPA in this syndrome after reviewing the original reports. The neurological signs (including opisthotonus) and enteritis observed in Turkey 'X' disease are not associated with aflatoxicosis but if CPA was also present in the Brazilian groundnut meal that caused the syndrome, then the clinical signs exhibited by the turkey poult could be totally explained (Cole, 1986). Bradburn *et al.* (1994) subsequently established that the turkey poult had ingested groundnut meal containing CPA as high as 31 µg kg<sup>-1</sup>.

**Table 21.1.** Field cases with suspected cyclopiazonic acid involvement.

Disease	Species	Feedstuff	Reference
Mortality	Cattle	Maize	Albright <i>et al.</i> (1964)
Mortality	Cattle	Barley	Harrison (1971)
Staggers	Cattle	Mixed feed	Hacking and Harrison (1976)
Kodua poisoning	Man, cattle	Millet	Rao and Husain (1985)
Turkey 'X' disease	Poults	Groundnuts	Cole (1986)
Mortality	Quail	Mixed feed	Stoltz <i>et al.</i> (1988)
Reproductive failure	Pigs	Sunflower	Ross <i>et al.</i> (1991)

## Effects in Poultry and Farm Animals

Cyclopiazonic acid is an indole tetramic acid and toxicological studies with CPA have been conducted in several species including rats, mice, chickens, pigs and dogs (for review see Bryden, 1991). Species vary in their susceptibility to acute exposure to CPA, with LD50 values ranging from 2.3 to 69.6 mg kg<sup>-1</sup> for the rat and quail, respectively (Bryden, 1991). The principal target organs are the liver, kidney and digestive system. The toxin acts primarily as an entero-nephrotoxin in chickens and pigs and primarily as an hepatotoxin in rats.

Dorner *et al.* (1983) found that diets containing 100 mg CPA kg<sup>-1</sup> significantly reduced chicken growth rate and feed intake. This dietary concentration approximates to a daily intake of 40 mg kg<sup>-1</sup> bodyweight or three times the LD50 of the chicken (Wilson *et al.*, 1989). In subsequent studies with growing chickens and laying hens intakes exceeding 5 mg kg<sup>-1</sup> bodyweight resulted in high mortality (Cullen *et al.*, 1988; Cole *et al.*, 1988; Suksupath, 1993). The discrepancy between the different studies presumably relates to the dosing protocol and the manner in which CPA was administered. In the study by Dorner *et al.* (1983) purified CPA was added to the diet, whereas in the latter studies, CPA was administered daily in gelatin capsules. The bolus of CPA administered when dosing with capsules may allow for greater gastrointestinal absorption of CPA. In contrast, addition of the purified toxin to feed is likely to reduce CPA availability through chelation with dietary constituents.

Broiler growth rate, feed intake and feed conversion efficiency are depressed by CPA (Dorner *et al.*, 1983; Wilson *et al.*, 1990; Suksupath, S., Bryden, W.L. and Cole, R.J., 1988, unpublished observations). Likewise, laying hens ingesting CPA reduce feed intake and egg production but egg weight was not changed (Cole *et al.*, 1988; Suksupath, 1993). The most noticeable feature of studies with laying hens is the marked deterioration of egg shell quality that accompanies CPA intoxication. Hens receiving daily doses of 5 mg kg<sup>-1</sup> bodyweight laid eggs that were either badly cracked or shell-less and all hens died within 7 days. No hens died when dosed with 2.5 mg kg<sup>-1</sup> day<sup>-1</sup> but their eggs had significantly thinner shells and were often visibly cracked (Cole *et al.*, 1988; Suksupath, 1993). Suksupath (1993) conducted a 28 day study in which hens were dosed daily with 2.5 mg CPA kg<sup>-1</sup> and artificially inseminated weekly. Bodyweight was unchanged during the study and feed intake, although depressed by 30% during the first week of dosing, returned to normal thereafter. Although egg production was reduced by 40% over the whole period it was reduced by 65% during week two and then began to increase. Egg shell thickness was reduced by CPA and this was reflected in a reduction in the number of eggs suitable for incubation. The fertility of hens was not diminished by CPA but hatchability dropped by 30%. Of the embryos that failed to hatch all appeared normal and hatched chicks grew normally. On the other hand, CPA reduced semen volume and sperm number and increased the number of abnormal spermatozoa in mature male chickens (Suksupath *et al.*, 1990).

Pigs are susceptible to CPA ingestion (Lomax *et al.*, 1984) but there have been no detailed studies of chronic CPA toxicosis in this species. Similarly, no long-term studies have been conducted with ruminants. Feed intake and milk production were significantly depressed within 24 hours of ewes receiving an oral dose of 5 mg CPA kg<sup>-1</sup> bodyweight (Cole *et al.*, 1988; Elrington, 1991). This is the only report of experimental CPA toxicosis in a ruminant species and the response of sheep suggests that further studies are warranted.

Another area of concern is the likely impact of CPA on immune function. The toxin did not affect delayed cutaneous hypersensitivity, complement activity, intracutaneous mitogen and *in vitro* lymphocyte blastogenesis in guinea pigs (Richard *et al.*, 1986; Pier *et al.*, 1989). Interestingly, CPA appears to restore the suppressive effects of aflatoxin in delayed cutaneous hypersensitivity response and *in vitro* lymphocyte blastogenesis (Pier *et al.*, 1989). It appears that CPA is without effect on cell-mediated immunity but studies in rats suggest that it may modulate antibody formation (Hill *et al.*, 1986) which would be consistent with the reduction in lymphoid tissue observed in dogs and chickens during CPA toxicosis (Dorner *et al.*, 1983; Nuehring *et al.*, 1985).

## Presence in the Food Chain

As noted above, CPA can occur in a range of plant commodities and represents a potential problem to farmers who feed their animals grain-based diets, especially those based on maize. The toxin has also been found in cheese and cured meats that have been subject to fungal spoilage (Bryden, 1991). Studies of the distribution and excretion of radiolabelled CPA in rats and chickens have shown that the toxin accumulates to a significant degree in skeletal muscle (Norred *et al.*, 1985, 1988). Accordingly, CPA may contaminate carcasses and meat products of animals ingesting this mycotoxin. This raises the possibility that CPA residues may be found in other animal products and studies in the authors' laboratory have demonstrated the presence of CPA in eggs of laying hens and milk of sheep dosed orally with CPA (Dorner *et al.*, 1994). In a series of studies Prasongsidh (1998) was able to demonstrate that CPA could be found in cheese curd, butter and cream manufactured from contaminated milk. He was also able to show that CPA could not be completely eliminated by heat-treatment during milk processing, storage or the processing and manufacture of dairy products. It can be concluded that animal products could be a source of CPA for humans.

A realistic evaluation of the risk presented by CPA in the food chain requires more detailed information on the toxicology of this mycotoxin and its occurrence. To date, difficulties with analysis have precluded large scale surveys (Bryden, 1991). Despite occurring naturally and being produced on a range of commodities by ubiquitous fungi, CPA remains neglected, although exposure to the toxin may be widespread. The demonstration of CPA residues in milk, eggs and meat further highlights possible exposure. Moreover, the clinical signs of CPA intoxication result from disturbance of cellular calcium metabolism following specific

inhibition of calcium dependent ATPase (for review see Riley, 1998; Norred and Riley, 2001). The importance of calcium homeostasis for normal cell metabolism would suggest that chronic or low level exposure to CPA may significantly impact on human and animal health.

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## Chapter 22

# Calystegines Isolated from *Ipomoea* spp. Possibly Associated with an Ataxia Syndrome in Cattle in North Western Australia

P.R. Dorling<sup>1</sup>, S.M. Colegate<sup>2\*</sup>, J.G. Allen<sup>3</sup>, R. Nickels<sup>4</sup>, A.A. Mitchell<sup>5</sup>, D.C. Main<sup>3</sup> and B. Madin<sup>6</sup>

<sup>1</sup>Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch WA 6150, Australia; <sup>2</sup>Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Victoria 3220, Australia; <sup>3</sup>Department of Agriculture, 3 Baron-Hay Court, South Perth, WA 6151, Australia; <sup>4</sup>Department of Agriculture, 283 Marine Terrace, Geraldton, WA 6530, Australia; <sup>5</sup>Northern Australia Quarantine Strategy, Australian Quarantine Inspection Service, C/o NT DPIF, PO Box 990, Darwin, NT 0801, Australia; <sup>6</sup>Department of Agriculture, 8 Hedland Place, Karratha, WA 6714, Australia

\*Author to whom correspondence should be addressed.

*Ipomoea muelleri* (morning glory) (Convolvulaceae) was suspected as a cause of cattle deaths in the north of Western Australia in the middle of the last century (Gardner and Bennetts, 1956). Then in the early 1960s, serious sheep losses in the western Gascoyne district of Western Australia were caused by consumption of this plant (Gardiner *et al.*, 1965). A disease with similar clinical signs, but caused by consumption of *Ipomoea* sp. Q6 (aff. *calobra*) (Weir vine), occurs in Queensland (Everist, 1974; Dowling and McKenzie, 1993). Until recently, lysergic acid amides were considered to be the causative toxins in both of these poisoning cases (Gardiner *et al.*, 1965; Dowling and McKenzie, 1993).

New studies into *Ipomoea* sp. Q6 (aff. *calobra*) revealed the presence of the glycosidase inhibitors swainsonine and calystegine B2 in the seeds. Furthermore, lesions present in the brain tissues of sheep and cattle poisoned by this plant in the field, were similar to those seen in swainsonine intoxication (Molyneux *et al.*, 1995). More recently, a neurological condition in goats in Mozambique, which manifested as ataxia, head tremors and nystagmus, was shown to be caused by the consumption of *Ipomoea carnea*, the leaves of which were found to contain swainsonine and calystegines B2 and C1 (de Balogh *et al.*, 1999) (Fig. 22.1).

Notable losses of sheep caused by the consumption of *I. muelleri* have not been reported since the 1960s. However, current pastoralists in the Gascoyne and West Pilbara areas of Western Australia believe that a transient staggering condition seen in some sheep being moved along stock routes is due to the

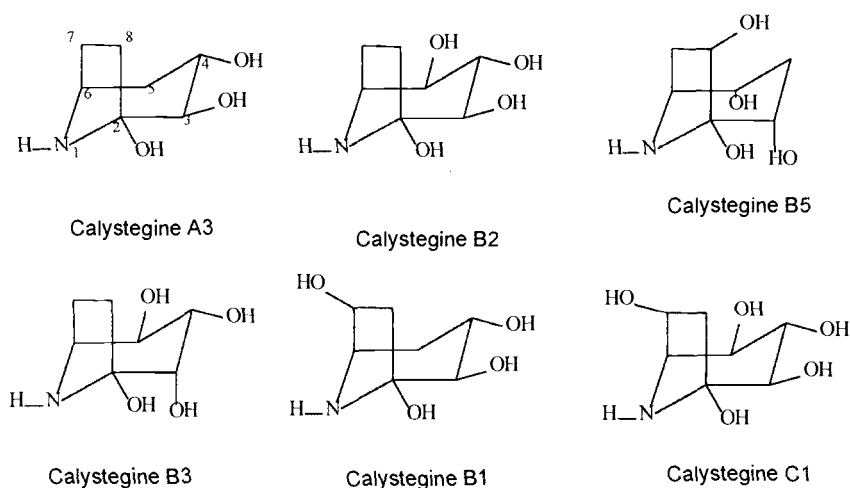


Fig. 22.1. Structures of calystegines found in *Ipomoea* spp.

consumption of this plant which grows along the stock routes (Peter Grey, Glenflorrie Station, 2001, personal communication). Additionally, on at least three occasions in the 1990s, a syndrome involving permanent hindquarter ataxia was reported in adult cattle grazing native *Ipomoea* spp. in the Gascoyne and West Pilbara areas of Western Australia. This condition in cattle sounded similar to the disease seen in sheep in the 1960s, and pastoralists described affected animals as appearing drunk.

These native *Ipomoea* spp. often provide the first green feed at the start of the wet season and the last green feed in the following dry season. Affected cattle were first observed during the wet season and usually perished during the following dry season. Veterinarians have observed affected animals but until very recently no samples have been collected for submission to laboratories. It was decided to collect samples of the plants and to examine them for the presence of swainsonine and calystegines.

## Phytochemical Investigation

Samples of *I. muelleri* and *Ipomoea lonchophylla* were collected from the West and East Pilbara and East Kimberley regions of north Western Australia, from areas where the ataxia syndrome had been observed, but not at the time of the poisonings.

The filtered, methanol extract of air-dried, finely ground whole plant was evaporated to dryness and the residue extracted with water. The filtered, acidified (pH 4) aqueous extract was applied to a strong cation exchange solid phase

extraction (SPE) column. The SPE column was then washed with deionised water and methanol. The adsorbed alkaloids were eluted with a solution of ammonium hydroxide in methanol (1% v/v). Transferred to a sample vial, the dried samples were treated with a pyridine (10): hexamethyldisilazane (HMDS) (9): trifluoroacetic acid (TFA) (1) solution (0.5 ml) at room temperature for at least 1 h before being qualitatively analysed using GC/MS and, for quantitation purposes, GC/MS/MS.

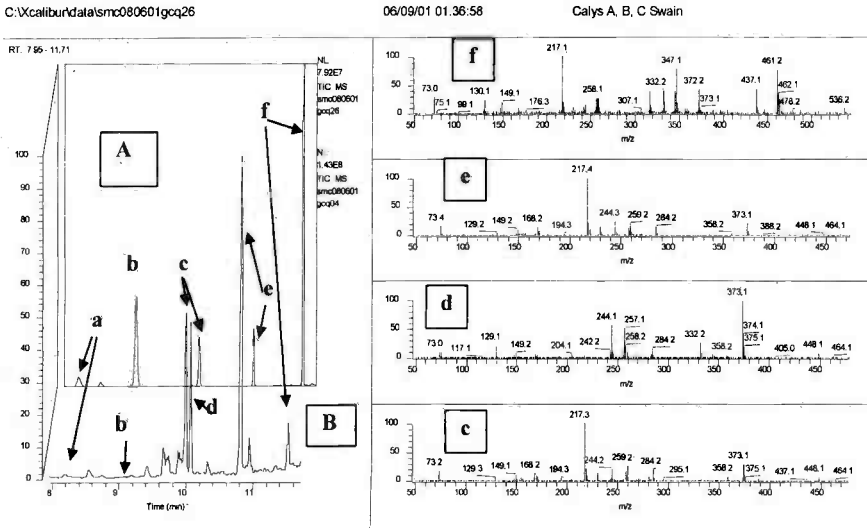
The MS/MS quantitation sequences utilized ions at  $m/z$  285 (tri-TMScalystegine A,  $M^+$  - TMSOH), 373 (tetra-TMScalystegine B,  $M^+$  - TMSOH), 461 (penta-TMScalystegine C,  $M^+$  - TMSOH) and 389 (triTMSswainsonine). Note that the use of the pyridine/HMDS/TFA reagent only derivatized the hydroxyl functional groups. Molyneux *et al.* (1995) noted the differences in the respective mass spectra obtained for the O-derivatized calystegines compared to the N- and O-derivatized compounds.

In addition to observing and quantitating those calystegines (A3, B2, B3 and C1) for which authenticated standards were available, another calystegine was observed in all the plants examined (Fig. 22.2). Examination of the mass spectrum of this unidentified calystegine indicated that it belonged to the B group ( $M^+$  463, 1%; 448, 8%,  $M^+$ -CH<sub>3</sub>; 373, 100%,  $M^+$ -TMSOH). The major difference between the mass spectra of the unidentified calystegine and the standard calystegines B2 and B3 was the lack of a strong ion at  $m/z$  217. The ion at  $m/z$  217 is due to TMSO-CH=CH-CH=O<sup>+</sup>TMS which is lost from calystegines (such as B2, B3 and C1) that possess a 1,2,3-trihydroxy entity (Molyneux *et al.*, 1995). Therefore, the unidentified calystegine is without this 1,2,3-trihydroxy character and is thus similar to B1 or B5 (Fig. 22.1). A related observation that this unidentified calystegine is the main calystegine in *Ipomoea violacea* (Colegate, S.M., Mills, J. and Dorling, P.R., unpublished results) will enable isolation and conclusive identification of this compound. The tentative assignment as a B1-like calystegine is supported by observation of an ion at  $m/z$  257, 57% (rather than  $m/z$  259, 20% as with calystegines B2 and B3). It is indicative of the loss of TMSOH from carbons 7 and 8 in the five-membered ring of the *nor*tropane skeleton.

A quantitative standard was not available for the unidentified B1-type calystegine, however, based on total ion response the levels seem to be about the same as observed for calystegine B3. This conclusion is supported by the GC/MS/MS response for the ion at  $m/z$  373 after making allowance for the observation that the 373 ion is about fivefold more relatively abundant from the unidentified calystegine B compared to that for B2 and B3. The estimated content of calystegines A3, B2, B3 and C1 in the plants (Table 22.1) are the means of duplicate analyses.

## Conclusions

GC/MS/MS analysis of extracts of *I. muelleri* and *I. lonchophylla* showed the



**Fig. 22.2.** Total ion chromatogram of derivatized calystegine and swainsonine standards (A) and base extract from *I. muelleri* (Accession number PRP838) (B). a: calystegine A3; b: swainsonine; c: calystegine B3; d: unidentified B1-type calystegine; e: calystegine B2; f: calystegine C1.

presence of calystegines A3, B2, B3, the B1-like calystegine and C1 (B2 > B1-like, B3 > C1 > A3; total calystegines between 1 and 10 mg kg<sup>-1</sup> dry weight (DW) of plant) and only a barely detectable trace of swainsonine. This is in contrast to the analyses of *I. carnea*, *I. sp. Q6* (aff. *calobra*) and *Ipomoea polpha* by others (Molyneux *et al.*, 1995; de Balogh *et al.*, 1999) where swainsonine was a major component of the plants.

Swainsonine and the calystegines are all glycosidase inhibitors (Molyneux *et al.*, 1993; Nash *et al.*, 1998), but only swainsonine has been shown experimentally to cause neurological disease. The calystegines *per se* have not been unequivocally associated with field intoxication syndromes. However, *Solanum dimidiatum* and *Solanum kwebense*, which cause Crazy Cow syndrome in Texas and Maldronksiekte (translated as mad-drunk disease) in South Africa respectively, both contain calystegines (Nash *et al.*, 1993). Both syndromes are characterized by neurological signs of cerebellar dysfunction such as incoordination and staggering. Histologically, the most striking change is severe vacuolation and degeneration of the Purkinje cells in the cerebellum.

When calystegine A3 was delivered to a mouse, via an implanted osmotic pump, at a rate of 10 mg kg<sup>-1</sup> day<sup>-1</sup> for 28 days, extensive vacuolation of the liver Kupffer cells was observed (Watson *et al.*, 2000). A similar effect was not observed when mice were dosed with calystegine B2.

In the dry season of 2001 (June) a 2-year-old Brahman heifer affected with a hindquarter ataxia condition on a property in the Gascoyne was necropsied. This heifer had consumed *I. muelleri* during the wet season, had then become

**Table 22.1.** Calystegines A3, B2, B3 and C1 content of *I. lonchophylla* and *I. muelleri*.

Plant sample Name (accession number)	Calystegine mg kg <sup>-1</sup> DW (% of total)				
	A <sub>3</sub>	B <sub>2</sub>	B <sub>3</sub>	C <sub>1</sub>	Total
<i>I. lonchophylla</i> (C61)	0.05 (2%)	2.69 (82%)	0.31 (9%)	0.25 (7%)	3.30
<i>I. muelleri</i> (PRP838)	0.18 (2%)	5.17 (70%)	1.27 (17%)	0.74 (10%)	7.36
<i>I. muelleri</i> (AAM5537)	0.01 (0.6%)	1.27 (83%)	0.10 (6.4%)	0.15 (10%)	1.53
<i>I. muelleri</i> (AAM4733)	0.12 (4%)	0.74 (88%)	0.17 (7%)	0.04 (1%)	1.07

clinically affected, and was deteriorating as the dry season progressed. There was no significant gross pathology. Microscopically there was apparent patchy loss of Purkinje cells, proliferation of Bergmann's glial cells, and very occasional spheroids in the molecular layer in the cerebellum. In the cervical, thoracic and lumbar/sacral cord there was mild to moderate Wallerian degeneration in all areas of the white matter, with no tracts being obviously more affected than others.

Pen feeding trials with the plants and experimental evidence that the calystegines can produce an ataxia syndrome in cattle will be necessary to establish that consumption of *I. muelleri* and *I. lonchophylla* by cattle may have caused the clinical signs observed.

## Acknowledgements

Dr R. Nash of the Institute of Grassland and Environmental Research in Aberystwyth, UK, supplied the authenticated samples of calystegines A3, B2, B3 and C1 used as standards in this study.

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## Chapter 23

# **Spectrofluorometric Analysis of Phylloerythrin in Plasma and Skin from Sheep Suffering from Hepatogenous Photosensitization**

E. Scheie<sup>1</sup>, B.L. Smith<sup>2</sup> and A. Flåøyen<sup>1,3</sup>

<sup>1</sup>*National Veterinary Institute, PO Box 8156 Dep., 0033 Oslo, Norway;*

<sup>2</sup>*AgResearch, Ruakura Research Centre, Private Bag, Hamilton, New Zealand;*

<sup>3</sup>*Norwegian School of Veterinary Science, PO Box 8146 Dep., 0033 Oslo, Norway*

### **Introduction**

In hepatogenous photosensitization diseases, hepatic excretion of phylloerythrin is decreased or ceased causing retention of phylloerythrin (Flåøyen, 1999). Phylloerythrin is a metabolic product of chlorophyll produced in ruminants by rumen microorganisms (Rimington and Quin, 1933; Quin *et al.*, 1935). Unlike chlorophyll, which has a long phytol side chain preventing it from being absorbed from the digestive tract, phylloerythrin has lost the side chain and can be absorbed from the gut. Normally, phylloerythrin is conjugated by the liver and excreted into the bile, but some types of liver damage or liver dysfunction impair the hepatic elimination of phylloerythrin. Thus, phylloerythrin enters the bloodstream and reaches the skin cells where it accumulates intracellularly (Slater and Riley, 1966). Phylloerythrin is a porphyrin (Fischer and Hilmer, 1925) and has its absorption (about 420 nm) in the visible region. An acute inflammatory response of the skin is induced when phylloerythrin is excited by sunlight. Excitation initiates reactions that require molecular oxygen. Excited phylloerythrin molecules transfer their energy to oxygen, thus causing the formation of toxic singlet oxygen or oxygen radicals. Singlet oxygen is believed to be the main cytotoxic agent of porphyrin-like molecules (Weishaupt *et al.*, 1976; Agarwal *et al.*, 1992).

The present work was undertaken to study the increase in phylloerythrin concentration in the plasma and skin of sheep dosed with sporidesmin, a mycotoxin known to cause liver damage resulting in phylloerythrin retention and photosensitization.

## Materials and Methods

Fifteen Romney x Polled Dorset weaned female lambs, approximately 5 months old were randomly selected from a commercial sheep farmer. Eleven of these lambs were dosed with 0.25 mg sporidesmin  $\text{kg}^{-1}$  bodyweight on each of 2 consecutive days. Four undosed lambs were kept with the sporidesmin-dosed lambs as controls. Both the sporidesmin-dosed lambs and the controls were kept in open pens and were exposed to natural sunlight for most of each day. After the onset of photosensitization the lambs were housed indoors in darkened conditions. The lambs were fed on green pellets of lucerne until day 12 and on fresh cut grass *ad libitum* for the remaining experimental period. The lambs were killed by captive bolt stunning and exsanguination on day 26 of the experiment. Tissue samples from the necropsies were taken, but data from that part of the study are not included in this chapter.

Sporidesmin was extracted (Mortimer and Ronaldson, 1983) from cultures of *Pithomyces chartarum* at AgResearch, Ruakura, New Zealand.

Phylloerythrin was provided by Porphyrin Products (Logan, UK). The dye was dissolved in methanol to a concentration of 1 mg  $\text{ml}^{-1}$  as a stock solution and stored at  $-20^{\circ}\text{C}$ .

Fluorescence emission and excitation spectra were measured by the means of a Perkin-Elmer LS-50B luminescence spectrometer (Perkin-Elmer, Norwalk, USA) equipped with the red-sensitive photomultiplier (red-sensitive PMT, Hamamatsu, R-928), to facilitate measurement in the spectral region from 400 to 900 nm.

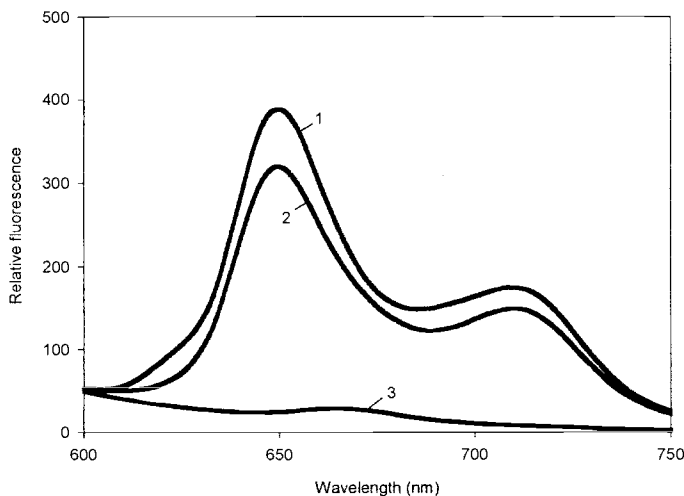
Plasma measurements were performed by direct spectrofluorometric examination in a 1 ml quartz cuvette. Skin measurements were done with an external fibre optic probe (Perkin-Elmer, Norwalk, CT) consisting of a 1 m-long fibre optic attached to the LS-50B. The readings were taken by an operator holding the free end of the optic probe on an unpigmented part of skin adjacent to but just to the area left of the vulva. Optimal sensitivity was achieved with a constant 1 cm distance between the fibre optic end and the sample, achieved by fixing the fibre optic end in a metal tube. All skin and plasma readings were conducted in darkened conditions.

## Results and Discussion

The two first cases of photosensitization from the outside lambs occurred 10 days after sporidesmin dosing. Eight out of 11 sporidesmin-dosed lambs became photosensitized during the experimental period. One became photosensitized on day 11, two on day 13, and one on days 14, 16 and 17, respectively.

The spectrum of the fluorescence from the specimens of plasma from photosensitized sheep was very similar to that obtained from plasma from healthy animals to which was added phylloerythrin (Fig. 23.1). The emission spectrum





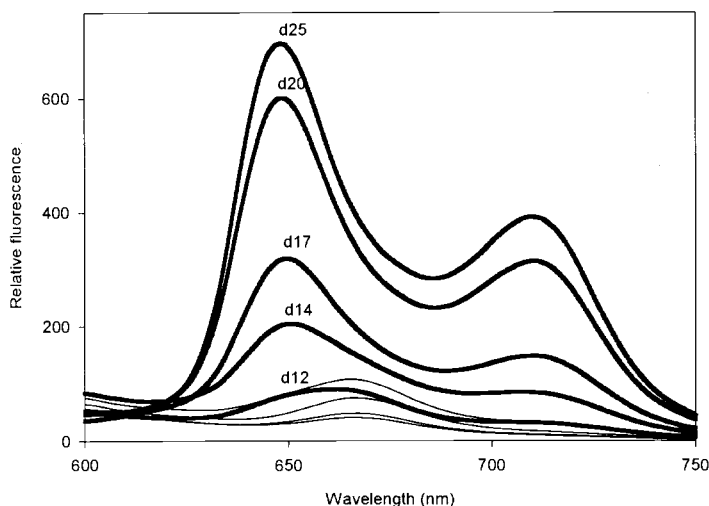
**Fig. 23.1.** The fluorescence spectra of plasma obtained from a healthy lamb to which phylloerythrin had been added (curve 1), from a photosensitized lamb 17 days after being dosed with  $0.25 \text{ mg sporidesmin kg}^{-1}$  bodyweight for 2 consecutive days (curve 2) and from a control (curve 3). The maximum of the shorter wavelength band occurs at 649 nm and the longer at 709 nm. Excitation wavelength was 422 nm for all spectra.

from plasma from a healthy animal without addition of phylloerythrin had a minor fluorescence at 670 nm and is shown in Fig. 23.1 as a baseline.

All the plasma obtained from photosensitized lambs exhibited strong phylloerythrin-like fluorescence having identical spectra; maximum of two main fluorescence bands at  $649 (\pm 1) \text{ nm}$  and  $709 (\pm 1) \text{ nm}$  (Fig. 23.2) and excitation maximum at 422 nm.

At the beginning of the experiment, all animals had a small fluorescence at 670 nm (Fig. 23.2). Then the peak gradually changed to a phylloerythrin spectrum with peaks at 649 and 709 nm, but the middle peak was still present. In most samples the intensity of the spectra from phylloerythrin increased compared to the peak at 670 nm, that it masked the peak totally. There are reasons to suspect that this middle peak is due to metabolized phylloerythrin. Phylloerythrin could be metabolized by the liver and excreted through the bile. Phylloerythrin is a very similar compound to bilirubin, which is conjugated with glucuronic acid in the liver (Kaneko, 1989); the same could be true with phylloerythrin.

From a model and comparison study, with spectra from plasma obtained from healthy sheep with addition of phylloerythrin, we estimated the concentration of phylloerythrin in the photosensitized animals from  $0.3$  to  $0.8 \mu\text{mol l}^{-1}$  the day they were clinically photosensitized. Figure 23.2 shows spectra from plasma of a lamb which became clinically photosensitized on day 14 after dosing, corresponding to



**Fig. 23.2.** Fluorescence spectra obtained from plasma from sporidesmin dosed sheep during the experimental period, last measurements being day 25 after dosing. The sheep was clinically photosensitized on day 14. Excitation wavelength was 422 nm for all spectra.

a concentration of  $0.8 \mu\text{mol l}^{-1}$  phylloerythrin. This increased to  $4.9 \mu\text{mol l}^{-1}$  on day 25.

The fluorescence in the skin showed similar spectra as in plasma. All the animals had a fluorescence signal with a maximum at 670 nm at the beginning of the experiment and in several animals this peak remained throughout the experimental period. The changes to the phylloerythrin-like spectra occurred in the skin 2 to 3 days after the plasma, and the phylloerythrin-like spectrum occurred only in the lambs that showed photosensitization early in the experiment. The excitation maximum of the phylloerythrin-like spectrum was at 421 nm and this gave rise to three fluorescence emissions peaks at 650, 670 and 710 nm respectively.

## Conclusion

Plasma fluorescence can be used to demonstrate the presence of phylloerythrin in hepatogenous photosensitization. The method may also be of value in demonstrating this photodynamic compound in cases in which liver injury may not be readily detected. Such cases may include chronic hepatic insuffering of very high phylloerythrin production.

All spectra obtained from photosensitized animals exhibited strong phylloerythrin spectra at the time they were clinically photosensitized. The

fluorescence spectra from plasma of photosensitized sheep were very similar to those seen in plasma from healthy sheep, to which phylloerythrin had been added. Skin measurements with a fibre optic probe showed phylloerythrin spectra too, but these were delayed compared to clinical signs and measurements in plasma.

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## Chapter 24

# Plants Toxic for Domestic Animals in the State of Paraíba, Northeastern Brazil

R.M.T. Medeiros<sup>1</sup>, F. Riet-Correa<sup>2</sup>, R.C. Barbosa<sup>1</sup>, E.F. Lima<sup>1</sup>,  
S.A.G. Neto<sup>1</sup> and I.M. Tabosa<sup>1</sup>

<sup>1</sup>Faculdade de Veterinária, Universidade Federal da Paraíba, Patos, PB, 58700-000, Brazil; <sup>2</sup>Laboratório Regional de Diagnóstico, Faculdade de Veterinária, UFPel, 96010-900, Pelotas, RS, Brazil

This chapter reviews the plants toxic for ruminants in the State of Paraíba, Northeastern Brazil. Intoxication by *Crotalaria retusa* and *Prosopis juliflora* are reviewed in separate chapters.

### Plants Causing 'Sudden Deaths'

*Mascagnia rigida* is the most important toxic plant of Paraíba. It causes 'sudden deaths', precipitated by exercise, in cattle. Clinical signs can be observed 24-48 hours after the animals have been exposed to the plant. Affected animals may suddenly stagger, tremble and fall with convulsions. They may die within 1-10 minutes after the first clinical signs. Animals less severely affected are reluctant to move and can recover if not forced to walk. Macroscopic lesions are not observed. The main histological lesion, in some animals, is vacuolation of epithelial tubular cells in the kidney. The plant has great variations in its toxicity and its toxic principle is unknown (Tokarnia *et al.*, 1961, 1990, 1994).

*Palicourea marcgravii* is the most important toxic plant in Brazil. It contains fluoracetate and clinical signs and pathology are similar to those described for *M. rigida* (Tokarnia *et al.*, 1990). Intoxication by *P. marcgravii* in the state of Paraíba has been observed in the municipality of Areias.

### Cyanide Poisoning

Cyanide poisoning caused by *Sorghum* spp. or the native trees *Piptadenia* (*Anadenanthera*) *macrocarpa* or *Manihot* spp. is occasionally observed in the state of Paraíba. Experimental intoxications with *P. macrocarpa* from other states from Northeastern Brazil demonstrated a variation in the toxicity of this tree (Tokarnia *et al.*, 1994, 1999). In experiments with *P. macrocarpa* leaves collected

in the municipality of Patos cyanide poisoning was reproduced in cattle with 10 g kg<sup>-1</sup> bodyweight.

Many species of wild *Manihot* are found in the state of Paraíba. At least two species, *Manihot glaziovii* and *Manihot piauhyensis* from other Brazilian regions are experimentally toxic for cattle (Canella *et al.*, 1968; Tokarnia *et al.*, 1994, 1999). Farmers mention that intoxication occurs mainly after the first rainfalls when the plant is sprouting.

### ***Thiloa glaucocarpa* (Cambretaceae)**

*Thiloa glaucocarpa* is a tree that causes nephrosis in cattle in Northeastern Brazil (Tokarnia *et al.*, 1981) and in Paraíba in the municipality of Itabaiana. The intoxication occurs 10-25 days after the first rainfalls after a dry period (Tokarnia *et al.*, 1981).

Clinical signs are characterized by subcutaneous oedema, anorexia, ruminal atony, constipation followed by passage of faeces containing mucus or blood, and nasal serohaemorrhagic discharge. Some animals do not have subcutaneous oedema, but had a dry muzzle with scab formation (Tokarnia *et al.*, 1981).

Gross lesions are characterized by anasarca, oedema of the gastrointestinal wall and mesentery, ascites, hydrothorax, hydropericardium, and ulcerations due to diptheroid necrosis in the mucosa of the larynx, trachea, pharynx and oesophagus. The kidneys are usually pale. The main histological lesion is a tubular nephrosis (Tokarnia *et al.*, 1981).

The tannins vascalagin, castalagin, stachyurin and casuarinin, were identified as the toxic substances responsible for the intoxication (Itakura *et al.*, 1987).

### ***Ipomoea* spp. (Convolvulaceae)**

*Ipomoea carnea* subsp. *fistulosa* is widely distributed in the state of Paraíba. The intoxication occurs during the dry season when the animals ingest large amounts of this plant as the main food for some weeks (Tokarnia *et al.*, 1960). Clinical signs are characterized by depression, weight loss followed by emaciation, weakness, muscular tremors mainly in the neck and head, nystagmus, ataxia, broad-based posture, paresis and final paralysis (Tokarnia *et al.*, 1960; Armién *et al.*, 1998). No significant lesions are observed at necropsies. Cytoplasmatic vacuolation of the neurons, macrophages and epithelial cells of various organs characterize histological lesions. Ultrastructurally the vacuoles are membrane bound containing amorphous membranous fragments or small amounts of granular material within an electron-lucent background (Armién *et al.*, 1998). *Ipomoea carnea* causes a lysosomal storage disease and plants from Paraíba contained swainsonine and calystegines B<sub>1</sub> and B<sub>2</sub> (R.J. Molyneux, Albany, 2000, personal communication).

*Ipomoea asarifolia* is also widely distributed in Paraíba and causes nervous signs in sheep, goats and cattle (Döbereiner *et al.*, 1960). In recent experiments *I. asarifolia* was administered to six goats at 10-37 g kg<sup>-1</sup> bodyweight daily during 7-17 days. All goats had clinical signs 4-7 days after starting ingestion; four died or were killed 7-17 days after the start of ingestion and two recovered after the suspension of the ingestion. Clinical signs were depression and tremors, similar to those caused by tremorgen mycotoxins and phytotoxins. No histological or ultrastructural lesions were observed in the nervous system or other organs. Samples of *I. asarifolia* used in those experiments contained almost undetectable amounts of swainsonine (0.001%) and calystegines (R.J. Molyneux, Albany, and D. Gardner, Logan, 2001, personal communication). These results suggest that *I. asarifolia* contains a tremorgen phytotoxin. Other samples of *I. asarifolia* from Paraíba contained an almost undetectable amount of swainsonine, but several calystegines including calystegines B<sub>2</sub> and C<sub>1</sub> (R.J. Molyneux, Albany, 2000).

### ***Plumbago scandens* (Plumbaginaceae)**

One outbreak of goat mortality was observed in a farm in the municipality of Santa Luzia, located in the semiarid region. The outbreak occurred 2 months after the introduction to the farm of a herd of 90 goats of different ages. Sixty goats died in a period of approximately 3 weeks. Clinical signs observed by the farmer were severe depression, bloat and marked foamy salivation. All affected animals died after a clinical manifestation period of 1-4 days. The farmer claimed that the animals were intoxicated with a plant later identified as *Plumbago scandens*. The fresh plant was given experimentally to four goats at 5, 10, 17.5 and 25 g kg<sup>-1</sup> bodyweight. Depression, anorexia, salivation with foamy saliva, bellowing, bruxism, humpbacked posture, bloat, ruminal atony, continuous lateral head movements, tachycardia, dyspnea and dark brown to black urine were observed in the goats intoxicated with 17.5 and 25 g kg<sup>-1</sup> bodyweight. The goats that ingested 5 and 10 g kg<sup>-1</sup> bodyweight had less severe clinical signs. The goat treated with 25 g kg<sup>-1</sup> bodyweight died approximately 18-20 h after administration. The others recovered in 3-9 days. At necropsy, the main lesions were the presence of a dark violet to black discoloration of the mucosa of the tongue, oesophagus, reticulum and ventral sac of the rumen, and gelatinous oedema in the visceral ruminal peritoneum. Histologically the reticulum and ventral sac of the rumen had diffuse epithelial necrosis and severe oedema and neutrophilic infiltration of the submucosa. Separation of the ruminal epithelium from the submucosa was also observed. Epithelial degeneration and necrosis was also observed in the omasum, oesophagus and tongue (Medeiros *et al.*, 2001a).

The toxicity of *P. scandens* was previously demonstrated experimentally in cattle causing similar signs and pathology to those observed in goats. The ingestion of the plant has been associated with cattle deaths in the state of Bahia, Northeastern Brazil (Tokarnia and Döbereiner, 1982). *Plumbago scandens* has

been used in Northeastern Brazil as a medicinal plant and it contains plumbagin (2-methyl-5-hydroxy, 1:4 naphthoquinone) (Lima *et al.*, 1968), which has anticarcinogenic properties and increases the macrophage bactericidal activity.

### ***Indigofera suffruticosa* Leg. (Papilionoideae)**

Farmers and practitioners from the semiarid region observed the occurrence of red urine in cattle and horses introduced to areas invaded by *Indigofera suffruticosa*. The animals recovered if they were removed from the pastures. Recently it was demonstrated that this plant causes haemoglobinuria in cattle in Northeastern and Northern Brazil (Barbosa Neto *et al.*, 2001).

### ***Aspidosperma pyricollum* (Apocinaceae)**

Farmers from Paraíba claim that *Aspidosperma pyricollum* causes abortion and skeletal malformations in goats. In field conditions malformations associated with the consumption of this plant are characterized mainly by forelimb flexures. In folk medicine *A. pyricollum* has been used to induce abortion in humans, and there is at least one narration in which the mother did not abort after the ingestion of an infusion of the plant and the child was born with leg malformations. In a preliminary experiment the aqueous extract of 20 g kg<sup>-1</sup> bodyweight of *A. pyricollum* was administered by gavage to one pregnant goat. Twenty days after the administration the goat aborted an approximately 4 months old fetus. In another experiment two pregnant goats received daily, during 30 days, 1 g kg<sup>-1</sup> bodyweight, dry *A. pyricollum*. One goat that received the plant between days 77 and 107 of pregnancy delivered two kids with lateral deviation of the metacarpus, which resolved spontaneously in 2 weeks. The other goat that received the plant between 80 and 110 days of pregnancy delivered two normal kids. Another goat that received 0.4 g kg<sup>-1</sup> dried *A. pyricollum* daily during days 85 to 149 of pregnancy delivered two normal goats. These preliminary experiments strongly suggest that *A. pyricollum* causes abortion and skeletal malformation in goats in field conditions.

### **Intoxication by Damaged Sweet Potatoes**

Atypical interstitial pneumonia in cattle associated with the ingestion of damaged sweet potatoes (*Ipomoea batatas*) was reported in the semiarid region. The sweet potatoes were severely damaged by the insect *Myzus persicae*, and had an obvious fungal infection characterized by white, black or grey growth over the surface. Eighteen milking cows, one bull and one steer were fed with approximately 400 kg of those sweet potatoes. Six days after the consumption 13 cows were affected.

Clinical signs were characterized by laboured abdominal breathing with extended and lowered head, coughing, expiratory grunt, salivation and protruded tongue. Six animals died and the others recovered in 4-7 days. At necropsy bullous emphysema and gelatinous exudates were observed in the interlobular, peribronchial and subpleural tissues. Microscopically, the pulmonary lesions were characterized by severe oedema and emphysema, and alveolar epithelial cell hyperplasia. Samples of sweet potatoes were cultured for fungi. *Fusarium* spp. was not isolated, probably because in 48 hours a zygomycete fungus covered the plates. However, it is probable that the sweet potatoes were also infected by some *Fusarium* spp. (Medeiros *et al.*, 2001b).

## Other Toxic Plants

Farmers mentioned the occurrence of haemoglobinuria in cattle caused by *Brachiaria radicans*, a plant intoxication well known in southeastern and southern Brazil. Outbreaks of photosensitization and abortion caused by the ingestion of the fruits of *Stryphnodendrom coriaceum*, *S. obovatum*, *Enterolobium contortissiliquum* and *E. gummiferum* are reported from other northeastern states and probably also occur in Paraíba. Outbreaks of photosensitization caused by *Brachiaria decumbens* are also observed. Other plants considered toxic by practitioners are *Ricinus comunis*, *Lantana* spp. and *Leucaena leucocephala*.

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## Chapter 25

# Occurrence of Cyanogenic Glycosides in Seeds and Seedlings of *Idiospermum australiense*, a Primitive Rainforest Angiosperm from Tropical Australia

V. Gordon<sup>1</sup> and P. Reddell<sup>2</sup>

<sup>1</sup>EcoBiotics Pty Ltd, PO Box 148, Yungaburra, Queensland, 4872, Australia;

<sup>2</sup>Cooperative Research Centre for Tropical Rainforest Ecology and Management, CSIRO Land and Water, PO Box 780, Atherton, Queensland, 4883, Australia

### Introduction

The 'idiot-fruit' tree, *Idiospermum australiense* (Diels) S.T. Blake, is endemic to complex lowland rainforest in northeastern Queensland. It is the sole member of the primitive angiosperm family Idiospermaceae (Blake, 1972) and is considered to be one of the most ancient of all living flowering plants (Lamont, 1974).

One remarkable and distinctive characteristic of this species is its very large seeds (60 to 100 mm in diameter) which have three to six cotyledons. These seeds are poorly dispersed and seeds and seedlings are found at very high densities under the parent trees. *Idiospermum* seeds are known to be highly toxic to domestic stock causing symptoms somewhat similar to those of strychnine poisoning (Everist, 1981). The specific toxic principles in *Idiospermum* seeds have not been demonstrated, although a variety of substances, including alkaloids, have been identified (Duke *et al.*, 1995).

Here we provide the first published report of the occurrence of cyanogenic glycosides in seeds and seedlings of *I. australiense*.

### Methods

#### Collection and storage of samples

Five recently fallen fruits and five seedlings (approx. 12 months old) of *I. australiense* were collected in complex lowland tropical rainforest being cleared for road access at Mt Hutchinson in northeastern Queensland. The material was placed in plastic bags in the field and transported back to the laboratory within 4 hours. In the laboratory, samples of pericarp, seed testa and seed interior were taken from each of the fruits, while mature leaves, immature leaves, stems and

roots were sampled from each seedling. All samples were then stored at  $-86^{\circ}\text{C}$  prior to analysis.

### Cyanide determination

Samples were prepared for extraction of cyanogenic glycosides based, with some modifications, on procedures described by Secor *et al.* (1976), Brinker and Seigler (1989) and Dahler *et al.* (1995). Two solutions were prepared immediately prior to the extractions, these were: (i) an enzyme stock solution consisting of 50 mg  $\beta$ -glucosidase (Sigma) and 50 mg  $\beta$ -glucuronidase (Sigma) in 5 ml 0.1 M phosphate buffer and (ii) 1 M NaOH (Merck). Extracts were made by weighing 0.5g of frozen plant tissue into 15 ml polypropylene tubes, adding 3 ml of phosphate buffer (0.1 M, pH 6.4) and then homogenizing the contents for 5 seconds (using a Polytron homogenizer with a PTA 10 s aggregate). The homogenate was poured into a glass scintillation bottle (30 ml capacity) and the 15 ml tube rinsed with 1 ml of phosphate buffer, which was also poured into the scintillation bottle. Enzyme stock solution (100  $\mu\text{l}$ ) was then added to each scintillation bottle to cause release of cyanide from the extract, and the bottles gently swirled to mix their contents. To capture HCN released from the extract, a 1.5 ml capacity Eppendorf tube with the lid removed and containing 0.5 ml of the 1 M NaOH solution was placed in an upright position in each scintillation bottle and the bottles sealed before being incubated overnight at  $37^{\circ}\text{C}$ .

Cyanide released during the overnight incubation was measured with a commercially available cyanide test kit (Merck Reflectoquant Plus). For this assay, 0.2 ml of solution from the Eppendorf tubes that had been sealed inside the scintillation bottles was added to the 5 ml reaction tube provided with the test kit. Deionized water (4.8 ml) and 55 drops of 0.05 M  $\text{H}_2\text{SO}_4$  (to adjust pH to 7) were also added to the assay tube and the contents mixed by swirling. A chlorinating agent (Merck CN-1 reagent) was then added and the contents swirled to dissolve the reagent and allow formation of cyanogen chloride. A second reagent (Merck CN-2) containing 1,3-dimethylbarbituric acid was then added immediately and dissolved by swirling the tube. After 5 min reaction time, the cyanide content of the solution was measured as  $\text{mg l}^{-1} \text{CN}^-$  by remission photometry using a Merck RQPlus reflectometer. Standards for this assay were prepared as 0, 0.1, 0.2, 0.4 and 0.8  $\text{mg l}^{-1} \text{KCN}$  (Fluka).

## Results and Discussion

With the exception of the fruit pericarp, cyanogenic glycosides were present in all tissues from all *I. australiense* seeds and seedlings examined in this study (Fig. 25.1).

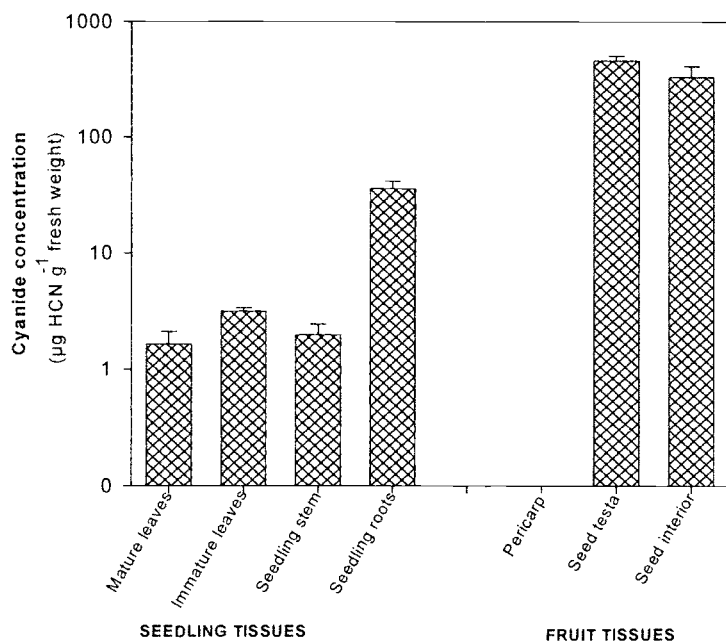


Fig. 25.1. Cyanide concentrations in tissues from seeds and seedlings of *Idiospermum australiense*.

Within *Idiospermum* seedlings, cyanogenic glycoside concentrations were highest in the roots (mean = 37  $\mu\text{g HCN g}^{-1}$  fresh weight) and immature leaves (mean = 3.6  $\mu\text{g HCN g}^{-1}$  fresh weight). Stems and mature leaves had the lowest concentrations and these were approximately half those found in immature leaves. Similar decreases in concentrations of cyanogenic glycosides with increasing leaf age and maturity are reported widely from other cyanogenic tree species (e.g. Hurst, 1942; Smeathers *et al.*, 1973; Dahler *et al.*, 1995).

Concentrations of cyanogenic glycosides in *Idiospermum* seeds were more than 10-fold higher than those found in seedling tissues (Fig. 25.1). Cyanogenic glycosides were more concentrated in the seed testa (mean = 448  $\mu\text{g HCN g}^{-1}$  fresh weight) than in the seed interior (mean = 367  $\mu\text{g HCN g}^{-1}$  fresh weight). These concentrations are within the middle of the range of values we have found in seeds of other cyanogenic species in the north Queensland's rainforest flora (e.g. 1145 and 364  $\mu\text{g HCN g}^{-1}$  fresh weight for seeds of *Macadamia whelanii* and *Syzygium gustavoides*, respectively).

The discovery of cyanogenic glycosides in *Idiospermum* adds to the variety of other putative defence chemicals known from this species. More than 40 terpenes (Brophy and Goldsack, 1992) have been previously isolated from *Idiospermum*

leaves, while Duke *et al.* (1995) reported on three alkaloids from *Idiospermum* seeds, including the piperidinoindoline (+)-calycanthine that comprised  $1\text{ g kg}^{-1}$  of seed dry weight. We have also found high concentrations of saponins in *Idiospermum* seeds (Gordon and Reddell, unpublished).

The potent defence chemistry of *Idiospermum* seeds probably explains their unattractiveness as a food source for the native mammalian and invertebrate herbivores that inhabit the lowland rainforests of northeastern Queensland. Although the large seeds of *Idiospermum* represent a significant potential nutritional resource for herbivores and seed predators, they remain largely ungrazed, even during periods of the year when other fruit resources are very scarce (e.g. April). The musky rat kangaroo (*Hypsiprymmodon moschatus*), an important mammalian frugivore in these forests, has been observed to pick up, chew the surface and quickly drop *Idiospermum* seeds (Drew and Spencer, 1998). Omnivorous rodents (*Uromys caudimaculatus*, *Melomys cervinipes* and *Rattus* species) that are significant consumers of other large-seeded species in these forests appear to completely avoid *Idiospermum* seeds. Cyanogenesis alone may be responsible for a major part of this aversion. For example, published lethal dose rates of cyanide for mammals (in mg HCN  $\text{kg}^{-1}$  bodyweight) range from 0.5 to 3.5 for humans (Halstrom and Moller, 1945) to 3.7 for mice and up to 10 for rats (Christensen, 1976). Based on the highest lethal dose rate in this range (10 mg HCN  $\text{kg}^{-1}$  bodyweight), ingestion of only 3 g of *Idiospermum* seed could be lethal to many of the small native rodents (*Rattus* species and *M. cervinipes*) that scavenge in these forests. Even *U. caudimaculatus*, the largest of the rodents in the lowland forests, with an average bodyweight of between 470 and 620 g (Wellesley-Whitehouse, 1983), would potentially need to ingest less than 15 g of *Idiospermum* seed to receive a lethal dose of cyanide. Similarly, the cyanide concentrations in *Idiospermum* seed may provide a significant deterrent to insects. Nahrstedt (1985) suggests 250 ng of HCN as a lethal dose for an 'average' 50 mg larvae; on this basis, ingestion of only 0.625 mg of *Idiospermum* seed would be toxic to such an organism.

Cyanogenesis may also contribute to the toxicity of *Idiospermum* seeds to cattle. Autopsies of poisoned animals found large amounts of partially masticated seeds present in the rumen (Everist, 1981). An average *Idiospermum* seed (with pericarp removed) weighs 155 g (Reddell and Gordon, unpublished). With the acute lethal dose rate for cyanide in cattle of approximately 2.0 mg HCN  $\text{kg}^{-1}$  bodyweight (Moran, 1954), ingestion of 15 to 20 *Idiospermum* seeds would be adequate to kill a cow. With high concentrations of neurotoxic alkaloids also present in seeds, even lower quantities of seed may be fatal because of the compounding effects of at least the two groups of poisons present.

The large resource 'investment' that *Idiospermum* makes in defence chemicals, especially for its seeds, may in part explain its survival success and status as a 'living fossil' from Gondwana's ancient rainforests (Keto and Scott, 1986).

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## Chapter 26

# Photosensitization in Cattle in Southern Brazil

A.L. Schild<sup>1</sup>, A.C. Motta<sup>2</sup>, F. Riet-Correa<sup>1</sup>, F.C. Karam<sup>3</sup> and F.B. Grecco<sup>4</sup>

<sup>1</sup>Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Pelotas University, 96010-900, Pelotas, RS, Brazil; <sup>2</sup>Faculdade de Veterinária, Universidade de Passo Fundo, Passo Fundo, RS, Brazil; <sup>3</sup>Faculdade de Veterinária, Universidade Rural da Campanha, Bagé, RS, Brazil; <sup>4</sup>Faculdade de Veterinária, Universidade de Cuiabá, 78015-480, Cuiabá, MT, Brazil

In Rio Grande do Sul, southern Brazil, data of the Regional Diagnostic Laboratory at Pelotas University showed that, from 1978 to 1998, an average of 10.6% of the conditions diagnosed in cattle were due to plant poisoning, varying annually from 5% to 25%. In that period *Senecio* spp. were responsible for 50.7% of the cattle deaths caused by toxic plants. Photosensitization was the main clinical sign observed in 27 (21%) out of 128 outbreaks of diseases due to toxic plants. In four outbreaks photosensitization was caused by *Senecio* spp., in two by *Echium plantagineum*, in one by *Enterolobium contortisiliquum*, in one by *Ammi majus* and in 19 outbreaks the cause of the photosensitization was not determined.

### Intoxication by *Senecio* spp.

In four of 66 outbreaks of intoxication by *Senecio* spp., the main clinical sign was photosensitization. In another six outbreaks photosensitization was observed in some cattle but was not the main clinical sign. Photosensitization due to *Senecio* spp. occurred in those outbreaks in which the clinical manifestation period was more prolonged (30-60 days). Morbidity in those outbreaks varied from 0.5% to 100% and case fatality varied from 5% to 34%. In other outbreaks of *Senecio* intoxication, photosensitization was not observed or occurred only in a few animals. The main clinical signs in most outbreaks of *Senecio* intoxication were diarrhoea, weight loss, abdominal straining, aggressiveness, incoordination and other nervous signs. Subcutaneous oedema, jaundice and rectal prolapsus were occasionally observed. In most cases the clinical manifestation period was of 1-4 days and case fatality rate was nearly 100%.

In Rio Grande do Sul the intoxication is caused by many species of *Senecio* including *S. brasiliensis* (the most important), *S. selloi*, *S. heterotrichius*, *S. cisplatinus*, *S. leptolobus*, *S. oxyphylus* and *S. tweediei*. Most outbreaks occur in

spring and winter (Méndez *et al.*, 1987; Barros *et al.*, 1987; Méndez and Riet-Correa, 1993, 2001).

### **Intoxication by *Echium plantagineum***

Two outbreaks of intoxication by *E. plantagineum* were observed during 1978-1998. One outbreak affected 28 out of 77 calves, two cows out of 85 and one heifer out of 40. Most affected calves had anorexia, abdominal straining, rectal prolapsus, diarrhoea, ataxia and ptyalism. The clinical manifestation period was of 1-7 days. Some calves with more prolonged manifestation period had photosensitivity. All the three adult cattle affected had photosensitivity and recovered (Méndez *et al.*, 1985).

Another outbreak affected three of 80 cows. One of the affected cows had severe photosensitivity (Riet-Correa *et al.*, 1983). In both outbreaks gross and histological lesions were characteristic of pyrrolizidine alkaloid intoxication. *Echium plantagineum* is a common weed in Rio Grande do Sul, found mainly in winter in crops of wheat, ryegrass and clovers. However, intoxication is rare because the plant only occasionally contains toxic levels of pyrrolizidine alkaloids.

### **Intoxication by *Enterolobium contortisiliquum***

An outbreak of hepatogenous photosensitization caused by *E. contortisiliquum* was observed in a flock of 21 Holstein dairy cows. In the paddock where the cattle were grazing two trees of *E. contortisiliquum* had been cut and the animals ingested large amounts of pods and leaves of the plant. Two days after ingestion six cows showed photosensitization characterized by dermatitis of the unpigmented skin, salivation and nasal and ocular discharge. Anorexia and abdominal pain were also observed. One cow in the sixth month of pregnancy aborted. The other animals were not pregnant. All cows recovered and the skin lesions disappeared in 4-5 weeks.

The pods of the plants were administered at 5 g kg<sup>-1</sup> bodyweight to one calf, 10 g kg<sup>-1</sup> to two calves, and 20 g kg<sup>-1</sup> to another calf. The experiment was authorized by the ethical committee of the Veterinary Faculty. All animals developed clinical signs. The two calves that received 10 and 20 g kg<sup>-1</sup> died 24 and 26 hours after dosing, and the other two recovered. Serum levels of AST, GGT and bilirubin were increased. Main clinical signs were anorexia, ruminal atony, diarrhoea or faeces with mucus or blood, and nasal and ocular discharge. One calf that ingested 10 g kg<sup>-1</sup> and recovered from the acute clinical signs had mild photosensitization and recovered in 14 days. The main gross lesion was the presence of a yellow liver with multifocal petechial haemorrhages. Haemorrhages were also observed on the gut and fore-stomachs. Multifocal haemorrhages and hepatic necrosis affecting mainly the periportal hepatocytes were observed



histologically (Grecco, 1999). No clinical signs were observed in a calf treated with 20 g leaves kg<sup>-1</sup> bodyweight.

Outbreaks of intoxication by different species of *Enterolobium* have been reported from other Brazilian regions (Tokarnia *et al.*, 1960; Deutsch *et al.*, 1965; Lemos *et al.*, 1998; Dantas *et al.*, 1999).

### **Hepatogenous photosensitization of unknown cause**

In 19 outbreaks of photosensitization the cause was not determined. The epidemiological and pathological aspects of those 19 outbreaks were studied. The disease occurred mainly in the spring and autumn with morbidity of 0.08% to 64% and mortality of 0 to 14% respectively. Cattle of all ages were affected. The pastures on which outbreaks occurred were characterized by low, flat and wet land, used alternately for rice or other crops and natural or cultured pastures of clover, oat and ryegrass.

The disease was characterized clinically by depression, anorexia, salivation and photosensitization of white or slightly coloured and hairless skin such as that on the muzzle, nostrils, eyelids, udder, groin and vulva. In severe cases the skin, mainly of dorsal midline and legs, was thickened, necrotic and sloughed revealing a raw area of subcutaneous tissue. Jaundice, yellow to orange discoloration and swelling of the liver, oedema of the gall bladder, excess serous fluid in body cavities and occasionally subcutaneous and peritoneal oedema were observed at necropsies.

Histological lesions were characterized by degenerative changes of hepatocytes that were swollen and had diffuse or zonal vacuolation. Individual or randomly scattered foci of hepatocellular necrosis, bile duct cell proliferation and portal fibrosis were also observed. Birefringent crystalloid material was not observed in the bile ducts or hepatic parenchyma, suggesting that lithogenic steroidal saponins were not responsible for the disease. Spores of *Phitomyces chartarum* were absent or in low number in the pastures. The most common plants found in the pastures were *Cynodon dactylon*, *Axonopus* spp., *Paspalum* spp., *Echinochloa cruzgalis*, *Juncus bufonios* and *Leersia hexandra*. Known toxic plants causing photosensitivity were not found in the pastures (Motta *et al.*, 2000).

### **Intoxication by *Ammi majus***

Primary photosensitization was observed in a herd of 12 cows and 12 calves grazing in a ryegrass pasture severely infected by *Ammi majus*. The disease occurred during December and January when *A. majus* was in bloom. All cattle were affected, but recovered after withdrawal from the pasture. The cows had lesions of dermatitis in the udder and their calves had dermatitis of the muzzle and keratoconjunctivitis. The localization of the lesion suggests that the

photosensitization was mainly due to contact of the skin with the seeds of the plant. The disease has been produced experimentally in calves by the administration of 1.7 to 8 g kg<sup>-1</sup> bodyweight (Méndez *et al.*, 1991).

## Acknowledgements

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## Chapter 27

# Transformed Root Cultures of *Solanum dulcamara* and Production of Secondary Metabolites

A. Marzouk<sup>1,2</sup>, A.I. Gray<sup>2</sup> and S.G. Deans<sup>1</sup>

<sup>1</sup> Aromatic and Medicinal Plant Group, Avian Science Research Centre, SAC, Ayr KA6 5HW, Scotland, UK; <sup>2</sup> Department of Pharmaceutical Sciences, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, Scotland, UK

Solanaceous alkaloids are commonly present in members of the Solanaceae family and are regarded as toxins. However, *Solanum dulcamara* (dogwood or bittersweet) is a recommended species for growing in the temperate regions of Europe as a source of steroid alkaloids (for example  $\beta$ -solaranine, see Fig. 27.1). These alkaloids are suggested to be alternatives for diosgenin in the commercial production of steroid pharmaceuticals (Mathé *et al.*, 1986). Steroid alkaloids like solasodine as well as its C-25 epimer tomatidenol can be easily converted to pregdienolone, which is an important intermediate in the synthesis of steroids. *Solanum dulcamara* exists in three chemovarieties that contain either solasodine, soladulcidine or tomatidenol glycosides (Willhun, 1966). These alkaloids are always accompanied by varying quantities of their corresponding oxygen analogues, that is the neutral saponins. Therefore, these chemovarieties can be more appropriately listed as tomatidenol/yamogenin, solasodine/diosgenin and soladulcidine/tigogenin types (Hegnauer, 1989). The tomatidenol-producing taxa are found in the humid Atlantic climate of Western Europe, the soladulcidine type occurs in the drier continental climates while the solasodine variety is comparatively rare (Sander, 1963; Mathé and Mathé, 1979). Only the solasodine and tomatidenol varieties are of interest, but the productivity of this species would not be comparable to that of other tropical or subtropical species such as *Solanum laciniatum*. Thus, if the steroid alkaloid content could be boosted by manipulation, *S. dulcamara* would be of interest for commercial growing due to its other qualities such as moderate cold hardiness, good growth on poor soils and perennial life cycle (Mathé *et al.*, 1986).

An alternative approach would be to produce these alkaloids intensively *in vitro*. Several *Solanum* species, and *S. dulcamara* itself, were subject to *in vitro* manipulation, but attempts, which involved techniques like cell suspension and callus cultures, failed to achieve the target. Secondary metabolite production, in general, needs a certain degree of tissue differentiation (Wink, 1989), something that is obviously lacking in those *in vitro* systems. For example, Ehmke and Eilert

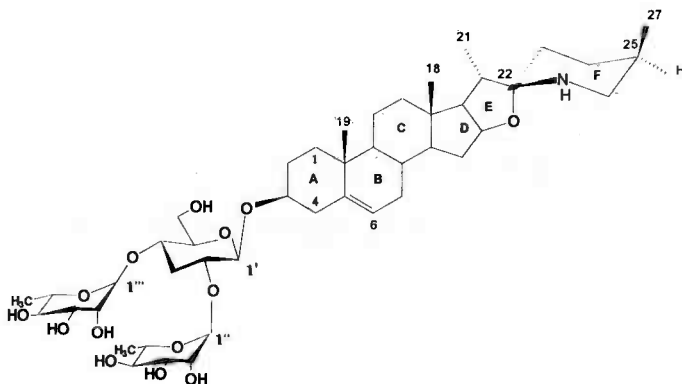


Fig. 27.1. Structure of  $\beta$ -solamarine.

(1993) did not detect any alkaloids in cell suspension cultures of *S. dulcamara* grown in the dark. These alkaloids started to be synthesized when the cultures were grown in light and began to show some differentiation and rooting, but again their level was not comparable to the original intact plant.

A more recent and promising technique, that is transformed or hairy root cultures, has been introduced as an alternative to the classical cell suspension culture. These roots are obtained by a natural genetic engineering mechanism in which the plant genome is altered by a specific gene sequence transferred and stably integrated by the Gram negative soil-borne plant pathogen *Agrobacterium rhizogenes*. The hairy roots produced are genetically and chemically stable and are characterized by high productivity of secondary metabolites, which in most cases mirrors that of the original non-transformed parent plant.

Few publications on hairy root cultures, which deal with the production of steroid alkaloids, are reported for *Solanum* species. Only those of *Solanum eleagnifolium* (Alvarez *et al.*, 1994), *Solanum mauritianum* (Drewes and Van Staden, 1995) and *Solanum aviculare* (Subroto and Doran, 1994; Yu *et al.*, 1996; Kittipongpatana *et al.*, 1998; Argolo *et al.*, 2000) could be traced. Other workers have also investigated the production of neutral steroid saponins from hairy roots of *Solanum aculeatissimum* (Ikenaga *et al.*, 1995). In this work, we describe for the first time, the production of steroid alkaloids from hairy root cultures of *S. dulcamara*.

## Materials and Methods

### General experimental procedures

Plant material was purchased from local nurseries; salts for media preparation were obtained from Sigma (UK); solvents for chromatography were reagent

grade;  $^1\text{H}$  and  $^{13}\text{C}$  spectra (400 and 100 MHz, respectively) were acquired on a Brüker DPX 400 spectrometer. Micrographs were taken using a Jeol JSM T220 scanning electron microscope (SEM). PCR reagents were obtained from Perkin Elmer (UK), primers from VH Bio Ltd (UK) and Perkin Elmer GeneAmp 480 thermal cycler was used for amplification.

### Establishment of transformed root cultures

Transformed roots were obtained by infecting surface-sterilized stem segments with *A. rhizogenes* strain A4. An overnight bacterial suspension in yeast mannitol broth (YMB) (Hooyakaas *et al.*, 1977) supplemented with 50  $\mu\text{M}$  acetosyringone was used for inoculation into freshly wounded explants. Infected samples were transferred to one-tenth Murashigue and Skoog (MS) agar solidified media (Murashigue and Skoog, 1962), kept in the dark for 48 h and then incubated under 16 h photoperiod at  $20 \pm 2^\circ\text{C}$ . The putative hairy roots, which appeared on the infected samples were excised and transferred to MS hormone-free, liquid media supplemented with 30  $\text{g l}^{-1}$  sucrose. Ampicillin, sodium salt at 500  $\text{mg l}^{-1}$ , was added until cultures were free from the residual bacteria. The axenic roots obtained were maintained in the same liquid media (50 ml in 250 ml flasks) on a gyratory shaker (90 rpm), at  $20 \pm 2^\circ\text{C}$  in the dark or under illumination of 16  $\text{h day}^{-1}$  at light intensity of approximately  $1.8 \text{ w m}^{-2}$ . The roots were sub-cultured every 2 weeks. For isolation of compounds, roots were inoculated into 5 l flasks containing 2 l of the same media, aerated with a bubble-type sparger and incubated for a period of 4 weeks either in the dark or under illumination. Some root samples were sub-cultured and maintained in other liquid media (Gamborg's B5 and Schenk and Hildebrandt) for the purpose of comparison.

### Confirmation of transformation by PCR

Genomic DNAs were extracted from 100 mg (fresh weight) of putative hairy roots and normal non-transformed roots (as controls) using a commercially available plant DNA extraction kit (Nucleon-Phytopure, UK). These DNAs were used as templates for the reaction. PCR was performed with *rol B* gene specific primers: 5'-ATG GAT CCC AAA TTG CTA TTC CTT CAA CGA-3' and 5'-TAA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3'. Amplification was carried out according to Hamill *et al.* (1990) with slight modification of the thermal cycle. Products of the reaction were run on 1.5% agarose electrophoretic gel stained with ethidium bromide, along with a standard DNA marker (1 kb Lambda DNA, Gibco, UK).

### Scanning electron microscopy

Fresh hairy root material was fixed with 1.5% glutaraldehyde (GA) in 0.05 M sodium cacodylate buffer, pH 7.0, for 45 min. After 1-2 min in vacuum (26 mm Hg, 3.46 k Pa) the fixative was substituted by 3% GA in 0.1 M cacodylate buffer,

pH 7.0 for 2 h. The material was then post-fixed in 1% aqueous solution (w/v) of osmium tetroxide for 2 h. All treatments were carried out at room temperature. The fixed material was dehydrated in graded ethanol series, dried by the critical point drying method and sputter coated with gold before observation in the SEM (Ascenão *et al.*, 1998).

### Extraction and isolation

Fresh root material (400 g) was extracted with cold methanol overnight (1 l x 3). The combined methanolic extracts were concentrated under vacuum at 40°C, partitioned between distilled water and petroleum ether, chloroform and finally with n-butanol. The steroid enriched n-butanol fraction (3.1 g) was fractionated on silica gel and Sephadex LH-20 columns using mixtures of chloroform-methanol (95:5-70:30). The final purification of compounds was performed on preparative TLC plates (silica gel 60 G F<sub>254</sub>, Sigma, UK) using chloroform-methanol-water (70:30:5) for development. <sup>1</sup>H and <sup>13</sup>C spectra were recorded in pyridine-d<sub>5</sub>.

<sup>13</sup>C spectral data of compound 1: (pyridine-d<sub>5</sub>, 100 MHz), δ 38.0 (C-1), 30.6 (C-2), 78.6 (C-3), 39.5 (C-4), 141.3 (C-5), 122.3 (C-6), 35.2 (C-7), 31.9 (C-8), 50.9 (C-9), 37.7 (C-10), 21.2 (C-11), 40.6 (C-12), 41.1 (C-13), 57.2 (C-14), 32.86 (C-15), 78.3 (C-16), 64.0 (C-17), 17.0 (C-18), 19.9 (C-19), 42.1 (C-20), 16.2 (C-21), 98.9 (C-22), 27.8 (C-23), 29.6 (C-24), 32.1 (C-25), 51.1 (C-26), 20.2 (C-27), 100.8 (C-1'), 78.4 (C-2'), 79.0 (C-3'), 79.1 (C-4'), 77.4 (C-5'), 61.8 (C-6'), 102.5 (C-1''), 73.0 (C-2''), 73.0 (C-3''), 74.4 (C-4''), 68.5 (C-5''), 19.0 (C-6''), 103.4 (C-1'''), 73.1 (C-2'''), 73.2 (C-3'''), 74.6 (C-4'''), 70.0 (C-5'''), 19.1 (C-6''').

## Results and Discussion

### Transformation

The putative hairy roots appeared on the infected stem segments as early as 1 week after infection, in some of the samples, and were complete within 2 weeks in the rest of them. Some roots appeared directly from or near the wounding site and some differentiated from small calli, which formed at the wounding sites. No hairy roots appeared on the infected leaf segments, instead green normal roots were developed from the cut ends of the midribs and from the basal parts of the infected as well as the control samples of the leaves and the stems. These normal roots failed to grow when transferred to liquid media lacking growth hormones, thus confirming their untransformed nature.

Although the solanaceous plants are reported to be highly susceptible to infection with *A. rhizogenes* (Porter, 1991), the transformation frequency obtained with our *S. dulcamara* (> 93%) far exceeded the published transformation rates for other *Solanum* species: one transformation event out of 80 infected samples for *S. mauritanum* Scop., whether acetosyringone was used

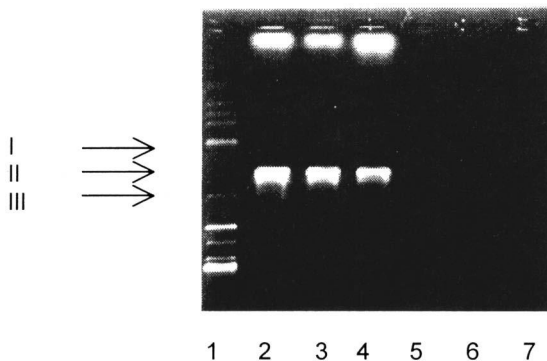
or not (Drewes and Van Staden, 1995) and 20-90 % transformation frequency for *S. aviculare* Forst. depending on the bacterial strain employed (Kittipongpatana *et al.*, 1998).

Roots incubated under illumination showed greening and those incubated under continuous light regenerated small shoots within 4 weeks. Roots grown in different liquid media, MS (Murashigue and Skoog, 1962), B5 (Gamborg *et al.*, 1968) and SH (Schenk and Hildebrandt, 1972) displayed typical properties of transformed roots, namely fast growth with almost no lag phase, high degree of branching and lack of positive geotropism. Insertion of the root-inducing plasmid was confirmed by PCR using primers specific to *rol B* gene. Results showed an amplification band expected for that gene (0.78 kb), while normal non-transformed roots used as controls did not show amplification bands (Fig. 27.2).

### Scanning electron microscopy

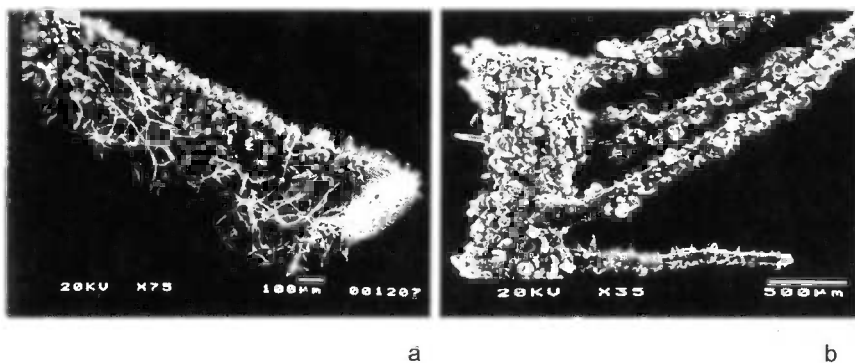
Micrographs of the transformed roots (Fig. 27.3a, b) showed a high degree of branching and large numbers of root hairs. Some of the hairs appeared as long unicellular tubes, while others were like small papillae covering the majority of the root surfaces, especially those growing near and out of the surface of the media. Cut surfaces of the roots (not shown) revealed the typical structure of young dicot roots with high amounts of starch granules and microcrystals of calcium oxalate in the cortical parenchyma.

These cell inclusions, which are characteristic features of plants belonging to the family Solanaceae (Metcalfe and Chalk, 1957) reflect genetic stability of the transformed cultures as they retained the histological fingerprints of the mother plant. The high degree of branching and the abundant root hairs are typical characteristics of transformed roots that are conferred by the root-inducing plasmid.



**Fig. 27.2.** Agarose gel showing PCR products. Lane 1, DNA marker; lanes 2, 3, 4, DNA from hairy roots (3, 2, 1  $\mu$ l respectively); lanes 5, 6, 7 DNA from normal roots (3, 2, 1  $\mu$ l respectively). I: 1.0 kb; II: 0.78 kb; III: 0.50 kb.





**Fig. 27.3.** SEM of transformed roots showing long hairs and short papillae (a); high degree of branching and short papillae only (b).

### Investigation of the cultures for secondary metabolites

Methanolic extract of the transformed roots was obtained in a yield of 10%, calculated on dry weight basis. TLC screening of the methanolic extracts of the transformed roots and different parts of the non-transformed plant (roots, aerial parts and fruits) showed, more or less, a similar pattern of secondary metabolites with three major alkaloidal spots and several minor ones. Analytical HPLC revealed much more complex profiles of the hairy root extracts. No qualitative differences were observed for roots grown in different liquid media or for roots grown in the dark and those grown in light, except for the formation of higher amounts of less polar compounds in the roots grown in the dark (higher percentage of chloroform extractives). Chromatographic separation of the butanol extract of roots grown in light, on silica gel and Sephadex LH20, lead to the isolation of two compounds.

Compound 1 is shown (Fig. 27.1), obtained as an amorphous solid, yield 0.055%, positive Dragendorff's and identified as (25*S*)-3-β-[[*O*-α-*L*-rhamnopyranosyl-(1→2)-*O*-α-*L*-rhamnopyranosyl-(1→4)]-β-*D*-glucopyranosyl-oxy}-22β*N*-spiroisol-5-ene (β-solamarine or tomatidenol-3-*O*-β-chacotrioxide), based on <sup>1</sup>H and <sup>13</sup>C spectroscopic data supported by APT measurements. The <sup>1</sup>H NMR spectrum exhibited signals due to two tertiary methyl groups (δ 0.83, 1.06, 3H, s), four secondary methyl groups (δ 1.10, 3H, d, *J* = 7.2 Hz; 0.81, d, *J* = 8.2 Hz; 1.64, d, *J* = 6.12 Hz; 1.78, d, *J* = 6.1 Hz), one olefinic proton signal (δ 5.34, br s) and three anomeric proton signals (δ 4.94, 5.86, s, 6.40, s). The <sup>13</sup>C spectrum displayed signals for 45 carbons including those for a spirocarbon (δ 98.85), olefinic carbon (δ 122.3) and three anomeric carbons (δ 100.8, 102.5, 103.4). These data collectively suggested a steroid glycoside of the spirosoleenol type.

The identity of the steroid part was confirmed by comparison of its spectroscopic data with those for standard samples of tomatine and solasodine recorded in the same solvent (experimental). The building blocks of the sugar moiety as well as the interglycosidic linkages were ascertained through HMQC and HMBC spectra.  $\beta$ -solamarine was reported before as one of the major steroidal alkaloids identified in the tomatidenol variety of *S. dulcamara* (Rönsch and Schreiber, 1966).

Preliminary study of the NMR data of Compound 2 revealed the same glycosidic moiety, but differences appeared in the chemical shifts for proton and carbon signals in ring F. Compound 2 is suggested to be the oxygen-containing furostanol type glycoside, but this still needs further confirmation. These results indicate the metabolic stability of the cultures where preliminary investigation showed no major differences between the hairy roots and the original plant. Other work (Ehmke *et al.*, 1995) revealed shifts of the metabolic pathway of another transformed system of *S. dulcamara*. Alteration from soladulcidine to solasodine type glycosides was observed in shooty teratoma cultures obtained by transformation via *Agrobacterium tumefaciens*.

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## Chapter 28

# Antibacterial Properties of Phytochemicals in Aromatic Plants in Poultry Diets

D.E. Cross<sup>1</sup>, K. Hillman<sup>2</sup>, D. Fenlon<sup>2</sup>, S.G. Deans<sup>1</sup>, R.M. McDevitt<sup>1</sup>  
and T. Acamovic<sup>1</sup>

<sup>1</sup>Avian Science Research Centre, SAC, Ayr, KA6 5HW, Scotland, UK; <sup>2</sup>Centre for Microbiological Research, SAC, Aberdeen, AB21 9YA, Scotland, UK

### Introduction

Plants contain a vast array of chemical compounds which have a variety of physiological and biochemical effects in animals (Wink, Chapter 1). These can be extremely toxic but depending on compound and concentration, may have beneficial effects (Panter *et al.*, this publication). A number of phytochemicals, especially the polyphenolics and terpenoids, have antimicrobial properties which may be important in animal production. Synthetic antimicrobials have been fed in poultry diets since 1949, providing economic benefits through increased productivity and better welfare, partially due to reduced occurrence and severity of disease. However, the extensive use of antimicrobials in agriculture may cause both a residual presence in meat and reduce the effective lifespan of medicinal antimicrobials, findings which have provoked intense speculation and debate for the future roles of the antimicrobials used in growth promotion (Young and Craig, 2001). These concerns have resulted in the legislative removal of several antimicrobials from animal feeds in the EU, with obvious implications for the competitive ability of the EU poultry industry on a global basis. A higher incidence of necrotic enteritis (NE) has already been associated with antimicrobial removal from poultry feeds in Norway (Schaller, 1998). The overgrowth of toxigenic *Clostridium perfringens* type A or C causes NE in the small intestine which results in a loss of gut wall integrity, and high bacterial loads associated with NE severely compromise broiler productivity (Porter, 1998; Lovland and Kaldusdahl, 2001).

With judicious use, secondary plant compounds can be exploited for their medicinal, aromatic and flavouring properties. Their uses in modern day medicine have declined since the discovery of lower-cost antimicrobial compounds produced by bacteria and fungi, and also the use of synthetic compounds (Cowan, 1999). The efficacy of plant secondary metabolites as alternatives for conventional growth-promoting antibiotics in the poultry industry requires

elucidation. Phytochemicals with antimicrobial activity include various terpenoid compounds formed by the acetate-mevalonate pathway. Terpenoids are the primary components of the essential oils and provide the flavour in herbs (Charai *et al.*, 1996). Antimicrobial activity has been reported *in vitro* for the essential oils of thyme, rosemary, marjoram (Smith-Palmer *et al.*, 1998; Hammer *et al.*, 1999) and oregano (Hammer *et al.*, 1999; Dorman and Deans, 2000). Yarrow has been shown to act against viruses and helminths *in vitro* (Cowan, 1999). However, incorporation of volatile essential oils into animal feeds may prove difficult, as their activity may be compromised by heat treatment in feed mills. This paper describes the effect of herbal supplementation of broiler diets on some prevalent species of gut microbes.

## Materials and Methods

### Animal experiment details and dietary treatments

Animal studies were conducted with the approval of the SAC Animals Experimental Committee. Ninety day-old Ross 308 broiler chicks (Ross Breeders, Newbridge, UK) were group reared for 7 days on litter, and then randomly transferred into five blocks of cages until the end of the experiment (day 29). Each cage contained three chicks, with each block serving as a treatment replicate. The birds were fed a basal wheat/soybean meal ration (13.4 MJ kg<sup>-1</sup> ME and 200 g kg<sup>-1</sup> CP), formulated to be low in vitamin E (< 50 mg kg<sup>-1</sup>). Each cage was randomly assigned to one of six dietary treatments on day 7 of the study. Oregano, marjoram, thyme, yarrow or rosemary (Green City, Glasgow, UK) were added as dried herb material to the basal diet at 10 g kg<sup>-1</sup> w/w to form five treatments, with the sixth treatment made up from the non-supplemented basal diet acting as a control. Diets did not contain antimicrobials, anticoccidials or in-feed enzymes.

### Sample collection and bacteriological analysis

Fresh faecal samples were collected for analysis on day 25 from three treatment replicates (18 cages), and caecal contents were removed from one bird in each of these 18 cages on day 29. The samples were immediately packed in ice on collection before bacteriological analysis. Faecal and caecal samples were subsequently serially diluted, and plated on to MRS agar (lactic acid bacteria), MacConkey agar No.3 (coliforms), Wilkins-Chalgren agar (anaerobes), Perfringens OPSP agar (*Cl. perfringens* spp.) and charcoal cefaperazone deoxycholate agar (CCDA) plates (*Campylobacter* spp.). After incubation, colonies were tested using oxidase, catalase and Gram stain tests for species confirmation. All bacterial counts were transformed by conversion to log<sub>10</sub>, and log counts were then analysed using GLM (General Linear Model) in Genstat Release 5.

**Table 28.1.** Bacterial counts from samples<sup>a</sup> of faecal and caecal material when broilers were fed diets containing herbal supplements.

Dietary supplement	Total coliforms		Total lactic acid bacteria		Total anaerobes		<i>Cl. perfringens</i>	
	Faecal	Caecal	Faecal	Caecal	Faecal	Caecal	Faecal	Caecal
Control	8.91	6.94	8.91	8.33	9.42	11.24	3.54	3.94
Marjoram	8.79	7.25	8.67	8.68	9.06	10.98	4.19	3.61
Oregano	8.83	7.50	8.21	8.27	9.13	11.36	3.77	3.57
Rosemary	8.62	7.24	8.51	8.15	9.21	11.12	2.41	2.80
Yarrow	8.60	8.40	8.76	8.63	8.62	10.99	2.57	2.00
Thyme	8.32	7.82	8.74	8.05	9.21	11.15	3.68	2.27
	NS	NS	NS	NS	NS	NS	NS	NS
S.E.D.	0.70	0.78	0.49	0.69	0.35	0.33	0.76	0.75

<sup>a</sup>n = 3. Comparisons in all cases were made between variables in the same column and values are expressed as log<sub>10</sub> colony forming units g<sup>-1</sup>.

## Results

As anticipated, concentrations of total anaerobes were higher in caecal samples compared with faecal samples ( $P \leq 0.001$ , data not shown). However, there was no effect ( $P > 0.05$ ) of treatment on the concentration of gastrointestinal bacteria compared to the control, irrespective of sampling site (see Table 28.1). Despite this some consistent trends were observed. The concentrations of *Cl. perfringens* in the caecum were lowest in birds fed diets containing supplements of thyme and yarrow (2.27 and 2.00 log<sub>10</sub> c.f.u. g<sup>-1</sup> respectively), and highest for control diets (3.94 log<sub>10</sub> c.f.u. g<sup>-1</sup>). In addition, birds fed the control diet had a numerically lower concentration of caecal coliforms at 6.94 log<sub>10</sub> c.f.u. g<sup>-1</sup>, compared to those fed diets containing either yarrow or thyme (8.40 and 7.82 log<sub>10</sub> c.f.u. g<sup>-1</sup> respectively). These two observations highlight the mixed effects of dietary herbal supplementation in this study.

Proportions of lactic acid bacteria to intestinal coliforms provide a good indication of gut health (Fuller, 1977). However, this experiment yielded no significant treatment effects on this ratio in either faecal or caecal samples (data not shown). Additionally, when diets supplemented with herbs were expressed as a ratio of the total bacterial count, no significant treatment effects were observed on the bacterial population ratios in this study (data not shown).

## Discussion

The lack of statistical significance between treatments in the present study may be a consequence of various factors, including the potential inactivity or reduced activity of the plant herb fraction, high variation between treatment replicates or the cleanliness of the cage environment for the birds. Dorman and Deans (2000) suggested that the active terpenoid phytochemicals in the whole plant may be

trapped within secretory gland structures, which may favour the antimicrobial activity of plant oils rather than plant herbs. Additionally, Cox *et al.* (2001) observed a higher activity of the active phytochemical terpinene-4-ol separately than when present as part of the essential oil fraction. More research is therefore required to determine the best form of supplementation of bioactive phytochemicals and their constituents. Dried plant herbs used in this experiment, although perhaps easier to incorporate into diets, were not composed of pure terpenoids and will contain other compounds that may reduce bioactivity. The herbal drying process may additionally have affected the constituent terpenoid concentration, and phytochemicals with less bioactivity may prevail within the plant due to their more stable nature. Thyme oil was relatively easy to incorporate into the oil portion of the diet, mixing fairly well with the vegetable oil fraction. However, the effect of heating the volatile oil fraction remains unknown in terms of dietary volatile oil content. Use of thyme oil in a related study on these same birds showed the greatest beneficial effects on productivity in terms of weight gained and feed consumed (unpublished data). As a result, the use of the essential oil component will form the focus of further work.

Despite a disappointing response in this experiment, oregano, containing various active phenolic components, is effective in promoting higher weight gain and feed conversion in pigs (Gill, 1999), although at smaller doses than in this study. Although work in pigs resulted in productivity benefits, work on a similar theme carried out in chickens reduced the incidence of coccidiosis without affecting productivity (Gill, 1999). Results have also been reported showing improvements in broiler productivity with associated anti-inflammatory and broncho-dilatory properties using a combination of Asian herbs based on Ayurvedic medicine (Cruickshank, 2001). Herbal supplements are thought to act by stimulation of the immune and cardiovascular systems, intestinal tract and various organ cell wall linings (McCartney, 2002). It may be possible that the faster intestinal transit and smaller volumes of digesta increase the variability of chickens in a study situation when compared with pigs, or the response of herbal supplementation is innately more variable than that of conventional antimicrobials. Thyme oil and oregano oil are both strongly phenolic compounds which are well known for their antimicrobial activity *in vitro*, although the dose rate and degree of replication in this study may have been insufficient to demonstrate significant effects *in vivo*, and therefore further studies are required.

In this study, birds fed diets containing all herbal supplements displayed increased caecal coliform populations compared with controls, suggesting that these herbs may select more against Gram positive than Gram negative bacteria. If this is the case, conditions suitable for the presence of *Campylobacter* or *Salmonella* may exist in birds on herb diets. However, it must be noted that *Campylobacter jejuni* was not a problem in these birds. This trial was possibly of insufficient length to fully appreciate any infection with *Campylobacter* spp. in these birds, and further trials should be taken to the full term of 42 days. Perhaps the best option eventually would be the use of various herbal therapy combinations, in order to combat both bacterial types effectively. Synergistic effects of the various components of essential oils have been shown for basil *in*

*vitro* (Lachowicz *et al.*, 1998) and for several constituents of tea tree oil *in vitro* (Cox *et al.*, 2001), which may even be of greater bioactivity than the complete essential oil fraction. One herb contains an extensive variety of phytochemicals, all with variable bacteriostatic or bacteriocidal activity, and use of herbal combinations may well prove to be the best course of action.

This initial experiment was mainly for screening purposes, and it focused on using herbs as natural antimicrobials in growing chickens in a caged experimental situation. From the results obtained, the emphasis in further work will be on thyme and yarrow treatments for reduction in concentrations of *Cl. perfringens* and the maintenance of healthy bird intestinal microflora ratios. With hindsight, there may have been an insufficient challenge against these birds for beneficial effects to become apparent. Previously, it has been shown that cage environments are the cleanest option in broiler chicken production in terms of helminth populations (Permin *et al.*, 1999) and red mite infestation (Höglund *et al.*, 1995). In future, it may prove more beneficial to raise birds under a different environmental situation, more representative of the commercial environment. Litter provides a substrate for pathogenic bacterial growth in conditions close to a neutral pH level (Pope and Cherry, 2000), and it may be more beneficial to use this or another substrate in the birds' growing environment. Survival rates within the two sample types should have been comparable in this experiment, yet certain differences exist between the caecal and faecal microflora. It may be the case that decreased sample exposure times to air would have resulted in the survival of a more stable and less sub-lethally injured bacterial population. Unfortunately, the samples had to be transported before analysis, which will have influenced the population composition, although all received similar treatment.

The use of more replicates in future feeding trials may reduce the high variation observed between bacterial counts, and therefore increase the likelihood of observing significant effects due to treatment. Several differences were observed within this experiment that could potentially be significant under a different experimental methodology. However, the lack of information contained within the literature on previous studies in this area provided complications at the design stage of this work.

The herbs used in the present study are generally recognized as safe and non-toxic, two of the main reasons why they are used in human cooking. There is an excellent range of bioactive phytochemical substances offered by natural herb plants, and terpenoid-containing volatile oils from these plants are among the most potent with regards their antibacterial properties. It is essential to focus on incorporating these chemicals solely or in combination into bird diets, in order that the level of antimicrobial protection required in intensive poultry production systems can be maintained. Further research on herbal supplements is therefore necessary to establish the potential or lack of potential for these treatments in poultry diets.



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## Chapter 29

# Microbial Metabolism of Tannins

J.D. Brooker<sup>1</sup>, L. O'Donovan<sup>1</sup>, I. Skene<sup>1</sup>, C. McSweeney<sup>2</sup> and D. Krause<sup>2</sup>

<sup>1</sup>*Animal Science Department, University of Adelaide, 5371, Australia;* <sup>2</sup>*CSIRO Tropical Agriculture, Brisbane, Australia*

### Introduction

Tannins are widespread in the plant kingdom (Haslam, 1998), where they form weak, pH dependent and reversible associations with a range of substrates including cellulose, proteins (Hagerman and Klucher, 1986), enzymes (Kumar and Singh, 1984), fats, nucleic acids and amino acids (Mole and Waterman, 1987), often making the substrate resistant to microbial attack. Several reports have suggested that the presence of condensed tannins (CT) at < 6% dry matter (DM) of the herbivore diet may result in improved animal performance (Barry and Manley, 1986) because less plant protein is lost as ammonia during ruminal digestion. More protein, therefore, passes to the abomasum and small intestine where dissociation of tannin-protein complexes can occur under acidic conditions (McSweeney *et al.*, 1988). In addition, the precipitation of soluble proteins effectively removes one of the primary factors involved in foam stabilization and, thus, reduces the likelihood of legume bloat (Waghorn *et al.*, 1990; Barry and McNabb, 1999).

In contrast, detrimental effects of CT in excess of 6% DM include decreased growth rate and bodyweight gain, perturbation of mineral absorption and inhibition of digestive enzymes (Butler, 1992; Yan and Bennick, 1995; Wood and Plumb, 1995). Kumar and Vaithyanathan (1990) have proposed that tannins inhibit rumen microbial function directly, by complexing with the bacterial cell envelope, or indirectly, by reducing the availability of protein nitrogen and sulphur for microbial use. Either would reduce the rate of plant fibre degradation in the rumen. McSweeney *et al.* (unpublished) have demonstrated selective inhibition of the cellulolytic ruminal species *Fibrobacter succinogenes* and *Ruminococcus* spp. compared with *Prevotella*, *Bacteroides* or *Porphyromonas*.

Animals that regularly browse CT-containing plants have developed populations of tannin-resistant ruminal microorganisms (Bernays *et al.*, 1989). Tannins have also been reported to induce changes in the morphology of several species of ruminal bacteria (Jones *et al.*, 1994; McAllister *et al.*, 1994). Ruminal transfers from tannin-adapted animals to domestic livestock have been demonstrated to promote tannin resistance in the recipients (Miller *et al.*, 1995).

Electron microscopy indicated that sainfoin proanthocyanidins were bound to cell coat polymers in *Streptococcus bovis*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola* and *Ruminobacter amylophilis*, but abnormal cell growth and division were observed only in *S. bovis* and *B. fibrisolvens* (Jones *et al.*, 1994). *Prevotella ruminicola* cells grown with CT were interconnected by condensed extracellular material which was absent from cells grown without CT. Bae *et al.* (1993) reported that *F. succinogenes* S85 cells grown in the presence of CT were also seen to possess large amounts of electron dense surface-associated material.

Several ruminal microorganisms that have the ability to degrade phenolic monomers have been isolated (Evans and Fuchs, 1988; Fuchs *et al.*, 1994) including *Eubacterium oxidoreducens* (Krumholz and Bryant, 1986) and strains of streptococcal and coprococcal (Tsai and Jones, 1975). Anaerobic and aerobic pathways of tannin degradation have recently been reviewed (Bhat *et al.*, 1998). A strain of *Selenomonas ruminantium* that expresses tannin acylhydrolase activity (Skene and Brooker, 1995) and *Streptococcus caprinus* (Brooker *et al.*, 1994; Nelson *et al.*, 1995; Sly *et al.*, 1997) have been isolated from feral goats' rumen samples, the latter being resistant to tannic acid (TA) at concentrations of up to 7% w/v and CT up to 4% w/v. In contrast, growth of *S. bovis* was inhibited by tannins at concentrations tenfold lower (Brooker *et al.*, 1994). This chapter reviews mechanisms of tannin tolerance in ruminal bacteria and also reports on changes in intestinal function as a result of tannin interactions.

## Materials and Methods

### Bacterial isolation

*Streptococcus bovis* type 2B was obtained from K. Gregg, University of New England, NSW, Australia. *Streptococcus caprinus* type strain 2.2 was originally isolated in our laboratory and is available through the Australian Collection of Microorganisms ACM3968. *Selenomonas ruminantium* K2 was isolated in our laboratory (Skene and Brooker, 1995). Modified brain heart infusion (mBHI) medium and minimal medium (NB) were prepared anaerobically as described (Brooker *et al.*, 1994 and Nili and Brooker, 1995 respectively). Crude extracts of acacia CT (ACT) were extracted from *Acacia aneura* leaves with 70% v/v acetone:water according to the method of Broadhurst and Jones (1978). ACT and tannic acid (TA, Sigma) were stored under anaerobic conditions and stock solutions were prepared immediately prior to use in anaerobic dilution solution (Ogimoto and Imai, 1984). Tannin solutions were neutralized, filter sterilized and added to broth cultures in Hungate tubes. Tannin content was analysed by the butanol-HCl method (Waterman and Mole, 1994).

Hungate tubes containing 10 ml of medium were inoculated under anaerobic conditions (95% CO<sub>2</sub>/5% H<sub>2</sub>) and were incubated at 39°C. In the presence of

tannins, growth was quantified on mBHI nutrient agar plates by serial dilution and viable colony counts.

#### **Gallic acid decarboxylase assay**

Bacteria were grown in mBHI alone or supplemented with 1.0% w/v of TA or gallic acid, or 0.5% w/v ACT, ferulic acid or *p*-coumaric acid and 50  $\mu$ l samples of whole cell suspensions, containing  $5 \times 10^5$  cells, were added to 1 ml of 0.1 M sodium phosphate buffer (pH 6.8), 2.5 mM dithiothreitol, 10 mM  $MgCl_2$  in a quartz glass cuvette and warmed at 37°C for 5 min. Gallate decarboxylase activity was determined as described in O'Donovan and Brooker (2001). Activity was expressed as mmoles phenolic acid decarboxylated  $min^{-1} mg^{-1}$  cell protein. Products of the gallate decarboxylase assay were analysed by Hewlett Packard gas chromatograph (GC) fitted with flame ionisation detectors and a BP-21 (SGE, 0.25) capillary column.

In liquid cultures, the presence of gallic acid was determined as described by Inoue and Hagerman (1988). Gallic acid and pyrogallol were also analysed by GC-mass spectrometry (GC-MS).

#### **Tannin acylhydrolase (tannase)**

Methods for assay and purification of tannase were as described in Skene and Brooker (1995).

#### **Removal of extracellular polysaccharides (EPS) from cells**

A 10 ml overnight culture of *S. caprinus* grown in the presence of 1% w/v TA was centrifuged at 12,000  $\times g$  for 30 min and the cell pellet was suspended in an equal volume of 1.5 mM sodium acetate, pH 4.2. The cells were mixed thoroughly to remove attached EPS (as described in Whitfield, 1988) and then re-centrifuged. The cell pellet was re-suspended in 5 ml of fresh medium and a 50  $\mu$ l aliquot was used to inoculate fresh medium. To determine the effect of TA on the production of EPS by *S. caprinus* and *S. bovis*, cells were grown for a period of 48 h in mBHI medium supplemented with increasing concentrations of TA. Tannic acid was removed by precipitation with 1% w/v polyvinyl pyrrolidone and overnight dialysis against CE (0.05 M  $Na_2CO_3$ , 0.025 M EDTA, pH 9.5) buffer. The EPS (expressed as glucose equivalents) was expressed as mg EPS  $mg^{-1}$  dry weight of the cells (after removal of EPS).

### Field emission scanning electron microscopy (FESEM)

*Streptococcus caprinus* or *S. bovis* were grown in mBHI medium in the presence of increasing concentrations of TA, ACT, gallic acid, ferulic acid, *p*-coumaric acid and pyrogallol. Throughout bacterial growth, samples were removed and the cells were centrifuged at 3000 x g for 10 min. Cells were re-suspended in fixative solution (Karnovsky, 1965) containing 0.15% w/v ruthenium red (Frehel *et al.*, 1988) and examined by FESEM (O'Donovan and Brooker, 2001).

### Transmission electron microscopy (TEM)

Bacterial cells were prepared as above, except that they were washed in citrate-EDTA (CE) buffer (50 mM Na<sub>2</sub>CO<sub>3</sub>, 25 mM EDTA, pH 10) and examined by TEM (O'Donovan and Brooker, 2001).

### Intestinal enzyme assays

For alkaline phosphatase (AP) sections were prefixed in formal calcium (1% w/v CaCl<sub>2</sub>, 8.75% v/v formalin in water) for 10 min, washed in 125 mM Tris-HCl (pH 9.2) at 39°C, and incubated in AP substrate (3.1 mg naphthol AS-BI phosphate, Sigma), 10 mg Fast Red (BDH Chemicals, UK), 60 µl dimethylformamide in 10 ml 125 mM Tris-HCl (pH 9.2) for 21 min.

For aminopeptidase-N samples were fixed in formal calcium (as above) at 4°C for 10 min, rinsed in 0.85% w/v saline solution and incubated in 0.1 M CuSO<sub>4</sub> for 2 min. Treated samples were incubated at 39°C in a substrate solution comprising 2 mg of L-alanine 4-methoxy-β-naphthylamide dissolved in 0.05 ml ethanol, 0.45 ml distilled water, 5 ml of 0.1 M sodium acetate buffer pH 6.5, 4 ml of 0.5% w/v saline, 0.5 ml of 13% w/v KCN and 5 mg of Fast Blue B (BDH Chemicals, UK). Assayed samples were stored at 4°C until analysis.

For each enzyme assay, precipitated reaction product was measured on a calibrated image analysis program.

### Statistical analyses

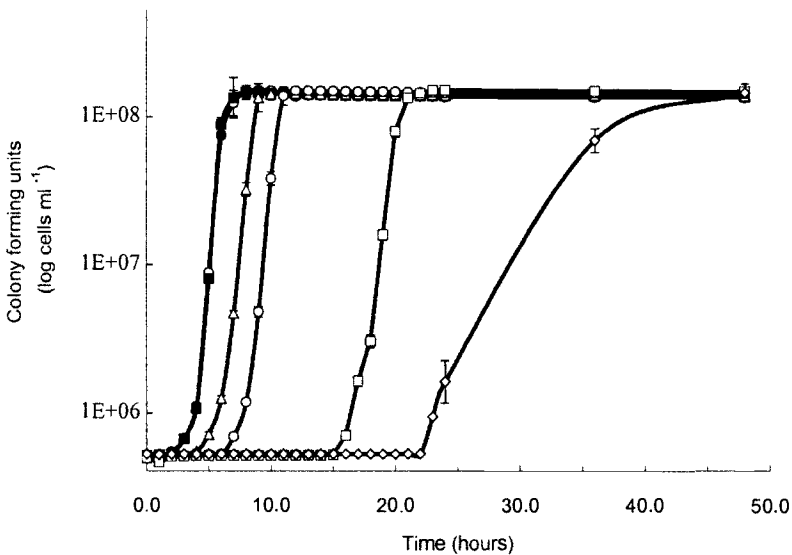
Differences in the levels of tolerance to phenolic acids between *S. caprinus* or *S. bovis*, cell and capsule sizes and in the amount of extracellular polysaccharide produced by the bacteria were determined using student's *t*-test. Slopes of exponential growth, determined by linear regression analysis, are reported as the growth rates and were analysed using analysis of variance for organisms-by-phenolic concentration interactions.

## Results

### Comparative growth *in vitro* of *S. caprinus* and *S. bovis* in tannin-containing medium

*Streptococcus caprinus* grew in up to 7.0% w/v TA or 4.0% ACT whereas *S. bovis* growth was inhibited by TA or ACT concentrations greater than 0.5% w/v. This response was consistent in either mBHI medium or in completely defined NB medium containing  $\text{NH}_4\text{Cl}$  as the sole nitrogen source. *Streptococcus caprinus* grew with average lag times of 4, 7, 16 and 23 h for TA concentrations of 1.0, 2.0, 3.0, and 5.0% w/v (Fig. 29.1). In the presence of up to 2.0% w/v ACT, the lag period was 10 h (Fig. 29.2).

Inoculation of fresh medium containing 2.0 % w/v TA with overnight TA cultures of *S. caprinus* or *S. bovis* decreased the lag time for both bacteria, although there was no change in the maximum tolerable level of tannin (results not shown).

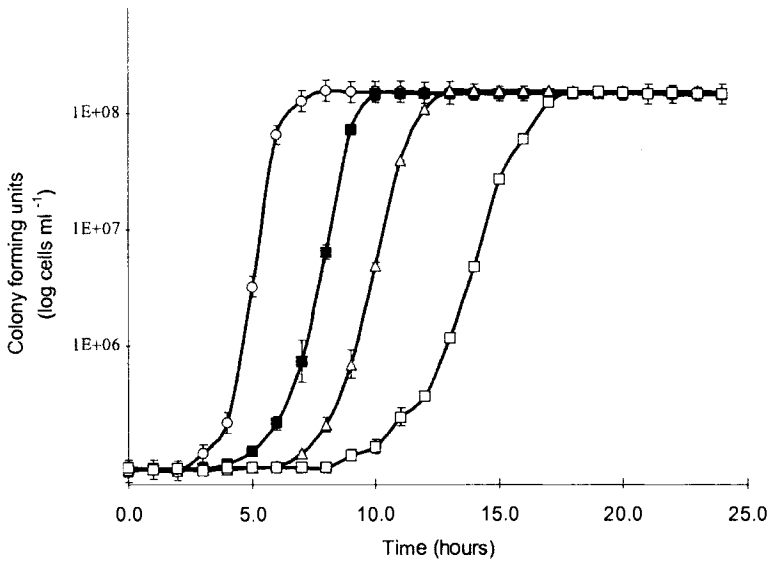


**Fig. 29.1.** Effect of tannic acid on the growth of *S. caprinus*.

*S. caprinus* was incubated in mBHI medium supplemented with increasing concentrations of tannic acid. At regular time intervals, samples were removed and growth was determined on mBHI plates by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.

Tannic acid concentration (% w/v): 0 ■, 0.5 ●, 1.0 ◻, 2.0 ◻, 3.0 ◻, 5.0 ◻.

† Significance from control (no tannic acid),  $P < 0.01$ .



**Fig. 29.2.** Effect of condensed tannin on the growth of *S. caprinus*.

*S. caprinus* was incubated in mBHI medium supplemented with increasing concentrations of ACT. At regular time intervals, aliquots were removed and growth was determined on mBHI plates by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.

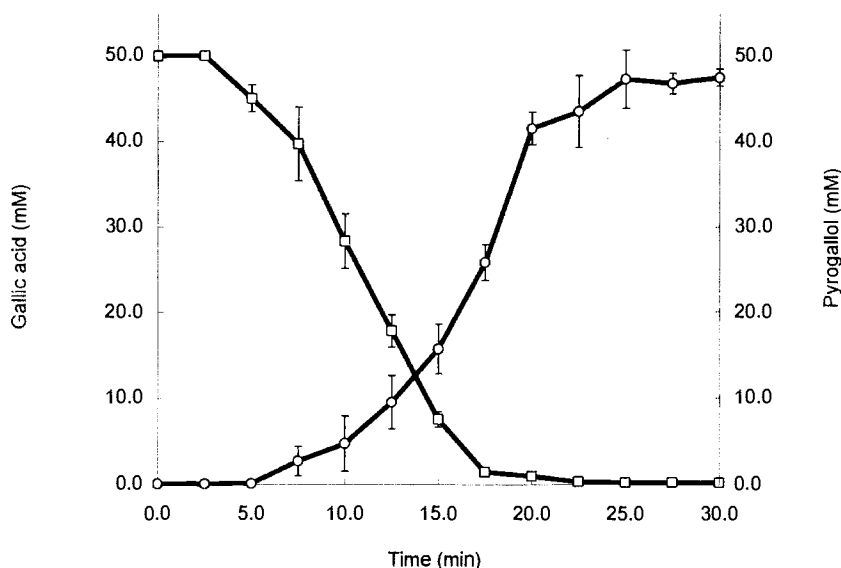
Condensed tannin concentration (% w/v): 0 ○, 0.5<sup>†</sup> ■, 1.0<sup>†</sup> △, 2.0<sup>†</sup> □.

<sup>†</sup> Significance from control (no condensed tannin),  $P < 0.05$ .

The logarithmic growth rate of *S. caprinus* remained unaffected by the addition of TA or ACT up to concentrations of 1% w/v, above which however, growth rates were significantly reduced. In 3% w/v TA or 2% w/v ACT, the growth rate of *S. caprinus* was reduced by 20-25%, whereas the growth rate of *S. bovis* was reduced by 50% and 85% in medium containing 0.5% and 0.75% w/v TA respectively.

### Gallate decarboxylase activity

Degradation of TA can occur by hydrolysis of ester and depside bonds, yielding gallic acid, which can be decarboxylated by gallate decarboxylase to yield pyrogallol (Krumholz and Bryant, 1986; Brune and Schink, 1992). In medium containing 1% w/v TA, after 16-20 h incubation, either in the presence of *S. bovis* or in the absence of bacteria, spontaneous release of gallic acid into the medium was measured at  $3.2 \text{ mg ml}^{-1}$ . In contrast, following incubation with *S. caprinus*, the gallic acid concentration increased to  $7.8 \text{ mg ml}^{-1}$  ( $P < 0.05$ ). Analysis of



**Fig. 29.3.** Degradation of gallic acid and corresponding pyrogallol production by *S. caprinus*.

Bacteria were incubated in mBHI medium containing 50 mM gallic acid at 25 °C for 30 min and samples were analysed by GLC. Points represent the average of triplicate trials. Bars represent the standard error.

Gallic acid degradation □, Pyrogallol production ○.

samples from a broth culture containing 1.0% w/v gallic acid (Fig. 29.3) shows that gallic acid disappeared during the incubation, coinciding with the appearance of pyrogallol. Addition of pyrogallol to culture medium had no effect on growth of *S. caprinus* or *S. bovis*.

The rate of decarboxylation of gallic acid by *S. caprinus* was calculated to be  $2.63 \pm 0.02$  mmoles  $\text{min}^{-1} \mu\text{g}^{-1}$  bacterial protein, but only in whole cell preparations under strictly anaerobic conditions. Growth of *S. caprinus*, gallic acid decomposition and pyrogallol production occurred together, with stoichiometric conversion of 50  $\mu\text{moles}$  of gallic acid  $\text{ml}^{-1}$  to 47  $\mu\text{moles}$  of pyrogallol  $\text{ml}^{-1}$ . *Streptococcus bovis* was unable to degrade gallic acid. Growth of *S. caprinus* in the presence of carboxylated compounds such as protocatechuic acid, 3,5-dihydroxybenzoic acid or hydroxybenzoic acid failed to produce their decarboxylated derivatives, catechol, resorcinol and phenol, respectively. When *S. caprinus* was grown in mBHI medium containing 1% w/v TA, gallate decarboxylase activity was increased 2.5-fold compared with TA controls and



**Table 29.1.** Gallate decarboxylase activity in *S. caprinus* following growth in the presence of phenolic compounds.

Gallate decarboxylase activity was determined in whole cell suspensions prepared from *S. caprinus* grown in mBHI and mBHI supplemented with the phenolic acids listed. Results are the averages of triplicate trials  $\pm$  the standard error.

† Significantly different from control (no phenolic acid),  $P < 0.05$ .

Medium	Gallate decarboxylase activity (mmoles pyrogallol $\text{min}^{-1}\mu\text{g}^{-1}$ )	Relative change
mBHI	2.63 $\pm$ 0.02	1.00
mBHI plus 1.0% tannic acid	6.44 $\pm$ 0.18 <sup>†</sup>	2.45
mBHI plus 1.0% gallic acid	10.96 $\pm$ 0.22 <sup>†</sup>	4.17
mBHI plus 0.5% ACT	3.28 $\pm$ 0.04	1.24
mBHI plus 0.5% ferulic acid	2.39 $\pm$ 0.05	0.91
mBHI plus 0.5% <i>p</i> -coumaric acid	2.56 $\pm$ 0.03	0.97
Boiled cells (control)	0.14 $\pm$ 0.003	—

4.1-fold when grown in the presence of 1% w/v gallic acid (significant at  $P < 0.05$ , Table 29.1). However, this did not occur in cells exposed to ACT, ferulic acid or *p*-coumaric acid.

### Effect of TA and ACT on bacterial cells

#### *Light microscopy*

Examination of *S. caprinus* and *S. bovis* by phase contrast microscopy showed homogeneous cocci, occurring mainly in pairs or short chains. Both bacteria stained Gram positive but this became variable with the addition of tannin. Average chain length was increased two- to threefold in cultures of *S. bovis* when the concentration of TA in the medium was greater than 0.2% w/v, or in *S. caprinus* when the TA concentration exceeded 1.5% w/v (figure not shown).

#### *Electron microscopy*

Using transmission electron microscopy (TEM) and field emission scanning

electron microscopy (FESEM), *S. bovis* was seen to increase chain formation and clumping when TA exceeded 0.2% w/v. When the TA concentration was increased to 0.75% w/v, increased numbers of abnormally shaped *S. bovis* cells were observed and lysed cells were present in every field of view. In some cells, multiple divisional planes suggested that the cells were undergoing unusual or incomplete cell division and blebs were visible on the cell surface (data not shown). In contrast, the size and shape of *S. caprinus* cells were unaffected by the presence of ACT or TA until concentrations reached 2.5 and 3.0% w/v respectively. Concentrations greater than these produced changes similar to those observed in *S. bovis*, although no abnormal cell divisions were detected and minimal cell lysis was observed.

A fibrous matrix extending from the surface of *S. caprinus*, but not *S. bovis*, was observed by TEM following growth of the bacteria in TA or ACT (not shown). When examined using FESEM (Fig. 29.4a and 29.4b), the extracellular material was globular and in patches on the surface of *S. caprinus* and increased in a concentration dependent manner such that when TA exceeded 2% w/v, the extracellular material completely encased the bacterium. In the absence of TA, *S. caprinus* produced little extracellular material.

### Characterisation of the extracellular matrix

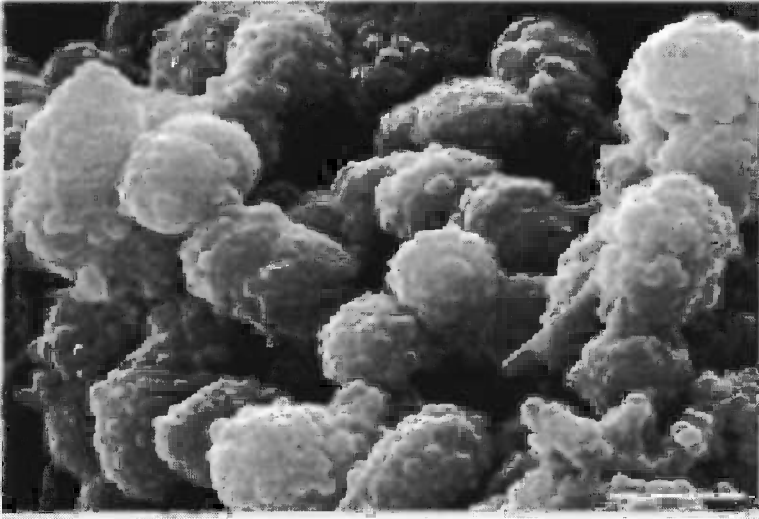
Extracellular matrix (EM) material was recovered by sodium acetate extraction of *S. caprinus* and *S. bovis* grown in the absence of TA. The average yield from stationary phase *S. caprinus* was  $1.25 \pm 0.12$  mg EM  $\text{mg}^{-1}$  cells (dry weight of cells following removal of EM) compared with  $1.30 \pm 0.06$  mg EM  $\text{mg}^{-1}$  cells isolated from *S. bovis*, and had an average molecular weight of  $2 \times 10^6$  daltons.

Colorimetric assays indicated that the material was composed predominantly of carbohydrate. However, analysis of the alditol acetate derivatives by GC and GC-MS showed the neutral sugar composition from *S. caprinus* and *S. bovis* to be different. In *S. caprinus*, glucose was primarily present, with trace amounts of mannose (glucose:mannose 1:0.2). Variable amounts (0 to 4.2%) of acyl and N-acyl residues were also detected. No uronic acids, proteins or hexosamines were found. In *S. bovis*, the neutral sugar composition was shown to consist of mannose, glucose and galactose in the ratio of 1:0.7:0.2. Larger amounts of acyl and N-acyl groups were detected (0 to 7.3%) and uronic acids were also found ( $2.7 \pm 1.2\%$ ). When the bacteria were grown in the presence of TA, no change in the carbohydrate composition occurred although the uronic acids increased in both *S. caprinus* and *S. bovis* by  $2.1 \pm 0.9\%$  and  $5.4 \pm 1.5\%$ , respectively.

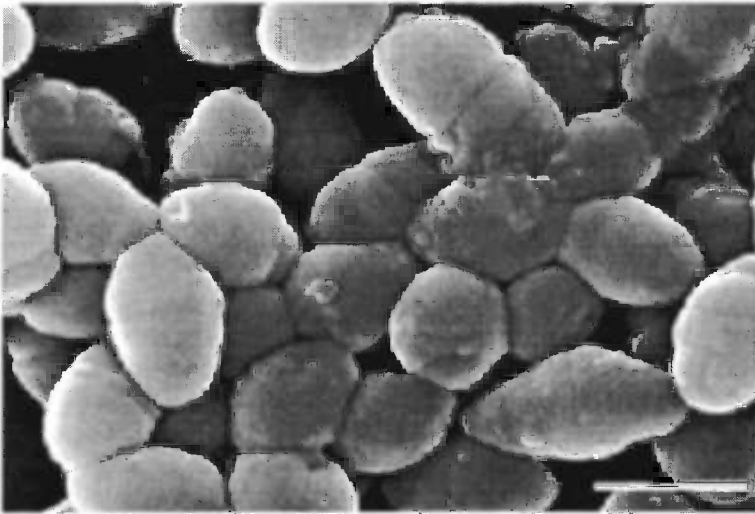
### Effect of tannin on the production of extracellular matrix

With the addition of  $> 0.5\%$  w/v TA or ACT, EM extracted from cultures of *S. caprinus* increased significantly ( $P < 0.05$ ); 5% w/v TA caused a sixfold increase in the amount of EM extractable from *S. caprinus* (Fig. 29.5). In the presence of

a



b



**Fig. 29.4.** FESEM of *S. caprinus* following growth in the presence or absence of tannic acid.

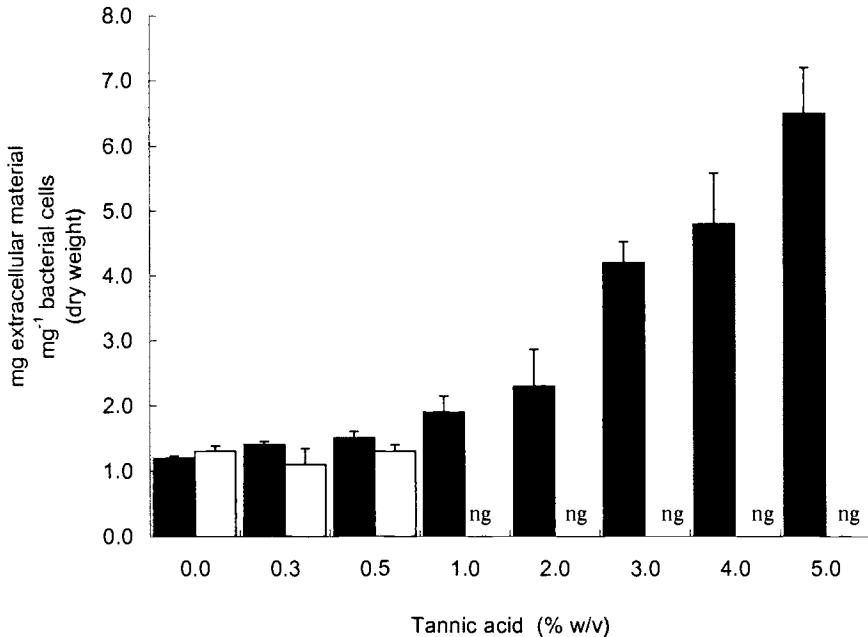
FESEM of *S. caprinus* after overnight growth in mBHI medium containing (a), or lacking (b), 2% w/v TA. Cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated as described in Methods. Preparations were viewed using a Phillips XL30FESEM. Arrow indicates extracellular matrix, bar represents 1  $\mu\text{m}$ .

2% w/v ACT, EM increased 2.5-fold (results not shown). Addition of phenolic acids such as gallic acid, *p*-coumaric or ferulic acid had no significant effect on the amount of EM produced by *S. caprinus*.

*Streptococcus bovis* produces excess EM when grown in the presence of sucrose. To determine whether EM alone would provide protection against TA, *S. bovis* was grown overnight in mBHI, then diluted and spread on plates containing 5% sucrose and 0, 0.2, 0.5, 1.0 or 1.5% w/v TA. In the absence of TA, colonies were extremely slimy with a spreading morphology. In the presence of 0.2% TA, there was no reduction in colony forming units (cfu) and colonies were white and shiny. There was no growth on TA > 0.5% w/v.

### *Selenomonas ruminantium* K2

Isolated on mBHI plates containing up to 5% w/v tannic acid, this organism was able to grow on either tannic acid or condensed tannin as a sole carbon source and was shown to produce gallic acid from tannic acid. Tannin



**Fig. 29.5.** Effect of tannic acid on the production of extracellular matrix by *S. caprinus* and *S. bovis*.

Extracellular matrix was isolated from *S. caprinus* and *S. bovis* following 48 h growth in mBHI medium supplemented with increasing concentrations of tannic acid. Data represents the average of triplicate trials; bars represent the standard error. ng: no growth.

*S. caprinus* ■, *S. bovis* □.

acylhydrolase (TAH) activity was demonstrated using gallic acid methyl ester (GAME) as an artificial substrate and activity was shown to increase by up to 35-fold when K2 was grown in the presence of tannic acid or GAME (Table 29.2), but not monomeric phenols. However, maximum specific activity of TAH was not observed until cells reached stationary phase at an  $A_{600}$  of greater than 1.0.

### Histochemistry of intestinal brush border enzymes

To determine whether tannins had effects downstream from the rumen, we examined enzyme profiles from regions of the intestine of sheep fed *Acacia aneura* (Mulga).

Alkaline phosphatase (AP) activity was measured in the duodenum, jejunum and ileum of sheep in five different groups. The results (Table 29.3) show that there was no significant difference in AP activity across the various regions of the intestine within treatment groups but between groups, activity in the Mulga-fed sheep was 50-60% lower than oaten hay chaff (OHC), OHC + polyethylene glycol (PEG) or Mulga + PEG-fed sheep.

Aminopeptidase-N (AP-N) specific activity was approximately threefold greater than AP activity, but the regional distribution was similar (Table 29.3). OHC, OHC + PEG and Mulga + PEG expressed similar AP-N activity, whereas activity in the duodenum, jejunum and ileum of Mulga-fed sheep was approximately 25% of the other treatment groups. The addition of PEG to the Mulga diet restored AP-N activity and regional distribution was demonstrated in the epithelial cells of the duodenum and the ileum.

**Table 29.2.** Effect of phenolic compounds on specific activity of tannin acylhydrolase (TAH) *in vivo*.

*Sel. ruminantium* K2 cells were grown in the presence or absence of phenolic compounds and cell-free extracts were assayed for TAH activity.

Growth medium	TAH activity
mBHI	0.13 ± 0.02
mBHI + 0.2 % tannic acid	4.52 ± 1.10
mBHI + 0.2% ferulic acid	0.12 ± 0.03
mBHI + 0.2% catechin	0.18 ± 0.04
mBHI + 0.1% GAME	2.10 ± 0.20
mBHI + 0.2% gallic acid	0.42 ± 0.09

**Table 29.3.** Effect of diet on alkaline phosphatase (AP) and aminopeptidase-N (AP-N) activities in the intestinal tract of sheep fed *Acacia aneura* (Mulga).\* Activities are expressed as mean absorbance per  $\mu\text{m}^2$  of microvillus membrane.

Enzyme and location	Enzyme activities versus diet				
	<i>Ad libitum</i>	OHC	Mulga	OHC+PEG	Mulga+PEG
Alkaline phosphatase*					
Duodenum	0.25 ± 0.04	0.28 ± 0.05	0.17 ± 0.02	0.25 ± 0.02	0.22 ± 0.02
Jejunum	0.33 ± 0.02	0.26 ± 0.02	0.16 ± 0.03	0.31 ± 0.02	0.24 ± 0.03
Ileum	0.26 ± 0.03	0.21 ± 0.03	0.17 ± 0.04	0.32 ± 0.03	0.31 ± 0.03
Aminopeptidase-N*					
Duodenum	0.79 ± 0.02	0.81 ± 0.02	0.15 ± 0.01	0.51 ± 0.11	0.59 ± 0.02
Jejunum	0.55 ± 0.08	0.80 ± 0.02	0.10 ± 0.01	0.77 ± 0.03	0.88 ± 0.03
Ileum	0.85 ± 0.03	0.81 ± 0.02	0.20 ± 0.02	0.65 ± 0.06	0.75 ± 0.02

## Discussion

*Streptococcus caprinus* and *S. bovis* were inhibited by the presence of tannins in the medium, shown by an extended lag phase before logarithmic growth and a reduced growth rate. Although both species exhibited an extended lag phase, *S. bovis* was approximately 30-fold more sensitive to TA or ACT than *S. caprinus*. At an ACT concentration of 2% w/v, the lag period for *S. caprinus* was only slightly increased (10 h) over TA (7 h).

These data demonstrate that both species are initially sensitive to tannins and that adaptation by enzyme induction or metabolic transformation of the tannin molecule is required. Only *S. caprinus* is able to achieve a level of adaptation to permit continued growth, albeit at a reduced rate, in the presence of > 0.75% w/v tannins. The fact that the lag period was tannin-concentration dependent suggests that growth in the presence of tannins may be at least partly dependent on metabolic transformation of the tannin or the development of a protective barrier. A reduction in the lag period following re-inoculation of tannin-grown *S. caprinus* supports the view that adaptation is associated with a time-dependent event such as enzyme induction (possibly gallate decarboxylase), and/or the synthesis of EM. Decreased growth rate is also consistent with the TEM and FESEM studies, which showed an increase in chain length and clumping for *S. caprinus* in the presence of TA.

We have demonstrated increased activity of gallate decarboxylase and the accumulation of pyrogallol as a result of exposure of *S. caprinus* to TA, but not ACT. Decarboxylation was specific for gallic acid. Since *S. caprinus* would

normally function in a mixed community, it is likely that other bacterial species would utilize the pyrogallol, thus maintaining a thermodynamic equilibrium in favour of gallate decarboxylation. Evidence for such a community approach is seen in the possible interaction between *Sel. ruminantium* K2, which produces gallic acid from hydrolysis of gallotannins (Skene and Brooker, 1995), and *S. caprinus* which decarboxylates gallic acid to form pyrogallol. Similar descriptions of *S. caprinus* and *Sel. ruminantium* K2 have been made by several other groups worldwide, strengthening the argument that there may be a bacterial community approach to the degradation of tannins in the rumen, and that these two organisms may be key members of that community.

The concept of a protective barrier is supported by the SEM studies that showed that *S. caprinus* produces elevated levels of EM in response to tannin and that this is found closely associated with the bacterial cell wall. Removal of EM from *S. caprinus* appeared to increase its sensitivity to TA, supporting the view that this material provides a protective barrier to the organism. In contrast, enhancement of EM synthesis by growth of *S. bovis* in a sucrose-rich medium did not increase its tolerance for TA. This suggests that EM biosynthesis alone is not enough to provide protection against tannins, and possibly, that induction of gallate decarboxylase is a critical factor in tannin tolerance by *S. caprinus*.

These results explain the widespread occurrence of *S. caprinus* and *Sel. ruminantium* K2 in the rumen of livestock that frequently browse tannin-containing forages (Brooker *et al.*, 1994), and it is likely that the presence of these and other tannin-resistant organisms provides a selective advantage to these animals, either through the metabolic transformation of gallate esters (and therefore decreased toxicity to other organisms) or the maintenance of rumen microbial biomass for the supply of bacterial protein for animal growth.

The results of histochemical studies clearly demonstrate inhibitory effects on abomasal and intestinal function, which are separate from effects on bacterial populations. Reduced activity of AP and AP-N were evident, and this was restored by the inclusion of PEG in the diet. The simplest explanation of this effect is that enzyme activity was inhibited by the protein-binding action of tannins and this was alleviated by pre-binding the tannins with PEG. However, an alternate explanation is that the tannins inhibited enzyme secretion by forming a lining on the intestinal mucosa, thus preventing the export of proteins from the intestinal epithelial cells. This second explanation is supported by additional data (not shown), which demonstrates that tannins induce histological changes in the intestinal mucosa including abnormal villous structure and disruption of cellular networks of communication.

Therefore these results indicate that tannins may inhibit several different processes including microbial and digestive tract functions, and that these effects may have an impact upon animal production over a range of grazing and browse feeds. However, recognition of the effects may be more problematic since the extent and duration of inhibition will depend upon a number of factors including diversity of forage available, intake, age of plant and other environmental

influences. Resistance to tannins may therefore occur at several levels – microbial tolerance, tannin degradation and intestinal tract adaptation.

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## Chapter 30

# **Modulation of *Escherichia coli* Shiga Toxin Activity on Vero Cells by the Use of the Secondary Plant Compound Swainsonine**

R.E. Droleskey, R.C. Anderson, T.R. Callaway, T.J. Anderson, K.M. Bischoff, C.L. Sheffield, R.B. Harvey and D.J. Nisbet  
*United States Department of Agriculture, Agricultural Research Service, Southern Plains Agricultural Research Center, College Station, TX 77845, USA*

### **Introduction**

Enterohaemorrhagic *Escherichia coli* (EHEC), such as O157:H7, produce Shiga toxins, STX 1 and STX 2, which are responsible for much of the diarrhoea and enterocolitis associated with disease caused by these pathogens. In some cases, Shiga toxins from enterohaemorrhagic *E. coli* can cause a potentially fatal haemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) as well as lesions of the central nervous system (Nataro and Kaper, 1998; Paton and Paton, 1998). The primary receptor on the epithelial cell surface for STX is the glycolipid receptor globotriaosylceramide, Gb<sub>3</sub> (Nataro and Kaper, 1998). The Shiga toxins STX 1 and 2 from *E. coli* are indistinguishable from *Shigella* Shiga toxins with respect to cytotoxicity, receptor specificity and affinity (Nataro and Kaper, 1998). Recent work has indicated that tunicamycin, an inhibitor of N-linked glycoprotein synthesis, reduced the expression of Gb<sub>3</sub> on the epithelial surface of HeLa cells in culture (Jacewicz *et al.*, 1989). Reduced expression of Gb<sub>3</sub> was accompanied by reduced cell cytotoxicity after exposure to Shiga toxin derived from *Shigella dysenteriae*. Swainsonine, like tunicamycin, is an inhibitor of N-linked glycoprotein synthesis with its action specific for lysosomal and Golgi mannosidase II (Dorling *et al.*, 1989; Bernhardt *et al.*, 1994). In the present report, we explored the possibility that swainsonine could also reduce the expression of Gb<sub>3</sub> and/or other potential low affinity STX binding sites. For our study we utilized a microtitre-based lactate dehydrogenase (LDH) cytotoxicity kit to detect toxin activity against Vero 76 cells, a cell line traditionally used to assay toxigenic *E. coli* for STX (Nataro and Kaper, 1998).

## Materials and Methods

Swainsonine (Sigma, St Louis, MO) was fully dissolved in Dulbecco's modified Eagle's medium (DMEM), filter sterilized, and diluted to a working concentration of 25  $\mu\text{g ml}^{-1}$  in either DMEM or Tryptic Soy Broth (TSB). Supernatant from a non-toxicogenic strain of O157:H7 (ATCC: 700728) (non-toxicogenic TSB) was prepared by centrifugation and filter sterilization of culture fluid from a culture grown overnight in TSB. Purified *E. coli* toxins STX 1 and 2 (provided by David Acheson, GRASP Center, New England Medical Center, Boston, MA) were prepared from frozen stock aliquots by re-suspension to the appropriate concentration in non-toxicogenic TSB. Cytotoxicity of Vero 76 cells (ATCC: CRL-1587) was determined by measuring release of LDH activity (Grif *et al.*, 1998). The assay was accomplished by seeding microtitre wells with  $10^4$  Vero cells in 200  $\mu\text{l}$  of DMEM and then incubating the cells overnight with or without swainsonine at 37°C under 5%  $\text{CO}_2$ . After this initial incubation, the media was replaced with fresh media as before and solutions of STX 1, STX 2 or appropriate controls were added. The plates were re-incubated for an additional 24 h, then 100  $\mu\text{l}$  of fluid from each well was transferred to a corresponding well in a new 96 well plate and reagents of the LDH cytotoxicity kit were added. The plate was then incubated at room temperature for 30 min and read in a Spectramax 340 PC spectrophotometer (Molecular Devices, Sunnyvale, CA). Assays performed with serial two-fold dilutions (0.005 to 2.5  $\text{ng well}^{-1}$ ) of STX 1 and 2 prepared in TSB yielded linear dose response curves ( $R^2 > 0.62$ ). The effects of swainsonine were assessed using Shiga toxins at 2.5 or 0.156  $\text{ng well}^{-1}$ , concentrations that represented a maximal and half maximal LDH effect, respectively. Cytotoxicity of Vero cells incubated in control wells with supernatant fluid from non-toxicogenic *E. coli* O157:H7 alone was  $\leq 5\%$  and was not affected by DMEM or TSB.

## Results and Discussion

Glycoconjugated proteins and lipids expressed on the host cell's surface often serve as receptors for numerous adhesions or toxins (Karlsson *et al.*, 1991). In the case of EHEC, binding of STX to  $\text{Gb}_3$  expressed by susceptible cells plays a critical role in haemorrhagic colitis (via killing of  $\text{Gb}_3$ -expressing epithelial cells or via vascular injury), in haemolytic uremia (via action against glomerular endothelial cells) and in central nervous system disorders (via binding to cerebral endothelial cells) (Neill *et al.*, 1994; Lingwood *et al.*, 1998; Nataro and Kaper, 1998; Tarr *et al.*, 2000; Eisenhauer *et al.*, 2001). Consequently, strategies are sought to interrupt this toxin-receptor interaction, particularly since, aside from intensive supportive therapy, there are few options currently available for treatment of these manifestations (Paton and Paton, 1998). For instance, antibiotic treatment of EHEC infections remains highly controversial due to concerns that such treatment will increase toxin concentrations in the gut lumen by lysing *E. coli* cells or by inducing prophage and/or transcriptional events (Paton and Paton,

1998). Research on the development of potential therapeutic strategies intending to bind and capture STX in infected individuals has begun and these may prove beneficial if administered very early in the course of the disease. For instance, oral administration of an oligosaccharide component of Gb<sub>3</sub> covalently linked to silica particles may bind STX in the gut lumen thus preventing further absorption (Armstrong *et al.*, 1991). Other approaches being investigated include using analogues of Gb<sub>3</sub> or 'humanized' anti-STX antibodies to bind toxin systemically (Lingwood *et al.*, 1998; Paton and Paton, 1998).

Alternatively, it may be possible to reduce cytotoxicity by reducing the number of available Gb<sub>3</sub> receptors on the cell surface. Tunicamycin, an inhibitor of N-linked glycoprotein synthesis, decreases expression of Gb<sub>3</sub> and reduces cytotoxicity in HeLa cells (Jacewicz *et al.*, 1989). Presently, we report that swainsonine, another inhibitor of N-linked glycoprotein synthesis, inhibited the cytotoxic effects of STX 1 and 2 in Vero cells (Table 30.1). While the effects of swainsonine appeared to be concentration dependent, they did not exhibit first order kinetics. For instance, in the absence of STX, swainsonine exhibited little cytotoxicity ( $\leq 2\%$ ) to Vero cells even when included at levels of up to  $2.5 \mu\text{g well}^{-1}$  (data not shown). However, in the presence of STX, the highest inclusion levels of swainsonine did not result in the greatest reductions in STX-associated cytotoxicity thus suggesting the existence of an as yet unexplained interaction between STX and swainsonine. In the case of cells challenged with  $0.156$  or  $2.5 \text{ ng well}^{-1}$  STX 1, maximum reductions in cytotoxicity (relative to that observed in control wells incubated without swainsonine) of 78% and 74% were observed with cells co-incubated with  $0.1563$  and  $0.625 \mu\text{g swainsonine well}^{-1}$ , respectively (Table 30.1). In the case of cells likewise challenged with STX 2, maximum reductions in cytotoxicity of 89% and 100% (relative to controls) were observed with cells co-incubated with  $1.25$  and  $0.3125 \mu\text{g swainsonine well}^{-1}$ , respectively (Table 30.1). It is also possible that the relatively low cytotoxicity observed in control wells lacking swainsonine may have confounded our results (Table 30.1). The reason for the low sensitivity of the Vero cells to the STX is not readily apparent but could be because the Vero cells used in our studies were obtained from ATCC which, if passaged more than 15 times, may not have expressed Gb<sub>3</sub> at levels adequate to allow optimal sensitivity to STX (Nancy Cornick, Iowa, 2001, personal communication).

Although the presumption that swainsonine caused a tunicamycin-like inhibition of Gb<sub>3</sub> expression on the cell surface seems plausible (Jacewicz *et al.*, 1989), there are at least two other possible pathways where swainsonine modulated Golgi mannosidase II inhibition may interfere with STX toxin activity. One possible mode of action would be interference with the formation of and internalization of coated pits (Lew *et al.*, 1994) containing the STX-Gb<sub>3</sub> complex (O'Brien and Holmes, 1996). The other possibility would involve interference with the routing of internalized STX-Gb<sub>3</sub> containing coated pits through the *trans*-face of the Golgi apparatus. Blockage, resulting from swainsonine-modulated inhibition of Golgi mannosidase II, could result in STX being either translocated to lysosome storage vacuoles or blockage of STX translocation through the Golgi apparatus to the endoplasmic reticulum. Translocation of

**Table 30.1.** Vero cell cytotoxicity (%) after co-incubation with swainsonine.

Swainsonine ( $\mu\text{g well}^{-1}$ )	Per cent cytotoxicity <sup>a</sup>			
	STX 1 (ng well <sup>-1</sup> )		STX 2 (ng well <sup>-1</sup> )	
	0.156	2.5	0.156	2.5
0	16.7 $\pm$ 1.5 (100)	18.5 $\pm$ 3.6 (100)	18.3 $\pm$ 3.5 (100)	29.9 $\pm$ 1.3 (100)
0.0098	14.0 $\pm$ 4.3 (84)	12.1 $\pm$ 3.9 (65)	8.9 $\pm$ 2.5 (49)	2.9 $\pm$ 0.6 (10)
0.0195	10.0 $\pm$ 3.7 (60)	13.1 $\pm$ 2.8 (71)	9.5 $\pm$ 1.9 (52)	10.0 $\pm$ 2.3 (33)
0.0391	9.2 $\pm$ 2.3 (55)	16.7 $\pm$ 3.4 (90)	7.6 $\pm$ 2.0 (42)	12.8 $\pm$ 3.1 (43)
0.0781	8.2 $\pm$ 2.3 (49)	17.0 $\pm$ 3.9 (92)	6.8 $\pm$ 1.5 (37)	8.6 $\pm$ 1.7 (29)
0.1563	3.6 $\pm$ 1.3 (22)	16.4 $\pm$ 6.0 (89)	6.7 $\pm$ 1.1 (37)	6.0 $\pm$ 0.9 (20)
0.3125	7.6 $\pm$ 2.1 (46)	9.1 $\pm$ 0.7 (49)	6.1 $\pm$ 1.2 (33)	0.0 $\pm$ 0.1 (0)
0.6250	6.4 $\pm$ 0.9 (38)	4.9 $\pm$ 0.3 (26)	3.9 $\pm$ 0.3 (21)	4.0 $\pm$ 0.5 (13)
1.2500	7.7 $\pm$ 0.3 (45)	10.4 $\pm$ 2.0 (56)	2.0 $\pm$ 0.3 (11)	5.4 $\pm$ 0.8 (18)
2.5000	14.1 $\pm$ 3.1 (84)	12.3 $\pm$ 2.5 (66)	12.9 $\pm$ 1.3 (70)	11.0 $\pm$ 2.4 (37)

<sup>a</sup> Mean  $\pm$  SD ( $n = 3$ ). Values in parentheses express cytotoxicity as a per cent relative to controls incubated without swainsonine.

internalized STX to the endoplasmic reticulum through the Golgi apparatus is reported to be an essential step in the STX toxin pathway (O'Brien and Holmes, 1996). Clearly, more work is needed to elucidate the molecular mechanisms behind the reduction in STX-associated Vero cell cytotoxicity. Nevertheless, the results obtained from our exploratory series of experiments empirically demonstrate the potential of swainsonine to inhibit glycoprotein processing of susceptible cells.

Whether or not practical interventions involving the use of swainsonine as a drug, which itself is somewhat toxic, can ultimately be developed to prevent attachment of STX to not only kidney cells, but perhaps to gut epithelial and cerebral endothelial cells as well, will depend on its effectiveness and safety *in vivo*. When consumed for extended periods, the toxic effects of swainsonine have been found to be transient and reversible (Dorling *et al.*, 1989), which thus provides support to the concept that short-term administration may be able to mitigate disease during critical periods of susceptibility. If swainsonine and perhaps other inhibitors of Golgi function and protein glycosylation can safely be administered as a drug to animals then these may be useful to temporarily inhibit the host's expression of other receptors used by a variety of pathogens. Examples include mannose-substituted proteins bound by type I fimbriae of *E. coli* and

*Salmonella* (Low *et al.*, 1996) or glycoconjugated receptors bound by adhesive K88 fimbriae of enterotoxigenic *E. coli* (Jin and Zhao, 2000). Swainsonine is being used to study the molecular properties of the poliovirus receptor (Bernhardt *et al.*, 1994) and, along with other inhibitors of protein glycosylation such as tunicamycin, deoxynojirimycin, deoxymannojirimycin and castrospirime, is being tested as a possible anti-tumour drug (Olden *et al.*, 1992; Demetriou *et al.*, 1995). Undoubtedly, their use in further elucidating receptor expression and epithelial cell development will provide valuable insights into mechanisms of pathogenesis that occur in host cells.

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## Chapter 31

# The Identification of Corynetoxin-like Tunicaminyuracil-glycolipids from Nematode Galls in *Festuca nigrescens* from North America and New Zealand

N. Anderton<sup>1</sup>, K.A. Beales<sup>1</sup>, Y. Cao<sup>1</sup>, S.M. Colegate<sup>1\*</sup>, J.A. Edgar<sup>1</sup>, A. Michalewicz<sup>1</sup>, I.T. Riley<sup>2</sup>, P.L. Stewart<sup>1</sup> and K.A. Than<sup>1</sup>

<sup>1</sup>Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Victoria 3220, Australia; <sup>2</sup>Applied and Molecular Ecology, Adelaide University, Glen Osmond, South Australia 5064, Australia

\*Author to whom correspondence should be addressed.

Corynetoxins (CTs), chemically and toxicologically related to the tunicamycins (TMs), are a group of tunicaminyuracil-glycolipids that are responsible for stock deaths associated with ingestion of nematode/bacterium-colonized grasses (Bryden *et al.*, 1994). As described by Riley *et al.* (Chapter 6 this volume), there has been speculation that early nematode-related episodes of stock poisoning in the USA might be similar to annual ryegrass toxicity (ARGT) and attributable to the presence of CT-like tunicaminyuracil-glycolipids. Nematode seed galls from *Festuca nigrescens* obtained from New Zealand and the USA were examined using an ELISA for the presence of an antigen derived from the CT-producing bacterium, *Rathayibacter toxicus* (Riley *et al.*, Chapter 6 this volume). Since the galls from New Zealand were only weakly positive, and the galls from the USA were negative for the presence of the *R. toxicus* antigen, it was important to determine whether CT-like material was present.

### Analysis of Galls

Galls (two from the USA, two from New Zealand and one from Western Australia as a positive control) and clean annual ryegrass (*Lolium rigidum*) seed (as negative control) were treated with 80% methanol in water in a manner proven to extract CTs from bacterium-colonized nematode galls of annual ryegrass seedheads collected in Western and South Australia (Cockrum and Edgar, 1985).

The extracts were then examined for cross-reactivity in a CT/TM-specific ELISA, for the presence of CT-like components using high pressure liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LCMS),

and for *in vitro* inhibitory activity of the enzyme N-acetylglucosamine-1-phosphate transferase (GPT), critical to protein N-glycosylation, which is irreversibly inhibited by TMs and CTs that act as substrate site analogues (Jago *et al.*, 1983).

### ELISA analysis

An indirect, competitive ELISA has previously been developed for the detection of CTs and TMs using polyclonal primary antibodies raised against a tunicamycin-derived hapten-protein conjugate (Than *et al.*, 1998; Than *et al.*, Chapter 59 this volume).

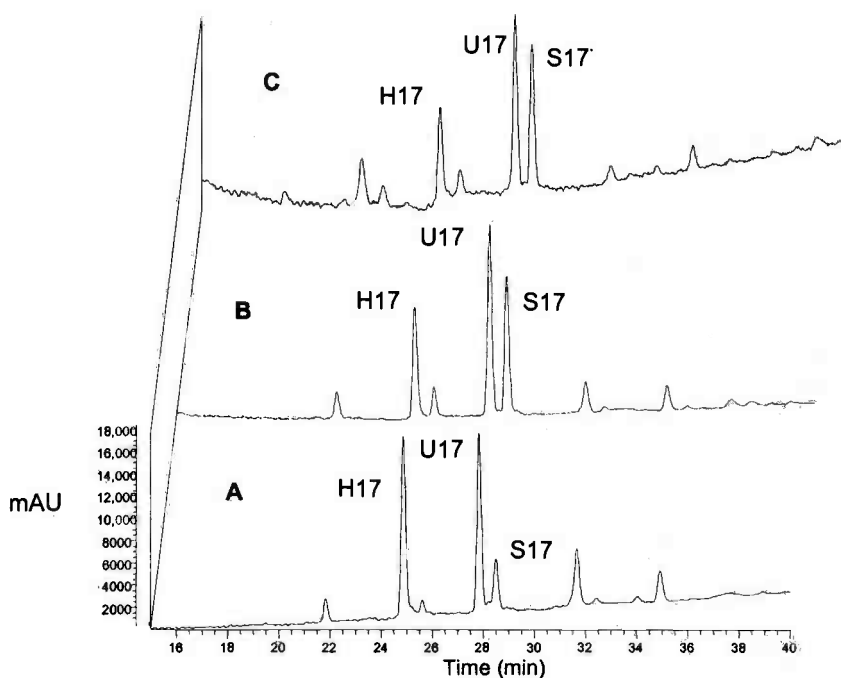
In an assay where the solvent blank and extract of clean ryegrass seed were devoid of ELISA cross-reacting material, the extract of bacterium-colonized nematode galls from annual ryegrass seedheads indicated about 1.6 µg CTs per gall. By comparison, the extracts of the galls obtained from *F. nigrescens* from New Zealand and the USA yielded cross-reacting components equivalent to about 1.1 µg CTs and 0.5 µg CTs per gall respectively.

### HPLC and LCMS analysis

High pressure liquid chromatograms (obtained using the method of Cockrum and Edgar, 1985) of the extracts from the New Zealand and USA galls clearly demonstrated the presence of CT and TM-like components both in terms of the retention times observed and the full scan UV absorption spectra of individual peaks (Fig. 31.1). Quantitation against a standard solution of purified CT H17a indicated a CT equivalent of 1.8, 0.9 and 0.4 µg per gall in the galls from Australia, New Zealand and the USA respectively.

Further confirmation of the identities of the individual components of the TM/CT-like cluster of HPLC peaks was obtained by directing the effluent of the HPLC column into the atmospheric pressure electrospray ionisation chamber of a Finnigan LCQ mass spectrometer. The M<sup>+</sup>+H adduct ions observed were the same for each of the corresponding peaks in the toxin profiles of the galls from Australia, New Zealand and the USA (Table 31.1).

While the peak profiles of the CT-like material from the USA and New Zealand galls were very similar to that of the Australian galls, there were some differences in relative intensities, especially of the major peaks (H17, U17 and S17, Fig. 31.1). However, experience has shown that the profile and relative intensities are consistent between samples of galls from different Australian sources and South Africa (Cockrum and Edgar, 1985). Therefore these observations could indicate a genetic difference in the bacterium species responsible for the toxins in the New Zealand and USA galls relative to that responsible for the Australian toxins. However, in support of the CT-like nature of the extractives, it should be noted that hydroxylated fatty acid chains (denoted by the descriptor, H in Table 31.1) have only so far been detected in the corynetoxin family of tunicaminylluracil-glycolipids.



**Fig. 31.1.** HPLC comparison (detection wavelength 260 nm) of extracts of (A) Australian galls from *Lolium rigidum*, (B) New Zealand galls from *Festuca nigrescens* and (C) USA galls from *F. nigrescens*. Peaks correspond to mass spectral data shown in Table 31.1.

### HPLC/ELISA immunogram

To confirm that the HPLC peaks assigned to CT-like compounds from the New Zealand and USA galls were also the source of the cross-reactivity observed in the CT ELISA, HPLC fractions were collected every minute during the analytical run. These fractions were then assayed using the ELISA. The peaks of ELISA response correlated with the elution of the CT peaks (see Than *et al.*, Chapter 59 this volume for an example). There was some cross-reactivity with earlier eluting material in each of the extracts of galls, possibly corresponding to more polar, CT precursors, but this was only a small part of the total cross-reactivity response and is a feature previously noted with CT extracts.

**Table 31.1.** Comparison of liquid chromatography mass spectral data for extracts of galls from Australia, New Zealand and the USA. Peak identification is based on nomenclature described by Cockrum and Edgar (1985).

Retention time (min)	Ions observed (M <sup>+</sup> +H)	Detected in galls			Peak identity
		Australia	New Zealand	USA	
19.77	831.2	+	-	-	U15
21.48	833.1	+	++	+	S15
21.55	863.2	++	+	+	H16
24.66	845.5	+	+	+	U16
24.54	877.4	+++++	++++	+++	H17
25.28	847.3	++	++	++	S16
27.5	859.3	+++++	+++++	++++	U17
28.13	861.5	++	++++	+++	S17
28.09	891.3	++	+	+	H18
31.25	873.3	+	++	++	U18
31.70	905.3	+	-	-	H19
34.43	887.4	++	++	++	U19
32.81	875.2	+	+	+	S18
35.73	889.0	+	+	+	S19

### N-acetylglucosamine-1-phosphate transferase inhibitory activity

The irreversible inhibitory effect of CTs and TMs on GPT provides a basis for determining *in vivo* exposure to these tunicaminyuracil glycolipids. This is achieved by examining the activity of hepatic microsomal GPT (Stewart and May, 1994; Stewart, 1998), and estimating the CT-like activity of an extract *in vitro* by examining the effect on hepatic microsomal GPT derived from a non-exposed sheep (Stewart, 1998).

In an assay in which the GPT activity of a control sheep liver microsomal preparation is set at 100%, solutions of TMs at concentrations of 2 ng ml<sup>-1</sup> and 12 ng ml<sup>-1</sup> caused a relative inhibition with a residual activity of 73% and 7% respectively. There was no adverse effect on the GPT activity by the solvent used to extract the gall samples (activity 108%). The extract of the galls derived from Western Australia inhibited the GPT by 98% (2% residual activity) and the extracts of the galls from New Zealand and the USA caused a relative inhibition of GPT of 98% (2% residual activity) and 96% (4% residual activity) respectively. These results clearly showed the presence of GPT-inhibition activity consistent with the presence of CT/TM-like compounds in the extracts of the gall material from Australia, New Zealand and the USA (1.3, 0.8 and 0.5 µg per gall respectively).

## Conclusions

Tunicaminyluracil-glycolipids, similar to the CTs produced by *R. toxicus* in Australia and South Africa, have now been identified in old nematode seed-galls from *F. nigrescens* from the USA (New Jersey) and New Zealand. The estimates of CT-like levels in galls by the ELISA, HPLC and *in vitro* enzyme inhibition were consistent.

Identification of the gall extractives as CT/TM-like tunicaminyluracil-glycolipids, including the presence of components possessing hydroxylated fatty acid side chains and characteristic of corynetoxins, was shown using HPLC/UV, HPLC/MS, cross-reactivity in an ELISA specific for tunicamycins and corynetoxins, HPLC/ELISA and *in vitro* inhibition of GPT.

While not identical to the corynetoxins profile produced by populations of *R. toxicus* from Australia and South Africa, the toxin profiles from the New Zealand and USA galls were similar enough to indicate that a geographical variant of *R. toxicus* or closely related species may be involved. Therefore it is concluded that the livestock deaths associated with the feeding of nematode-infested and bacterium-infected screenings of *F. nigrescens* in Oregon, USA in the 1940s to the 1960s may also have been caused by CT-like toxins produced by the bacterium. A case of poisoning with ARG-T-like clinical signs described in California (Galey *et al.*, 1997) may be an indication of the continued presence, albeit low level of occurrence, of tunicaminyluracil-glycolipid induced disease in the USA.

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## Chapter 32

# The Metabolism of Saponins from *Yucca schidigera* in Sheep

A. Flåøyen<sup>1,2</sup>, A.L. Wilkins<sup>3</sup> and M. Sandvik<sup>1</sup>

<sup>1</sup>National Veterinary Institute, PO Box 8156 Dep., 0033 Oslo, Norway;

<sup>2</sup>Department of Large Animal Clinical Sciences, Norwegian School of Veterinary Science, PO Box 8146 Dep., 0033 Oslo, Norway; <sup>3</sup>Chemistry Department, School of Science and Technology, The University of Waikato, Private Bag 3105, Hamilton, New Zealand

### Introduction

*Yucca schidigera* is a desert plant native to the southwestern USA and Mexico that is widely used in the food and feed industries as well as in the drug and cosmetic industries (Cheeke, 1998). *Yucca* products are used as anti-stress agents in man and in poultry, to promote weight gain in cattle, to inhibit ammonia formation in poultry and pig waste (Kaneda *et al.*, 1987). They are also used in very large quantities as foaming agents in beverages. Steroidal saponins are considered to be the active ingredient of extracts of *Y. schidigera* (Kaneda *et al.*, 1987; Hostettmann and Marston, 1995).

Steroidal saponins are spirostanol or furostanol glycosides bearing one or more sugar chains, usually one at the C-3 carbon and one at C-26 (Hostettmann and Marston, 1995; Osbourn, 1996). The classical definition of saponins is based on their surface activity, for many saponins have detergent properties and give stable foams in water (Hostettmann and Marston, 1995). The aglycone or non-saccharide portion of the saponin is called the *genin* or *sapogenin*. The steroidal portion is derived from the cyclization and rearrangement of six isoprene units.

Chemical structure analyses of *Y. schidigera* saponins have shown the dominant genin components to be sarsasapogenin and smilagenin, together with lower levels of markogenin, samogenin, gitogenin and neogitogenin (Kaneda *et al.*, 1987; Miyakoshi *et al.*, 2000).

This paper describes the metabolism and excretion of *Yucca* saponins in sheep. Two 3-month-old Dala breed lambs, 15.5 and 21 kg live weight were dosed with 26 mg kg<sup>-1</sup> sapogenins day<sup>-1</sup> for 11 consecutive days, assuming an average 48% sapogenin contribution to the *Yucca* saponins, based on the relative molecular mass of sarsasapogenin (416 Daltons), and that of the dominant *Yucca* saponin (872 Daltons). The ratio (mass basis) of sarsasapogenin to smilagenin in the dosed material was 85 to 15. For 14 days before dosing commenced, and throughout the dosing period, the lambs were fed with sheep concentrate and hay

*ad libitum*. One of the dosed lambs was killed by captive bolt stunning and immediate exsanguination 5 hours after being dosed with the last dose. The second lamb was killed 1 week later. No adverse effects were observed in the two animals after dosing started.

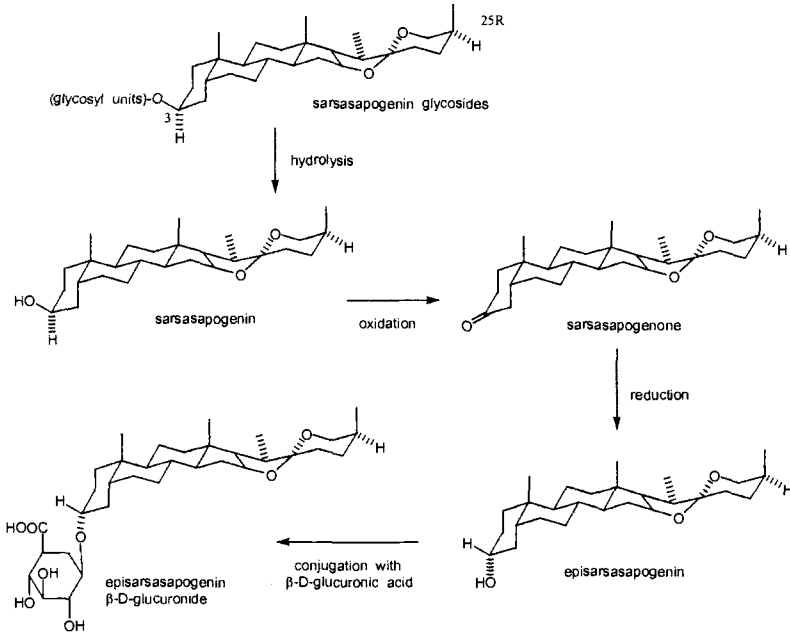
## Metabolism

The total levels of free and conjugated saponinins found in GI-tract samples from one of the *Yucca*-dosed lambs are presented in Table 32.1. The results obtained in

**Table 32.1.** Total free and conjugated saponinins (mg kg<sup>-1</sup> dry matter) and % episaponinins found in GI-tract and liver samples following the dosing 11 consecutive days of a *Yucca* saponin extract.

	Extract	Total saponinins	Percentage episaponinins of total saponinins
Rumen	Free	263	23
	Conjugated	6.7	18
Omasum	Free	842	22
	Conjugated	79	19
Abomasum	Free	441	25
	Conjugated	25	19
Duodenum	Free	84	19
	Conjugated	3.4	21
Jejunum	Free	118	16
	Conjugated	6	59
Ileum	Free	299	91
	Conjugated	40	89
Caecum	Free	1063	75
	Conjugated	155	74
Colon ascendens	Free	1177	75
	Conjugated	175	73
Faeces	Free	1507	70
	Conjugated	187	68
Liver	Free	21	27
	Conjugated	1.1	100





**Fig. 32.1.** Proposed metabolism of *Nartheceium ossifragum* and *Yucca schidigera* sarsasapogenin glycosides (saponins) to afford episarsasapogenin  $\beta$ -D-glucuronide.

the dosing trial were comparable to those obtained in other dosing experiments in which sarsasapogenin and smilagenin saponins from *Nartheceium ossifragum* were dosed to sheep (Flåøyen and Wilkins, 1997; Flåøyen et al., 2001).

### Fore-stomachs and abomasum

We propose that rumen microbes quickly hydrolyse the *Yucca* saponins almost completely to the parent sapogenins, i.e. sarsasapogenin and smilagenin. In the rumen 15 to 30% of the sapogenins undergo oxidation and reduction at C-3 to afford episarsasapogenin (Fig. 32.1) and epismilagenin. Epimerization is arrested when the sapogenins arrive in the omasum and abomasum indicating that, in the fore-stomachs, rumen microbes are primarily responsible for the conversion of sapogenins to episapogenins.

### Small intestines

The concentration of sapogenins in the dry matter drops significantly in the upper (small) intestines indicating that this is probably the major site for absorption of sapogenins.

## Liver

Sarsasapogenin and smilagenin, which have not been oxidized and reduced in the fore-stomachs, appear to be oxidized and reduced to the corresponding episapogenins in the liver, prior to conjugation with D-glucuronic acid and excretion into the bile. The oxidation and reduction processes can be classified as phase I metabolism whereas the conjugation is a typical phase II process.

## Large intestines and faeces

The ratio of conjugated and unconjugated (free) sapogenins seems to be reasonably stable throughout the large intestines. In the animals studied so far, conjugated sapogenins contribute between 5 and 15% of the total sapogenins.

## Urine

To date we have not detected free or conjugated sapogenins in urine samples following the dosing of *N. ossifragum* or *Yucca* saponins.

## Discussion

Saponins from *N. ossifragum*, together with saponins from at least nine other plants, have been suggested to cause hepatogenous photosensitization diseases of sheep (Flåøyen, 1999). These plants are *Agave lecheguilla*, *Tribulus terrestris*, *Brachiaria decumbens*, five *Panicum* spp. and *Nolina texana*. Among these plants only *N. ossifragum* and *A. lecheguilla* contain saponins predominantly derived from sarsasapogenin and smilagenin (Flåøyen, 1999) – the same sapogenins found in *Y. schidigera*.

The ovine metabolism of *Yucca* saponins appears to be identical to that of *N. ossifragum* saponins (Flåøyen and Wilkins, 1997; Flåøyen *et al.*, 2001, 2002). We therefore suggest that saponins from *Y. schidigera* are likely to be equally toxic to sheep as are saponins from *N. ossifragum*.

*N. ossifragum* is a small plant, and it is time consuming to collect enough plant material for a dosing experiment. Commercially available *Yucca* saponins may therefore replace *N. ossifragum* saponins in future studies of *N. ossifragum* associated hepatogenous photosensitization of sheep.

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## Chapter 33

# Ecological, Physiological and Behavioural Interactions between Marsupial Folivores and *Eucalyptus* Antifeedants

W.J. Foley<sup>1</sup>, I.R. Lawler<sup>1, 2</sup>, B.D. Moore<sup>1</sup> and I.R. Wallis<sup>1</sup>

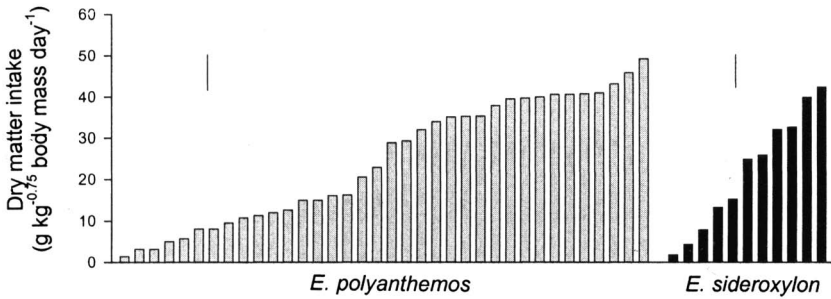
<sup>1</sup>*School of Botany and Zoology, Australian National University, Canberra 0200, Australia;* <sup>2</sup>*Current: Department of Tropical Environment Studies and Geography, James Cook University, Townsville 4811, Australia*

### Introduction

*Eucalyptus* dominates Australian forests and woodlands in both the temperate and tropical zones (Williams and Woinarski, 1997). There are about 800 species of eucalypt (all but two are evergreen) ranging from trees of about 100 m through to small shrubs. In spite of this dominance, few insects and even fewer mammals feed to a large extent on *Eucalyptus* foliage. The mammalian fauna are all marsupials and include the koala (*Phascolarctos cinereus*) an obligate eucalypt folivore (6–13 kg liveweight), the greater glider (*Petauroides volans*), a smaller (1–1.5 kg) obligate eucalypt folivore, common ringtail possum (*Pseudocheirus peregrinus*, 0.7–1.0 kg) and common brushtail possum (*Trichosurus vulpecula*, 2–5 kg) both of which feed on a range of other species as well as eucalypts. In addition several of the terrestrial kangaroos (e.g. the swamp wallaby, *Wallabia bicolor* and the Tasmanian pademelon, *Thylogale billiardieri*) also feed partly on eucalypt leaves and are regarded as serious pests of young seedling trees by the wood-fibre industry in some parts of the country (McArthur *et al.*, 2000).

### Intraspecific Variation and the Patchiness of *Eucalyptus* Forest for Folivorous Marsupials

*Eucalyptus* foliage is a highly variable and patchy resource for herbivores at a range of spatial scales. For example, at the landscape scale folivorous mammals are distributed patchily in large areas of contiguous forest and there is evidence that this pattern is associated with soil and foliar nutrients measured as concentrations of N, P and K (Cork and Catling, 1996). At the finest scale, animals discriminate between individual trees within a species (Pass *et al.*, 1998;



**Fig. 33.1.** Variation in dry matter intake by common ringtail possums fed *Eucalyptus polyanthemos* or *Eucalyptus sideroxylon* in two separate experiments. Grey shaded bars are *E. polyanthemos* and black shaded bars are *E. sideroxylon*. Vertical bars above each group of data are least significant differences (5%) from ANOVA or REML analysis.

Lawler *et al.*, 1998a, 2000) and between branches within a single tree (Edwards *et al.*, 1990; Foley, unpublished observations).

It is the intraspecific variation that is of most relevance because it forces us to stop regarding taxonomic species of trees as food resources for these animals. It has been said before that 'animals don't eat Latin binomials' and this is particularly true of *Eucalyptus* and folivorous marsupials. For example in *Eucalyptus polyanthemos* and in *Eucalyptus sideroxylon*, the range of voluntary dry matter intakes of fresh foliage by common ringtail possums ranged from 50 g kg<sup>-0.75</sup> day<sup>-1</sup> to 4 g kg<sup>-0.75</sup> day<sup>-1</sup> for 36 individual trees (Fig. 33.1) (Lawler *et al.*, 2000).

Experimental evidence has shown that all species of folivores examined show a similar ranking of individual trees within a species with respect to dry matter intake (Lawler *et al.*, 1998a; McIlwee *et al.*, 2001). When koalas and common ringtails were fed a number of individual trees of both *Eucalyptus ovata* and *Eucalyptus viminalis*, koalas ate significantly more foliage from the 'worst' individuals yet the ranking of trees in terms of intake from best to worst was the same for each species (Lawler *et al.*, 1998a). This pattern has also been demonstrated for the kangaroos *W. bicolor* and *T. billardieri* (Lawler and Foley, 1999).

### Chemical basis of feeding inhibition

Eucalypts contain a wide variety of chemical compounds, many of which can be toxic to herbivorous mammals. These include terpenoids, tannins and other phenolic compounds, as well as cyanogenic glycosides. Numerous studies have attempted to link the occurrence of these compounds to food choices by marsupials. However, most studies have focused on tree species rather than

individual trees. The amount of intraspecific variation illustrated in Fig. 33.1 suggests why these approaches have not yielded definitive results.

Detailed studies addressed at the intraspecific level of variation have shown that the differences in feeding by marsupials is largely explainable by the occurrence and concentration of a newly described but well-characterized group of plant secondary metabolites called formylated phloroglucinol compounds (FPCs) (Pass *et al.*, 1998; Lawler *et al.*, 1998a, 2000) (Fig. 33.2).

For example, in *E. polyanthemos*, 86% of the variation in dry matter intake of common ringtail possums is explained by the concentration of a single plant secondary metabolite called sideroxylylonal A. Sideroxylylonal A is one of a group of diformyl phloroglucinol compounds (Fig. 33.2) that are terpene–phenol adducts (Lawler *et al.*, 2000) and that are widely distributed in eucalypts (Pass *et al.*, 1998). Sideroxylylonals are active against other marsupials as well. In koalas fed *Eucalyptus melliodora* foliage, sideroxylylonal A concentration explained 70% of the variation in intake (Moore and Foley unpublished). Related compounds (Fig. 33.2) occur in other species and act as effective defences against common ringtail possums (Pass *et al.*, 1998; Lawler *et al.*, 1998a). For example in *E. ovata*, the concentration of macrocarpal G explains much of the difference in intake of both koalas and common ringtails (Lawler *et al.*, 1998a).

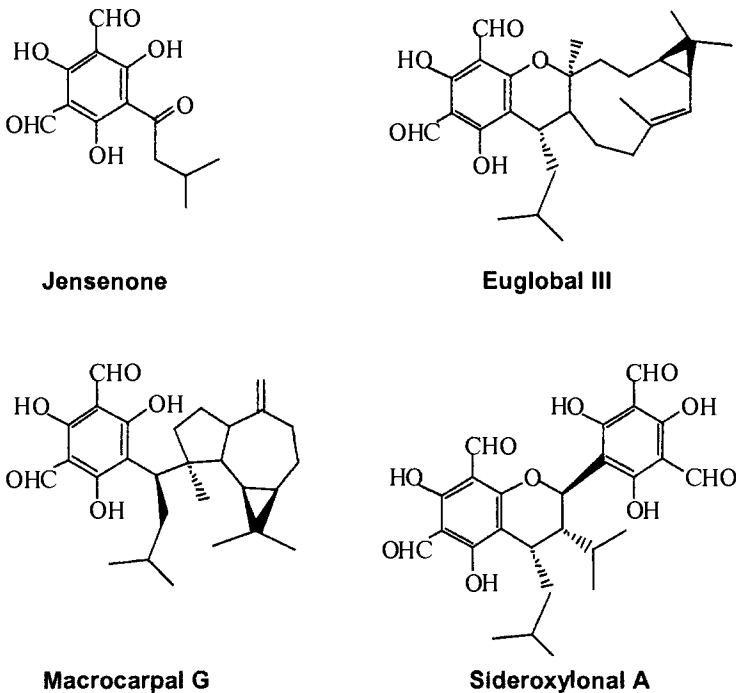


Fig. 33.2. Formylated phloroglucinol compounds from *Eucalyptus* foliage.

Most FPCs (e.g. the euglobals and macrocarpals: Fig. 33.2) contain both terpene and phenolic moieties. The phenolic part of the compound is derived from phloroglucinol and this is coupled to a common foliar mono- or sesquiterpene such as  $\beta$ -phellandrene, or bicyclogermacrene by a Diels-Alder reaction. Other compounds such as sideroxylonals and jensenone contain only a C5 side chain. Although the biosynthesis of the FPCs is complex, it was not surprising to find that there is a tight correlation between the concentration of FPCs and terpenes in eucalypt leaves. For example, Lawler *et al.* (2000) showed that the concentration of 1,8-cineole, the major foliar terpene in *E. polyanthemos*, explained more than 80% of the variation in foliar sideroxylonal concentration. This relationship is important because it allows animals to use the concentration of volatile terpenes as a cue to the concentration of the non-volatile sideroxylonals. We will return to this point later.

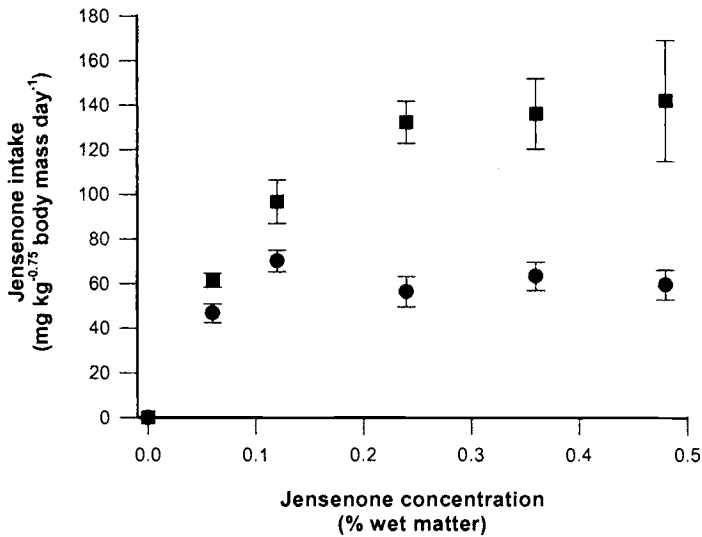
### **Evidence that the Intake of FPCs is Regulated**

The concentration of FPCs in the diet effectively sets a threshold to the amount of food that is consumed. For example, Lawler *et al.* (1998b) showed that both common ringtail and brushtail possums adjusted their total food intake as the concentration of jensenone in the diet increased. This resulted in no change in jensenone intake over a tenfold difference in the concentration of dietary jensenone (Fig. 33.3). Common ringtail possums eat about 105 mg kg<sup>-0.75</sup> body mass day<sup>-1</sup> of sideroxylonal even though the concentration of the compound varied between 5 and 30 mg g<sup>-1</sup> dry matter.

These thresholds may be absolute and animals are unwilling to ingest more FPC even when their energy requirements are elevated (such as during prolonged exposure at 4°C) (Stapley *et al.*, 2000). This suggests that feedback resulting from ingestion of sideroxylonals and related compounds could override feedback from other sources.

### **How do FPCs act to reduce food intake?**

Any argument invoking plant secondary metabolites as an important factor in regulating feeding by mammals has to identify and test a feedback mechanism (Foley *et al.*, 1999). Feedback mechanisms are the link between the physiological effects of a secondary compound and the behavioural responses of adjusting intake or seeking an alternative food. Although the toxic actions of sideroxylonal and related compounds have not yet been identified, part of the feedback that signals the animals to limit their feeding is mediated through serotonin (5HT<sub>3</sub>) receptors (Lawler *et al.*, 1998b). Administration of a selective 5HT<sub>3</sub> antagonist



**Fig. 33.3.** The effect of varying concentrations of dietary jensenone on the intake of jensenone in common ringtail and brushtail possums. Squares are ringtail possums, circles are brushtail possums.

(ondansetron) resulted in significantly greater intakes of FPC than in controls. Since 5HT<sub>3</sub> receptors play such a key role in mediating vomiting in other animals (and the FPCs are in themselves emetogenic at high concentrations) we suggest that nausea plays a role in limiting the feeding of animals on these compounds (Lawler *et al.*, 1998b). However, it is unlikely that nausea is the only feedback that modulates feeding.

### Translating Physiological Effects into Behaviour

We have already described the relationships between the concentration of foliar terpenes and foliar FPCs. Correlative studies initially implicated terpenes in the differential palatability of *E. ovata* (Pass *et al.*, 1998). However, bioassay experiments have shown that the tolerance of marsupial browsers for terpenes is sufficient to allow high food intakes when terpene concentrations exceed those seen in resistant trees (Lawler *et al.*, 1998a, 2000). Therefore we asked what was the real role of foliar terpenes in the *Eucalyptus*-marsupial interactions.

Many studies have noted that marsupial folivores smell leaves before eating or rejecting the foliage and this has been generally regarded as evidence that a volatile cue indicates the palatability of a leaf (Hindell *et al.*, 1985). We hypothesized that because of the correlation noted above between FPC and terpene concentrations, marsupials could use the odour of the leaf terpenes as a



proximal cue to assess the likely post-ingestive consequences of FPCs in a leaf (Lawler *et al.*, 1998a, 2000). Such an association between a taste or smell and post-ingestive consequences is termed a conditioned flavour aversion (CFA).

Animals in the wild may have existing CFAs to natural foods and so testing this concept is not straightforward. However, CFAs are dynamic and they can be extinguished by repeated exposure to the taste stimulus alone. If this can be done, then the formation of a CFA can be tested in the conventional manner.

We therefore designed a series of experiments to test three hypotheses:

1. Wild-caught folivorous possums held in captivity will show a strong aversion to *Eucalyptus* terpenes, due to their previous experience;
2. This aversion can be reduced or removed by feeding the animals an artificial diet to which increasing amounts of terpene are added;
3. Once removed, the aversion can be 'reconditioned' by giving the animals a diet consisting of both the terpene and a FPC known to produce negative post-ingestive effects in corresponding concentrations (Lawler *et al.*, 1999).

All three hypotheses were supported. When a common *Eucalyptus* terpene (1,8-cineole) was added to an artificial diet, both common ringtail and common brushtail possums reduced their food intakes (note though that this response occurred at cineole concentrations higher than those found in browser-resistant foliage). We then fed one group of possums (the 'test group') an artificial diet to which we added cineole in increasing concentrations for about 2 weeks (final concentrations were 10.5% DM and 16.5% DM for ringtail and brushtail possums respectively). Subsequent experiments showed that acclimation led to an increased willingness to ingest cineole (such that it was indistinguishable from the intake of the untreated diet) while control animals that had not been acclimated to cineole in their diet substantially reduced their intakes in response to the same cineole concentration. We then fed the animals that had been acclimated to cineole a diet containing both jensenone (an FPC, Fig. 33.2) and cineole in corresponding concentrations. We anticipated that the effect of jensenone would be to reduce the animals' food intakes by an amount similar to that previously observed with cineole only. We did this for 6 days using three pairs of concentrations of jensenone and cineole, each being offered to each animal twice. We then tested whether a CFA to cineole had been reconditioned by those animals that had learnt to associate the taste of cineole with jensenone.

We found that in both species of possum, the response of the test group to cineole was once again indistinguishable from the control group. We concluded that one important role of terpenes in marsupial folivore-*Eucalyptus* interactions may be to act as a cue to the level of toxins in the leaf, rather than to act as toxins in their own right (Lawler *et al.*, 1999).

## **Ecological Consequences**

We have shown that marsupials eat significantly different amounts of foliage from different trees within a species. In order to scale these observations up to animal habitat level, we need to know what kind of variation exists in a contiguous patch of forest. This involves making many measurements and the only feasible way of doing this is to use near-infra-red reflectance spectroscopy (NIRS). Foley *et al.* (1998) and McIlwee *et al.* (2001) showed that NIRS was a suitable tool for measuring nutrients and secondary metabolites in dried, ground eucalypt leaves. Lawler *et al.* (2000) found that FPCs were normally distributed in the leaves of a plot of 80 *E. polyanthemos* trees and the same has been shown to be true for 150 *E. melliodora* trees (Foley and Wallis, unpublished). This suggests that most trees that an animal will encounter in the forest will have a mid-range FPC concentration. If this mid-range concentration exceeds the animal's threshold for the FPC, then the majority of the apparent food resource is unavailable. Accordingly, it is likely that we will need new statistical approaches when we attempt to correlate tree-use with leaf chemistry. For example, there may be many reasons why animals do not use individual trees irrespective of their content of FPCs. Traditional correlative approaches are likely, therefore, to underestimate the importance of FPCs in feeding of wild marsupials.

A second issue is the scale over which measurements of canopy chemistry are made. We have extended the NIRS method to measurements of whole, fresh leaves in the field (Foley, unpublished; S. Dury and W.J. Foley, unpublished). Furthermore, we are now testing whether foliar components such as nitrogen and sideroxylonals can be detected from an airborne remote-sensing platform. If so it will become possible to map the nutritional variability in the habitat of leaf-eating marsupials on a landscape scale. The studies described above show the absolute necessity for such measurements. If we restrict ourselves to assessing habitat quality and abundance as a function simply of the occurrence of known food species, we may substantially overestimate the amount of the resource that is truly available to the folivore of interest.

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## Chapter 34

# PCR Amplification for Detection of *Synergistes jonesii*, the Ruminal Bacterium that Degrades the Toxins of *Leucaena leucocephala*

T.J. Anderson<sup>1</sup>, R.C. Anderson<sup>1</sup>, M.J. Williams<sup>2</sup>, R.O. Elder<sup>1</sup> and D.J. Nisbet<sup>1</sup>

<sup>1</sup>USDA-ARS, Southern Plains Agricultural Research Center, Feed and Food Safety Research Unit, 2881 F&B Rd, College Station, Texas 77845, USA;

<sup>2</sup>USDA-ARS, Subtropical Agricultural Research Station, Brooksville, Florida 34601, USA

### Introduction

Large scale ranching requires a reliable continuous supply of livestock forage but due to the poor soil conditions and periods of overall low rainfall experienced in many areas, native grasses in south Texas are not always able to supply the animal's feed demands. *Leucaena*, *Leucaena leucocephala*, is a highly digestible, high quality forage legume (Jag Jiwan Ram *et al.*, 1994; Aschfalk *et al.*, 2000) that has the potential to be used as forage in this area due to its drought tolerance, disease resistance and ability to grow in poor soils (Akingbade *et al.*, 2001). In some areas of south Texas where leucaena has been planted, it thrives, growing up to 10 m within 2 years if not grazed (personal observation). However, leucaena contains mimosine, a toxic non-protein amino acid analogue of tyrosine (Hegarty *et al.*, 1964; Jones, 1985; Hammond, 1995) and under certain conditions has caused poisoning of Texas cattle (Anderson *et al.*, 2001). During mastication and rumen digestion, mimosine undergoes enzymatic degradation to 3-hydroxy-4(1H)-pyridone (3,4-DHP) and its isomer 2,3-dihydroxy pyridine (2,3-DHP), both of which are also toxic (Jones, 1985; Hammond, 1995). Symptoms of leucaena toxicosis include a partial loss of appetite, emaciation, poor live weight gains, alopecia (especially near the tail switch, near the base of the tail and at brand sites), scaly skin, ear and eye lesions, mouth ulceration and drooling viscid saliva (Jag Jiwan Ram *et al.*, 1994).

## Detoxification of Mimosine

In areas of the world where leucaena is native (St Croix, US Virgin Islands and Haiti) or has been well established (Hawaii, Indonesia and Venezuela), ruminants consuming leucaena do not experience leucaena toxicosis, even when consuming diets containing more than 50% leucaena (Jones and Megarrity, 1986; Hammond, 1995). The rumens of these animals are colonized by specialized bacterial populations capable of collectively degrading mimosine and its DHP metabolites (Allison *et al.*, 1987, 1990, 1992; Dominguez-Bello and Stewart, 1990, 1991). While bacteria possessing either or both 3,4-DHP- and 2,3-DHP-degrading activities have been isolated, detoxification appears to be accomplished primarily by bacteria that degrade the more toxic 3,4-DHP (Jones and Lowry, 1984; Jones and Megarrity, 1986; Hammond, 1995). Some of the DHP-degrading microbes have not yet been fully identified (Dominguez-Bello and Stewart, 1990, 1991), however, at least one bacterium, *Synergistes jonesii*, has been extensively studied (Allison *et al.*, 1987, 1990, 1992; McSweeney *et al.*, 1993). Unlike most ruminal bacteria that use carbohydrates as energy sources, *S. jonesii* appears to ferment only 3,4-DHP, 2,3-DHP, arginine and histidine (Allison *et al.*, 1992). Persistence of *S. jonesii* within the rumen is dependent on the presence of these substrates and ruminants that have never had or had no recent access to leucaena may lack or harbour low numbers of *S. jonesii*, a condition enhancing their risk to toxicosis. Consequently, in order to safely manage leucaena, livestock producers need to know the *S. jonesii* colonization status of their animals prior to grazing leucaena. Also, they may at times need to inoculate this important detoxifying microbe into at least a few sentinel animals, which consequently serve to disseminate the bacterium to herd mates via natural, yet unknown, mechanisms.

## Detection of *Synergistes jonesii*

The current methodology to specifically detect DHP-degrading bacteria is very time consuming and tedious. *Synergistes jonesii* is an obligate slow growing anaerobe not easily grown in the laboratory and the toxic substrate 3,4-DHP used as a medium component has to be hydrolysed from mimosine and column purified (Allison, 1991). The presence of *S. jonesii* is inferred through the disappearance of the substrate mimosine or 3,4-DHP from the culture medium using a colorimetric method based on acidified ferric chloride (Allison *et al.*, 1990; Allison, 1991; Hammond *et al.*, 1992). Alternatively, indirect detection of DHP-degrading activity within ruminants can be determined by a colorimetric urine analysis method (Jones, 1985; Jones and Megarrity, 1986). To simplify and expedite detection methods for *S. jonesii*, Yang *et al.* (1999) developed PCR primers to a unique 0.9 kb fragment of *S. jonesii* DNA, which they used to detect *S. jonesii* in rumen fluid samples.

In our laboratory, we found that primer sequences used by Yang *et al.* (1999) were highly specific for the detection of *S. jonesii*. Using chromosomal DNA

prepared from a pure culture of *S. jonesii* (2.5 pg of template DNA per reaction), we obtained a PCR product of the correct size. Also, a PCR product could be detected by adding 400-fold (1 ng) or 4000-fold (10 ng) excess bacterial DNA to the PCR. Total chromosomal DNA (1 ng or 10 ng) prepared from rumen fluid or faecal samples from cattle never fed a diet containing leucaena failed to generate any PCR product thus indicating the absence of potential cross-reacting DNA. All samples were PCR positive when spiked with whole cells of *S. jonesii* and then extracted, or by addition of 2.5 pg of *S. jonesii* DNA, therefore, validating the extraction method and the absence of PCR inhibitors in the reaction mix. The rumen fluid of a cow known to be culture positive for the presence of *S. jonesii* was positive by PCR. Unfortunately, *S. jonesii* could not be detected from the faeces of the same animal suggesting that the number of bacteria in the faeces, if any, was below the detection limit for PCR. Considering that the collection of rumen fluid from extensively grazed animals may be inconvenient in the field, we assessed the practicality of sampling and testing bovine saliva for the presence of *S. jonesii* DNA. Saliva samples were readily collected from naive animals via a commercial swab kit (Catch-All™, Epicentre, Madison, WI, USA) but produced no PCR product unless first spiked with whole cells or total chromosomal DNA. Ongoing studies will test the saliva of known *S. jonesii* culture positive animals and if successful, should yield a less invasive sampling technique for testing ruminants for the presence of *S. jonesii*. The presence of *S. jonesii* in saliva may also confirm a suspected mode of transmission of the bacterium between animals.

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## Chapter 35

# **Bracken Fern (genus *Pteridium*) Toxicity – a Global Problem**

B.L. Smith

*New Zealand Pastoral Agriculture Research Institute Ltd, Ruakura Research Centre, Private Bag 3123, Hamilton 2001, New Zealand*

Bracken fern (genus *Pteridium*), a plant of ancient lineage, is as widespread as any plant on our planet. Its distribution is restricted only by the extremes of temperature and moisture. In some areas bracken is aggressively encroaching on to farmland. Such factors as the nature of land tenure, the size of land holdings, subdivision and changing farm economies contribute to a lack of grazing pressure and the spread of bracken. Livestock readily consume bracken. Man also consumes bracken or its toxins either directly, as in the case of the croziers or the rhizomes, or indirectly through the consumption of milk or meat from animals grazing on bracken fern.

Bracken causes a range of well-defined syndromes in farm animals. These include thiamine deficiency of monogastric animals, acute haemorrhagic syndrome associated with bone marrow aplasia and upper alimentary ulceration, 'bright blindness', a progressive retinal degeneration and two neoplastic disease syndromes. The latter include neoplasia of the urinary bladder (enzootic haematuria) and a syndrome involving upper alimentary carcinomata. The major carcinogen appears to be the sesquiterpenoid glucoside, ptaquiloside. Other carcinogens and mutagens may be implicated in some of the syndromes especially in association with papilloma viruses.

Man has consumed the rhizomes and croziers, the latter still being consumed as traditional food or by necessity in some areas. The carcinogen of bracken passes to the milk of bracken-fed cattle. Epidemiological evidence suggests that some cancers in man might result from the direct or indirect consumption of bracken carcinogens. Ptaquiloside forms adducts readily and it reacts with DNA with affinity with certain base sequences, especially those associated with adenine. It mutates codons associated with known oncogenes and hypotheses for its role in the pathogenesis have been proposed.

### **Natural Syndromes of Bracken Poisoning in Animals**

A number of distinct and well-recognized syndromes will occur in livestock after bracken herbivory and their occurrence will depend on a number of factors. These



include the quantity of bracken available, its content of toxins, the stage of growth and time of year, the consumption rate and the length of time consumption occurs, and the species, sex and age of the animals. It is important that these different syndromes and their pathogenesis be recognized in any work on the aetiology of bracken poisoning. Previous reviews have also dealt with these syndromes as well as the implication for man of bracken consumption (Fenwick, 1989; Hopkins, 1990; Smith, 1990, 1997; Smith and Seawright, 1995; Shahin *et al.*, 1999).

### **Bracken-induced thiamine deficiency**

Bracken fern causes a nervous condition in animals, particularly monogastric species such as the horse and pig. Its earliest signs consist of anorexia and ataxia but these may proceed to opisthotonus, convulsions and death with irreversible lesions of polioencephalomalacia present in the brain. The early signs respond well to thiamine administration.

This syndrome is caused by a type I thiaminase (Evans, 1976) which splits thiamine into its two component ring structures. The thiaminase activity of bracken is highest in rhizomes, especially in the summer, and in the croziers but declines with maturation of the plant. The natural condition has been recorded in the horse (Evans *et al.*, 1951) and pig (Evans *et al.*, 1963) and, although it has been produced in the sheep experimentally (Evans *et al.*, 1975), is considered to be rare in the field. Ruminants synthesize their own thiamine in the rumen.

### **Acute haemorrhagic disease**

This syndrome occurs often in weaned calves, especially if they have been recently introduced to bracken fern, but is also known to occur in older cattle and sheep occasionally (Moon and McKeand, 1953). The acute disease is the sudden clinical manifestation of a degenerative change in the more rapidly growing cells in the body, especially in the bone marrow. Epithelial cell degeneration of calves also gives rise to severe necrosis of the pharynx, larynx and small intestine, the so-named laryngitic and intestinal forms.

The megakaryocytes, which produce the platelets, are depleted during the bone marrow aplasia and a profound thrombocytopaenia occurs. This causes a severe acute haemorrhagic crisis. Other stem cells of the haemopoietic system are affected especially those leading to the formation of granulocytes and lymphocytes and a severe leukopaenia occurs. In cattle neutropaenia is common (Evans *et al.*, 1958) while in sheep a profound lymphocytopaenia has been reported (Sunderman, 1987). It usually takes more than three weeks for the thrombocytopaenia to develop sufficiently to cause a haemorrhagic crisis. This feature enables the use of ruminants (especially sheep) to control regrowth of bracken.

### **Bright blindness**

In some areas of the UK sheep fed on bracken develop a progressive degeneration of the retina that results in blindness (Watson *et al.*, 1972a, b). This retinal atrophy leads to an increased reflectance of the tapetum lucidum, seen especially in semi-dark conditions, which has been given the name 'bright blindness'. A stenosis of the retinal vessels was also observed.

### **Enzootic haematuria**

This syndrome is named from the clinical and epidemiological manifestations of multiple mixed tumours in the urinary bladder. Some of these tumours are of epithelial or connective tissue origin but many are of vascular origin and these, in particular, give rise to intracystic haemorrhage. The condition occurs mainly in cattle but also occurs in sheep (Harbutt and Leaver, 1969). Both conditions occur after prolonged ingestion of bracken. For cattle the time of exposure to bracken is usually a minimum of two to three years. This syndrome occurs worldwide.

### **Upper alimentary carcinoma**

Carcinomata of the oral cavity, nasopharynx, oesophagus and fore-stomach of ruminants have been reported from UK, Kenya and Brazil. Cattle are most commonly reported with this syndrome but sheep also have been reported with fibrosarcomas of the mandible, maxilla and papillomata of the rumen. These cattle tumours are believed to be caused by the malignant transformation of bovine papilloma virus (BVP) Type 4 papillomata (Jarrett, 1987). An association between urinary bladder carcinoma and BPV-2 has also been suggested. Bracken fern has been identified as a source of cofactors. Cofactors such as quercetin and immunosuppressive agents exist in bracken and these can contribute to tumour progression. The relevance of bracken as an environmental co-carcinogen of BVP-4 to papilloma progression and the presence of BPV-2 bovine urinary bladder (Campo *et al.*, 1992) has been investigated especially in relationship to quercetin (Connolly *et al.*, 1998). The relative importance of quercetin to the more recently discovered carcinogens of bracken in both domestic herbivores and experimental animals is still unknown.

## **Experimental Reproduction of the Syndromes by Bracken or Ptaquiloside**

Bracken has been established as the cause of the naturally occurring conditions listed above. Administering bracken has experimentally reproduced thiamine deficiency of horses and rodents, acute haemorrhagic disease, bright blindness and enzootic haematuria or neoplasia in ruminants and experimental animals. The administration of ptaquiloside to a calf caused the profound thrombocytopenia

and bone marrow hypoplasia of the acute syndrome but, because the supply of ptaquiloside was limited, the haemorrhagic crisis did not occur (Hirono *et al.*, 1984c). The progressive retinal neuroepithelial degeneration has also been reproduced by feeding bracken to sheep (Watson *et al.*, 1972b), rats and rabbits and by the administration of ptaquiloside directly into the small intestine of sheep (Hirono *et al.*, 1993). Bracken feeding has reproduced the carcinogenic syndromes of cattle (Rosenberger and Heeschen, 1960; Pamukcu *et al.*, 1967) and the urinary bladder carcinomas in rats (Hirono *et al.*, 1987; Smith *et al.*, 1988) and guinea pigs (Bringuier *et al.*, 1995). It has also produced neoplasms in other organs such as the ileum, mammary gland and lungs of rodents (Pamukcu and Price, 1969; Evans *et al.*, 1986; Smith *et al.*, 1988). Oral feeding of ptaquiloside causes the urinary bladder and small intestinal tumour of rats (Hirono *et al.*, 1984a, b). The parenteral administration of activated ptaquiloside caused the formation of mammary carcinomas of rats (Shahin *et al.*, 1998a).

### **Carcinogens and Toxins in Bracken and Other Ferns**

Two groups of workers almost simultaneously published the structure of a major bracken carcinogen, now generally known as ptaquiloside. The first (Niwa *et al.*, 1983; Hirono *et al.*, 1984a, b) showed ptaquiloside to be carcinogenic to rats by oral and parenteral dosing of increasingly refined fractions of bracken. Ileal and urinary bladder neoplasms were found in the dosed rats. The second paper used a modified Ames mutagenicity test using *Salmonella typhimurium* as a bioassay and named the same compound aquilide A (Van der Hoeven *et al.*, 1983). Subsequent animal studies have shown that ptaquiloside causes the preclinical pathological changes of the acute haemorrhagic disease (Hirono *et al.*, 1984c), the bright blindness in sheep (Hirono *et al.*, 1993) and neoplasia in various laboratory animals (Hirono *et al.*, 1984a; Shahin *et al.*, 1998a, b). Ptaquiloside has been shown to be mutagenic, clastogenic and carcinogenic.

Different taxa of *Pteridium* have been compared for differences in ptaquiloside concentration. All taxa examined contained ptaquiloside although very large variations in concentration were identified. Higher concentrations of ptaquiloside were associated with higher latitudes in eastern Australia (Smith *et al.*, 1994a) and higher altitudes in Venezuela (Villalobos-Salazar *et al.*, 1999). In New Zealand quite large differences in ptaquiloside concentration have been recorded consistently between two locations (Smith *et al.*, 1988, 1993). Bracken fern collected from the vicinity of Hamilton were consistently low (< 100 ppm) while fern collected from 250 km further south from an area where bovine enzootic haematuria is common usually had much higher concentrations of ptaquiloside (most often > 2000 mg kg<sup>-1</sup>). The difference was maintained when rhizomes of bracken were transferred to a common glasshouse environment and soil types changed. These differences were maintained for three consecutive years (Smith *et al.*, 1992), and pellets made from these two sources of ferns were substantially different in their ability to cause cancer in rats (Smith *et al.*, 1988).

These findings suggest a genetic component to the different brackens or some other factor such as an endophyte being transferred with the rhizomes.

Ptaquiloside concentrations are highest in the young growing parts of the plant. In composite samples taken from a uniform patch of bracken it was found that the tips of the croziers and immature fronds had the highest levels. As samples were taken from increasingly mature pinnae towards the base of the lamina, so the concentrations of ptaquiloside diminish. The lowest levels were found in the dark green pinnae of more mature plants (Smith *et al.*, 1993). All samples of rhizome, the apices of the shoots or frond primordia taken from below the soil surface gave no ptaquiloside. However, very immature croziers only a few centimetres above the surface showed the presence of ptaquiloside (B.L. Smith, unpublished). The emergence of the crozier apices and exposure to light may influence the concentration of ptaquiloside. These results are at variance with other findings in which rhizomes were shown to be either capable of causing the acute haemorrhagic syndrome in pigs and cattle (Evans *et al.*, 1961, 1963) or to contain ptaquiloside (Saito *et al.*, 1989). The reasons for this apparent anomaly are not known but could include the contribution of, as yet, unknown other toxins, regional differences in location of ptaquiloside within the plant, different harvesting techniques or condition of rhizome material. Exposure of rhizomes to light could lead to the production of ptaquiloside as it appears in the plant immediately after emergence. Highest levels of ptaquiloside in bracken are found in the springtime with the levels declining throughout the winter (Smith *et al.*, 1992).

The presence of ptaquiloside in spores of bracken fern remains to be demonstrated. Several experiments in which carcinogenicity of spores (Evans *et al.*, 1986; Villalobos-Salazar *et al.*, 1995) or their ability to form DNA adducts (Povey *et al.*, 1996) have suggested that carcinogenic or mutagenic compounds are present. Ptaquiloside was not found in bracken spores from one source (Saito *et al.*, 1989), either by mutagenicity testing or by two-dimensional thin-layer chromatograph. However *Cheilanthes sieberi*, which causes the acute haemorrhagic disease and the urinary bladder neoplasia of ruminants, has been shown to contain ptaquiloside in parts of the fern but not in the spores (Smith *et al.*, 1989).

Other fern genera have been shown to contain ptaquiloside. *C. sieberi*, which has been shown to cause the syndromes of thiamine deficiency, acute haemorrhagic disease and enzootic haematuria in Australia (Clark and Dimmock, 1971; McKenzie, 1978), has been shown to contain ptaquiloside (Smith *et al.*, 1989).

Various ptaquiloside-like compounds or compounds showing mutagenic properties have also been found in bracken and other ferns. Ptaquiloside itself has been isolated from *Histiopteris incisa* and *Pteris cretica* (Saito *et al.*, 1990). Ferns showing mutagenic contents include the genera *Histiopteris*, *Cheilanthes*, *Cibotium*, *Dennstaedtia*, *Hypolepis*, *Pteris* and *Pityrogramma* (Saito *et al.*, 1989). Bracken fern also contains ptaquiloside and other illudane type-B glucosides, isoptaquiloside and caudatoside with structural similarities to ptaquiloside (Castillo *et al.*, 1997). Other ferns too have been shown to contain similar

illudane-type compounds. *Hypolepsis punctata* and *Dennstaedtia hirsta* contain a number of illudane-type sesquiterpene glucosides (Saito *et al.*, 1990) including hypolosite A. It is probable that in some locations some of these ferns, like *Cheilanthes*, may cause similar syndromes to those caused by bracken.

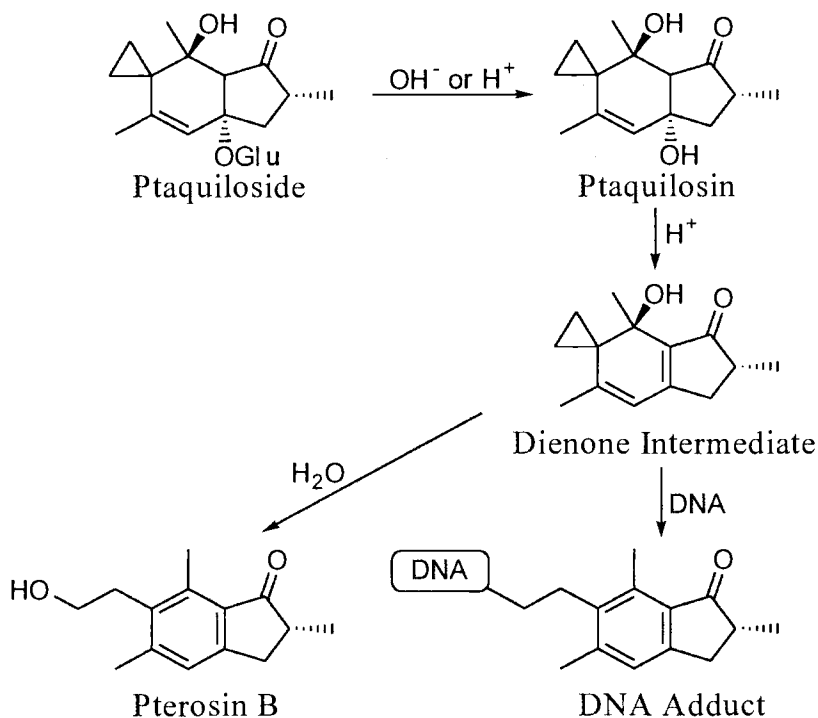
Prior to the discovery of ptaquiloside other compounds were implicated in the carcinogenicity of bracken. Two of these, shikimic acid and quercetin, are found widely in the plant kingdom. One experiment strongly suggested that quercetin, in bracken, caused intestinal and urinary bladder tumours in rats (Pamukcu *et al.*, 1980). Subsequent work has failed to substantiate this finding but its mutagenicity has been confirmed (Stoewsand *et al.*, 1984). The carcinogenicity of quercetin has been specifically discussed and the evidence found to be insufficient (Ito, 1992; Hirono, 1992). However, quercetin is recognized as a co-carcinogen with BPV (Campo *et al.*, 1999).

### Chemistry: Extraction and Analysis of Ptaquiloside

Ptaquiloside is an illudane-type sesquiterpene glucoside and is unstable under both acidic and basic conditions. Degradation is more controlled under basic conditions, where the glucoside is hydrolysed to produce a conjugated dienone adjacent to a cyclopropyl ring. This highly reactive compound can react further under mild acid conditions in the presence of suitable nucleophiles to produce one of a number of stable aromatic compounds, the pterosins (Van der Hoeven *et al.*, 1983). This facile reaction sequence can also lead to DNA adduct formation (Fig. 35.1).

Ptaquiloside and related compounds can be extracted from bracken, and other plant materials containing them such as *C. sieberi*, by water or by organic solvents such as methanol or chloroform. The labile nature of the glycoside makes degradation of a proportion of the compounds inevitable, and the recovered crude extracts also contain pterosins. It seems likely that at least a part of the pterosins reported from plant extracts, are artefacts resulting from the particular extraction and cleanup protocols employed. For analytical purposes, aqueous extraction followed by processing in minimal time is desirable. For the bulk extraction of ptaquiloside, which involves lengthier procedures, various organic solvents and their azeotropes have been employed as part of clean-up procedures to avoid degradation (Hirono *et al.*, 1984a; Oelrichs *et al.*, 1995).

The unstable nature of ptaquiloside makes accurate analysis a challenge. Indirect methods have been devised which react a semi-purified extract to the more stable pterosin B prior to analysis (Agnew and Lauren, 1991; Alonso-Amelot *et al.*, 1992). A direct method for ptaquiloside analysis by Agnew and Lauren (1991) uses water extraction followed by simple cleanup through polyamide resin and analysis by HPLC. The polyamide removes much non-polar material including any pterosins present in the extract, and affords a suitable solution for estimation of ptaquiloside and related compounds. The authors also describe a controlled method for reacting the recovered ptaquiloside to pterosin B



**Fig. 35.1.** The proposed scheme of ptaquiloside reactions to form pterosin B or DNA adducts.

with subsequent HPLC analysis. This procedure can be used as a confirmatory step or for greater sensitivity. This second step was found useful for samples of *C. sieberi* where coextractives interfered with quantitation of ptaquiloside directly (Smith *et al.*, 1989). A modification of this method has been used for scouting bracken extracts for ptaquiloside-like compounds (Castillo *et al.*, 1995, 1997). After water extraction followed by polyamide cleanup, subsamples both without and with base-acid reaction are analysed by HPLC using a solvent gradient. By comparing the peaks in both chromatograms the presence of ptaquiloside-like materials (i.e. reactive products) can be deduced.

### Actions of Ptaquiloside

Ojika *et al.* (1987, 1989) were the first to propose that ptaquiloside carcinogenicity is expressed via initial DNA damage. They showed that

ptaquiloside alkylated N3 of adenine mononucleotide via the reactive cyclopropyl ring. Since then it has been shown the ptaquiloside alkylates DNA preferentially at N3 of adenine in the minor groove (Kushida *et al.*, 1994; Smith *et al.*, 1994b; Prakash *et al.*, 1996). Further work showed that it preferentially alkylated adenines occurring in 5'-TAG and 3'-A in 5'-AA-3' sequences (Prakash *et al.*, 1996).

In many instances, alkylation of DNA is believed to be the first step in the initiation of chemically induced carcinogenesis. Modified bases in specific codons of a proto-oncogene lead to point mutations, which in turn lead to protein products with deleterious effects. For example, methylnitrosourea modifies guanine in codon 12 leading to G-A transition while activated dimethylbenzanthracene alkylates adenine in codon 61 leading to A-T transversion (Kito *et al.*, 1996). Freitas *et al.* (2001) have shown that the adducts induced by bracken in mouse tissues are different to those induced by ptaquiloside. The significance of this remains obscure as the adducts were formed in different species, and resulted from different methodologies, one *in vivo* and the other *in vitro*.

Campo *et al.* (1990) reported the presence of H-ras oncogenes in the papilloma virus-associated carcinomas of the upper alimentary tract in bracken-fed cattle. Later, our study with bracken-fed calves showed the presence of ptaquiloside-DNA adducts and activated H-ras harbouring mutation in the adenine residue of codon 61 in the target organ, ileum (Prakash *et al.*, 1996). In our recent work we found that rats dosed with activated ptaquiloside by iv route developed mammary gland and ileal adenocarcinomas (Shahin *et al.*, 1998a, b). We further showed the presence of DNA adducts (Shahin *et al.*, 1998a) and H-ras mutations in codon 58 and 59 in the preneoplastic tissues (Shahin *et al.*, 1998c). The importance of these findings is suggested by *in vitro* mutagenesis studies which have shown that mutations occurring in codons 12-13 and codons 59-63 reduce the GTPase activity of ras protein by altering its conformation which in turn triggers uncontrolled cell growth associated with neoplasia (Benjamin and Vogt, 1991).

## Bracken and Human Health

Man may consume the toxins of bracken either directly or indirectly. In Japan and several other countries the croziers are eaten directly. In Japan they are steeped in water or treated with wood ash or sodium bicarbonate to preserve the colour and reduce toxicity. This treatment has been tested and found to reduce but not eliminate the carcinogenicity of the croziers (Hirono *et al.*, 1972). The rhizomes too have been a traditional food in Japan. Their starch is extracted and mixed with wood ash as part of the drying procedure. The bracken rhizomes have been consumed in other parts of the world, notably the indigenous peoples of Australia, New Zealand and North America where they also consume the croziers.

Some epidemiological evidence associates the direct consumption of bracken with cancer in man. In Japan, despite the traditional treatment of bracken, a significantly greater proportion (x2.1 for men; x3.7 for women) of the population consuming bracken developed carcinomas of the oesophagus (Kamon and Harayama, 1975). In Brazil, there is recent epidemiological evidence of an association between crozier eating and gastric (x3.45) and oesophageal (x3.40) cancer in man (Marliere *et al.*, 1998).

The ingestion of bracken spores by experimental animals has been linked with cancer and the presence of adducts in tissues. However, although humans in certain environmental situations are often exposed to heavy spore concentrations (Povey *et al.*, 1996), no link has yet been established between the accidental intake of spores and human ill health.

The indirect consumption of bracken toxins through the milk of cattle has been the subject of some interest. Milk from bracken-fed cattle (Evans *et al.*, 1972; Pamukcu *et al.*, 1978; Villalobos-Salazar *et al.*, 1990) has been shown to cause cancer in rodents and ptaquiloside has been shown to be present in such milk (Alonso-Amelot *et al.*, 1996, 1998; Smith *et al.*, unpublished).

In North Wales, where this gastric cancer is particularly prevalent, there was a significantly increased risk (x2.34) among people who spent their childhood in bracken infested areas compared with matched controls (Galpin *et al.*, 1990). With the advent of bulked milk distribution this risk is no longer thought to be present. In mountainous areas of Costa Rica epidemiological studies have shown a correlation between the prevalence of bovine enzootic haematuria and gastric and oesophageal cancer in man (Villalobos-Salazar *et al.*, 1989). Recently, it has been shown that higher rates of gastric cancer in man occur mainly in upland Venezuela where there is more bracken fern and bovine enzootic haematuria in cattle. Milk was suggested as a possible vehicle for the carcinogen (Alonso-Amelot and Avendano, 2001).

Risks to man from consumption of bracken fern croziers are reduced, but not eliminated (Hirono *et al.*, 1972), by the treatments applied to them. Prolonged consumption of bracken croziers by populations must be considered a risk, and epidemiological evidence to support this is accumulating. Likewise, long-term ingestion of milk from bracken-consuming cows must also be considered a risk where the consumption is long term in a non-migratory population. Supporting this is the evidence that ptaquiloside is present in the milk of bracken-fed cows, the ability of such milk to cause cancer in laboratory animals and a growing volume of epidemiological evidence from studies in man.

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## Chapter 36

# **Histochemical Studies of Bracken Fern: Localization of Phenolic and Tannin Defences in Bracken Accessions along an Altitudinal Gradient in the Neotropics**

M.E. Alonso-Amelot, E. Arellano, A. Oliveros Bastidas and M.P. Calcagno

*Grupo de Química Ecológica, Departamento de Química, Facultad de Ciencias, Universidad de Los Andes, Mérida 5101, Venezuela*

### **Introduction**

Bracken fern (*Pteridium aquilinum*) synthesizes and accumulates a variety of toxins belonging to different compound families (Fenwick, 1988; Alonso-Amelot, 2002) which amount to over 100 known compounds. These materials contribute, each one in its own way, to fending off invertebrate and vertebrate predators. Particularly, quantitative studies of phenolics and tannins (P/T) in bracken are limited in number but nevertheless they suggest that a negative correlation between total phenolics and insect herbivory exists (Tempel, 1981). Also, there is abundant evidence in connection with the role of tannins as feeding deterrents and their part in the disruption of digestive processes in mammals. At least two additional aspects in bracken chemistry need to be addressed for a better understanding of the ecological role of P/T accumulated at considerable metabolic cost. Firstly, if preliminary quantitative analyses of P/T show considerable variance with season (Graham, 1991; Vogt *et al.*, 1991; Chaves *et al.*, 1993), it is not entirely clear how this variation correlates with the biotic and abiotic variables encountered in ecological gradients. Secondly, the particular part within the segments where specific compounds are located has not been determined and might provide information in assigning a specific role as toxicants according to the attacking species.

Our long interest in *P. aquilinum* ecological chemistry (e.g. Alonso-Amelot, 2002) led us to:

1. Determine quantitatively the content of monomeric and polymeric phenolics – anthocyanidins – in the neotropical varieties of bracken growing in a range of habitats with contrasting abiotic conditions and predator pressure; and

2. Examine the total phenolic distribution within the pinnae segments using histochemical techniques, in plant populations distributed along altitudinal gradients.

The present work offers the preliminary results of this investigation.

## Materials and Methods

### Plant sampling

Two varieties, *caudatum* and *arachnoideum* of *P. aquilinum* grow in the neotropics and are altitudinally discriminated: (*caudatum* 0–2000 m above sea level (asl), *arachnoideum* 1700–3100 m asl). Only *arachnoideum* was analysed for comparative studies of phenolics content in the present portion of the work because it embraced a greater variety of habitats in the mid to upper mountain levels, and also because there were significantly lower amounts of phenolics in *caudatum*. For P/T assays only the second pinna from the bottom up of sun exposed fully grown fronds were selected (N = 12 per site). All samples were placed fresh in plastic click bags, stored and taken to the laboratory in a cooler at 10–15 °C within 3 hours of collection and processed fresh (Orians, 1995). Sampling sites for the P/T analyses were at three locations (sites I, II and III) chosen from 2200 to 2930 m asl, in the Andean mountain range of the state of Mérida, western Venezuela. Fronds were collected in February (dry season) 1996 and 1998.

### Extraction procedure

The second pinna from the bottom up of freshly collected fronds were excised, vacuum dried at 0.2 mm Hg (Hagerman, 1988; Orians, 1995) for 24 h and stored frozen for a few days before use. An exact weight of sample (2–11 g depending on content of P/T) was blended at high speed in 70% acetone in distilled water (70 ml) at room temperature for 30 min, followed by sonication for an additional 30 min in an ice-water bath. The procedure was repeated twice until all plant materials were extracted. After centrifugation and solvent evaporation below 30°C the volume of the remaining aqueous solution was brought to 100 ml from which a 2 ml aliquot was drawn and passed through Sephadex LH-20. Elution with 90% methanol (90 ml) yielded monomeric phenolics, and aqueous acetone 70% (90 ml) furnished the condensed tannins. Organic solvents in each fraction were evaporated *in vacuo* and subjected to the standard Prussian blue assay with readings at 550 and 725 nm (Price and Butler, 1977) for P and T, respectively. Previously purified (Asquith and Butler, 1985) quebracho (*Schinopsis quebracho-colorado*) tannin was used as standard for tannin quantitation, whereas salicylic acid was used as standard for simple phenolics.

### Anatomical and histochemical samples

Bracken swards from three sites representing altitudinal extremes of the ecological distribution of *caudatum* and *arachnoideum* were sampled. The sites were:

- A (60 m asl, 18–35°C, average 27°C);
- B (1850 m asl, 10–25°C, average 18°C), both *caudatum* and *arachnoideum* grow sympatrically in the area;
- C (2930 m asl, Andean páramo, 0–18°C, average 7°C), the site represents the highest altitudinal limit of the entire *Pteridium* taxon.

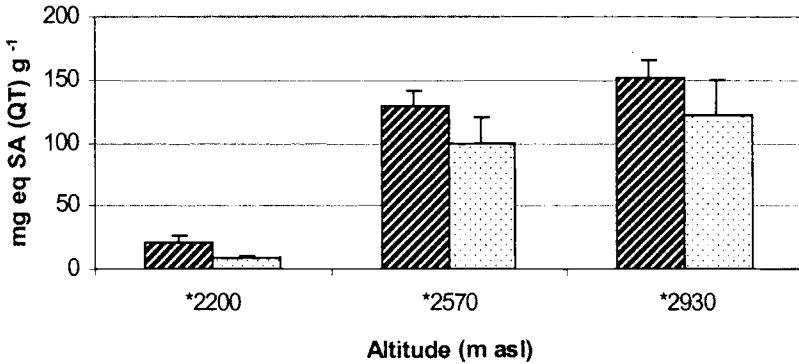
## Results and Discussion

### Variation of phenolic derivatives in ecological gradients associated with altitude

Tropical bracken of the *arachnoideum* variety contains a considerable quantity of phenolic material, distributed between various phenolic carboxylic acids and flavonoids. The second pinnae of sun-exposed fronds contained 14–36 mg of salicylic acid equivalents  $\text{g}^{-1}$  of biomass (mg eq SA  $\text{g}^{-1}$ ). Condensed tannins amounted to 5.5–75 mg of quebracho tannin equivalents  $\text{g}^{-1}$  of biomass (mg eq QT  $\text{g}^{-1}$ ) in the same samples. These amounts were consistent with these values in several samples collected at and below 2200 m asl, taken from a variety of habitats where insect pressure is widely variable. This observation implies that these quantities must confer enough protection against herbivore predation since little if any herbivore activity on bracken could be observed. However, in examining the phenolics and tannin contents in bracken fronds collected along the altitudinal transect, a contrasting result emerged (Fig. 36.1). Monomeric phenolics increased dramatically with altitude, going from 21.02 mg eq SA  $\text{g}^{-1}$  at site I to 152.01 mg eq SA  $\text{g}^{-1}$  at site III, representing a 7.2-fold rise. A parallel result was obtained in the condensed tannin load of fronds, ranging from 8.69 mg eq QT  $\text{g}^{-1}$  at the lower site to 122.22 mg eq QT  $\text{g}^{-1}$  at the páramo location, corresponding to a 14-fold increment. Herbivore pressure cannot explain this result because it is known that species diversity and number wane with altitude. Also, it is unlikely that plant competition has any contribution since plant density and height is considerably greater at sites I and II and less so at site III. Soils at the three locations vary in composition and texture, and probably microbiota as well, but soils of the several other sites also vary where the concentration of phenolics and tannins was found to be constricted within a lower and well-defined band.

Ecological gradients associated with high altitude mountains pose several challenges to the components of the ecosystem. Sites I and II continued to receive moisture during the dry months in the form of mist and dew and occasional light rains carried by dominant east winds and condensed by mountain-forced updrifts,





**Fig. 36.1.** Variation of monomeric phenolics (dark bars), expressed in mg eq SA g<sup>-1</sup>, and condensed tannins (light bars), expressed in mg eq QT g<sup>-1</sup>, of *Pteridium aquilinum* var *arachnoideum* fourth stage fronds growing along an altitudinal gradient in the central Andean range of western Venezuela. Error bars are standard errors of means (N = 12).

whereas site III, located almost 400 m above II, experienced greater desiccation in the same period as it was situated above the level of condensation. The absence of higher vegetation proper of the perennial forests that form a well-defined ecotone some 200 m below, attested to this phenomenon. Nevertheless, bracken in site II also produced 6 and 11 times the amount of phenolics and tannins, respectively, in comparison with site I (Fig. 36.1). Therefore, a stress other than hydric-based strain must be in operation there. In high altitudes the lower particle and moisture content of the atmosphere makes it more transparent to solar radiation. Sunlight induces the synthesis of phenolics in leaves (Woodhead, 1981; Larsson *et al.*, 1986; Mole *et al.*, 1988). At the same time, there is a considerable increase of damaging ultraviolet (UV) photon flux as altitude escalates in high mountains. This UV light is a potent inducer of flavonoid production in plants (Tevini *et al.*, 1983; Chapell and Hahlbrock, 1984; Tevini and Teramura, 1989; Vogt *et al.*, 1991; Panagopoulos *et al.*, 1992; Cen and Bornman, 1993; Liu *et al.*, 1995; Chaves *et al.*, 1997). However, plants respond with great variability to excess UV-B radiation (Sullivan *et al.*, 1992), some remaining completely unaffected and others showing substantial anatomical (Robberecht and Caldwell, 1978) and chemical modification (Teramura and Sullivan, 1991; Jansen *et al.*, 1998), as we now see with *P. aquilinum*.

#### **The structure of bracken segments and sites of accumulation of P/T**

The internal structure of the bracken leaflet is composed of a thick cuticle, one or two rows of medium sized epidermal cells, a well-organized palisade and a store parenchyma. The lower epidermis and cuticle are much thinner than those in the

adaxial surface. A clearly discernible vascular bundle appears along the central vein flanked above and below by the collenchyma that is formed by a honeycomb pattern of thick walled polygonal cells. About 30–40% of the volume in these cells is occupied by the cytoplasm and vacuole.

The structure of the segment shows considerable variation depending on *P. aquilinum* variety and site of collection. In general, *caudatum* displays glabrous leaflets with little or no trichomes and the width of the mesophyll is narrower than in *arachnoideum*. Comparisons within the *caudatum* populations also led to differentiation. Plants collected in the megathermic site (A) possess thin soft segments with a very narrow adaxial cuticle, only one row of palisade cells, a thick store parenchyma and an insubstantial collenchyma. But the segments of *caudatum* fronds from mountain site (B) near the upper ecological distribution of this variety have a much thicker cuticle and epidermis, frequently with two rows of palisade cells, thinner store parenchyma, much more robust collenchyma, and gnarled trichomes growing in the abaxial surface. *Caudatum* shares all these traits with *arachnoideum* but the latter grows even stronger thicker structures.

Plant phenolics were visualized by various colour reactions, including ferrous sulphate, ferric chloride and the Prussian blue oxidation. We applied these tests to segments by shortly immersing thin slices of fern leaflets in appropriate solutions followed by distilled water washing and observation at 400x magnification. The location of phenolics stood out covering most of the mesenchyma, upper cuticle and epidermis. This was most notable in *arachnoideum* exposed to the hardships of excess UV radiation in sites around 3000 m asl. Greater magnification allowed us to locate these compounds in cell walls and the vacuoles in the palisade. The vacuoles of the collenchyma also contained a striking amount of phenolic derivatives as well as the collar of the vascular bundle sheath, probably to protect phloem and xylem carried materials from excess radiation and the extreme temperatures experienced at such altitudes.

The case of bracken fern examined here appears to fit the model of synthesizing a greater amount of phenolic material as a protective device against this excess radiation. Considering that the electromagnetic spectrum of these compounds shows efficient absorption in the UV-A/B wavelength range but not in the photosynthetically active radiation (PAR) sections of the solar spectrum, the phenolics would guard the segments by filtering out this damaging radiation while the PAR components would still be allowed to reach the chloroplasts in the palisade (Robberecht and Caldwell, 1978; Caldwell *et al.*, 1983; Les and Sheridan, 1990).

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## Chapter 37

# **Chronic Toxicity in Cattle due to *Pteridium aquilinum* (Bracken Fern) in Tarija Department, Bolivia: an Interdisciplinary Investigation**

E. Marrero<sup>1</sup>, C. Bulnes<sup>1</sup>, L.M. Sánchez<sup>1</sup>, I. Palenzuela<sup>1</sup>, R. Stuart<sup>2</sup>, F. Jacobs<sup>3</sup> and J. Romero<sup>3</sup>

<sup>1</sup>National Center for Animal and Plant Health, CENSA, Apdo. 10, San José de las Lajas, La Habana, Cuba; <sup>2</sup>Institute of Animal Science, ICA, San José de las Lajas, La Habana, Cuba; <sup>3</sup>AUTAPO Project, Tarija, Bolivia

### **Introduction**

In the course of both intensive and extensive cattle production the full implications of plant toxicity have not always been considered. For example, factors such as the indigenous flora of the region, the characteristics of the soil and the climate of the region each play a part in the management of the animals. Animals may be grazed in areas infested with undesirable plant species that contain natural chemical toxicants (Seawright, 1995). Such plant species may also be very resistant to unfavourable environmental conditions and they may also proliferate and replace established pasture plants (Thompson *et al.*, 1995). One example of such a toxicity scenario for cattle is that currently affecting bovines in the 'Humid Chaco' in the Department of Tarija, Bolivia where bovine enzootic haematuria (BEH) due to ingestion of *Pteridium aquilinum* (bracken fern) is prevalent (Romero, 1982).

*Pteridium aquilinum* can give rise to both acute and chronic intoxication in cattle (Smith, 1997) but it is the chronic form of the disease, namely BEH which is of more significance in regions of Central and South America (Villalobos-Zalazar *et al.*, 1989). In Bolivia in particular there has been a marked increase in the incidence of this disease, mainly in the Tarija Department where it is also often associated with tumours of the upper alimentary tract. BEH in this region of Bolivia is currently responsible for appreciable economic loss, affecting both meat and milk production, particularly at the family farm level. The present report describes a recently conducted study of the disease in the Tarija Department of Bolivia.

In the 'Humid Chaco' region two phases of grazing practice are employed during the year. In the dry season corresponding to April to October, animals are allowed to graze in the mountainous areas which are dominated by wet jungle. In the wet season from November to May the cattle are brought down from the mountains to the

pampas where there is by then abundant pasture.

## Materials and Methods

During the months of October and November 1999 a project was initiated to study epidemiological, botanical, phytochemical, nutritional, clinical and pathological aspects of BEH in five regions of the 'Humid Chaco', Tarija, Bolivia. Taxonomic studies were carried out on botanical specimens of *P. aquilinum* (Page, 1976; Humphrey and Swaine, 1997). Ptaquiloside was assayed in bracken fern samples from the different localities by High Performance Liquid Chromatography (HPLC) methods (Ojika *et al.*, 1985; Yamada *et al.*, 1998). In particular, plant material was air-dried, ground and extracted with water and organic solvents, and the ptaquiloside determined indirectly by measurement of pteridin B generated by alkaline hydrolysis of the ptaquiloside aglycone.

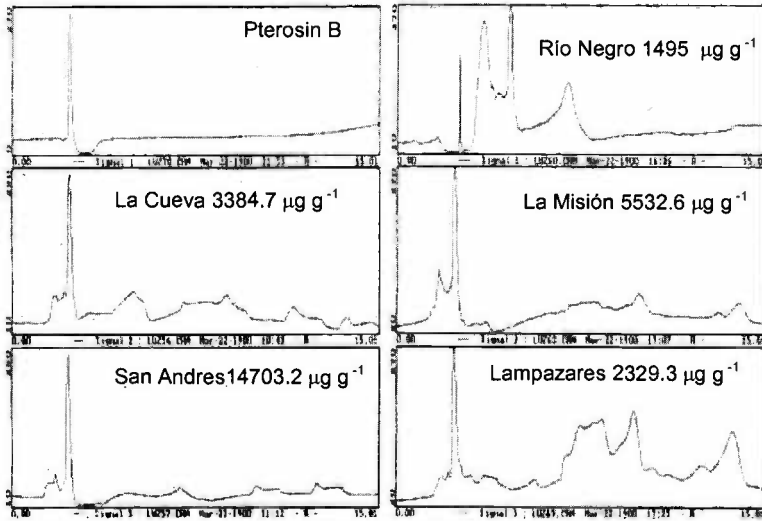
Meteorological data was obtained from weather stations located in the areas designated for study. Herd management and nutrition were evaluated on BEH affected farms. A questionnaire was circulated to farmers in the study areas in order to obtain disease incidence data, particularly in relation to BEH. Clinical examinations were conducted on 21 cattle suffering from BEH. These animals ranged in age from 3 to 10 years with a median age of some 5.8 years. For each animal, rectal temperatures and respiratory and heart rates were recorded. Jugular blood samples were collected for haematological examination. Necropsies were carried out on seven animals and various tissues were collected into 10% buffered neutral formalin for histopathological examination. Paraffin sections were prepared and stained with haematoxylin and eosin.

## Results

Botanical studies revealed extensive colonization of the mountainous areas (up to 50% of the area available to the cattle) with bracken fern. Two varieties of *P. aquilinum* were found there, namely *P. aquilinum* var *arachnoideum* and *P. aquilinum* var *caudatum* with additional sub-varieties of these also possible, depending on the specific location.

Phytochemical analyses of bracken samples from the five regions under investigation all revealed the presence of ptaquiloside (Fig. 37.1). These locations were between 1990 and 2010 m of altitude and BEH was prevalent in each of the regions sampled.

At the time of the study, the availability of alternate pasture in these mountainous areas was very low and the cattle were observed to be avidly ingesting the bracken fern. Other toxic plants were also observed growing in this region and included



**Fig. 37.1.** High Performance Liquid Chromatograms showing concentration of 'ptaquiloside' from *P. aquilinum* samples collected in five different localities affected by BEH in Tarija, Bolivia. Determined indirectly as pteroin B.

*Cestrum* spp., *Senecio* spp., *Senna* spp., *Eupatorium* spp., *Solanum* spp., *Baccharis coridifolia* and *Bohemeria cordata*. The survey of the farmers indicated that the incidence of BEH in the area had increased over the past 20 years. In addition, the age at which cattle were observed to become sick was as low as 2 years. The present incidence of BEH in the cattle was calculated to be 10.6%. Dysphagia associated with upper alimentary tract tumours was also relatively common and because of feeding difficulties cachexia among these animals was common. Pallid mucous membranes associated with urinary tract blood loss were also commonly observed. In all 21 cases of BEH studied there was anaemia and leucopaenia although recordings of respiratory and heart rates and rectal temperature were variable but unremarkable.

As was expected, neoplasms were present in the bladders of all animals and in the upper alimentary tract in 50% of them. The bladder wall in general was thickened and the mucosa was roughened and hyperaemic and contained angiomatous and solid tumour masses. Histologically there were transitional cell carcinomas, angiosarcomas and fibrosarcomas while in the oesophagus there were squamous cell carcinomas characterized by epithelial plaques and profuse fibrous stroma infiltrated by inflammatory cells.

## Discussion

The present study confirmed the presence of BEH caused by the chronic ingestion

*P. aquilinum* in Tarija, and that its incidence in the cattle herds is around 10.6%. Compared with the incidence of the disease given in an earlier report (Romero, 1982), there appears to be a significant increase over the last 20 or so years. In addition to the bladder neoplasia, which characterizes BEH, affected cattle in this region also have a high incidence of upper alimentary tract neoplasia as has been reported in the UK, Kenya and Brazil (Dobereiner *et al.*, 1967; Plowright *et al.*, 1971). The latter lesion is considered to be associated with the malignant transformation of bovine papilloma virus type 4 (Campo *et al.*, 1994). Bovine papilloma virus type 2 has also been implicated in the bladder neoplasia in the disease (Campo *et al.*, 1992). The present incidence of BEH in this area of Bolivia of 10.6% may not represent the true incidence of the disease. The management system of the cattle in the region may obscure the real incidence of cases as at the first sign of haematuria, farmers are known to slaughter such animals (Marrero *et al.*, 2001). The intermittent exposure of the cattle to bracken in the diet due to the operation of the two separate phases of management may also be a factor in determining the disease incidence on some farms. Under the appropriate conditions of pH, ptaquiloside is converted to an alkylating intermediate and this is thought to be the basis of the carcinogenic mechanism of this compound and the preference for its action in specific bovine tissues such as the oesophagus and the bladder (Smith, 1997). The disease does not lend itself to specific therapy and its control will have to depend on prevention through the appropriate management of bracken-infested pastures. This is going to be difficult in the mountainous jungle regions of the 'Humid Chaco' in which complex ecological conditions including alternative feed availability, climate and soil-related factors will determine the availability of the plant. However, improved nutritional conditions for cattle such as mineral and nitrogen supplementation, and even with compounds such as sulphur amino acids in the diet to promote the detoxication of ptaquiloside (Yamada *et al.*, 1998) could assist in the prevention of the intoxication. There is the additional public health concern of the potential for excretion of ptaquiloside in milk, which has been suspected in some regions as playing a part in the aetiology of human cancer (Smith, 1997).

Large numbers of cattle in the upper Amazon regions of South America are affected with BEH and this should justify the mounting of a comprehensive research programme aimed at the control of this disease. If further justification of such a programme is needed, the disease has a global distribution, bracken toxin is recognized as a carcinogen and may cause human cancer in some populations.

## Acknowledgements

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## Chapter 38

# Ptaquiloside in Bracken and in Topsoil in 20 Danish Bracken Populations

L.H. Rasmussen<sup>1</sup>, H.C.B. Hansen<sup>1</sup>, J.C. Frisvad<sup>2</sup> and S. Kroghsbo<sup>2</sup>

<sup>1</sup>The Royal Veterinary and Agricultural University, Chemistry Department, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark; <sup>2</sup>Technical University of Denmark, BioCentrum-DTU, Søtofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark

### Bracken as an Environmental Hazard?

Bracken (*Pteridium aquilinum* (L.) Kuhn) contains several toxic compounds causing a wide range of diseases in animals and humans, ranging from induced thiamine deficiency to cancer (Hirono *et al.*, 1973; Smith, 1997; Castillo *et al.*, 1998). The norsesquiterpene glycoside ptaquiloside is thought to be the main carcinogenic compound in bracken (van der Hoeven *et al.*, 1983). Ptaquiloside can be transferred to animals or humans when bracken is used for food, but contamination of watersheds with bracken leachates may also cause ptaquiloside contamination of the human diet (Galpin and Smith, 1986; Galpin *et al.*, 1990).

In this study, it is hypothesized that ptaquiloside uptake may occur not only through food but also via drinking water, as ptaquiloside is likely to leach from the bracken ecosystem to recipients. Ptaquiloside is highly water-soluble and has a low  $\log K_{ow}$  value (estimated to  $-1.04 \pm 0.51$  (Ojika *et al.*, 1987; ACD, 1995)) and therefore can be transferred to the soil and aqueous recipients when dead or living bracken substances are leached by rain. Rasmussen *et al.* (2003) found ptaquiloside contents between 0.22 and 8.49  $\mu\text{g g}^{-1}$  dry matter in Oi-horizons at four bracken locations in Denmark. This finding indicates the possibility for ptaquiloside to exist in the soil environment for a prolonged time.

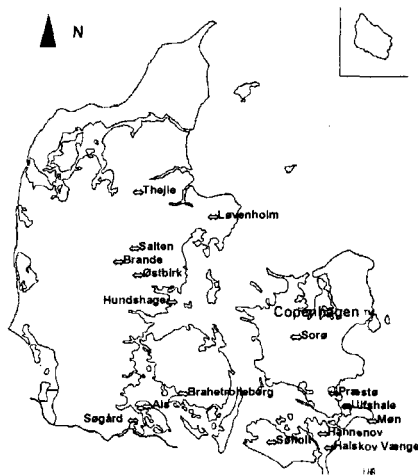
Ptaquiloside is unstable under both acidic and alkaline conditions and will transform to pterosin B, which is harmless (Nagao *et al.*, 1989; Burkhalter *et al.*, 1996). In topsoil material (epipedons) of unmanaged natural or forest soils, where bracken is found, pH values between 3 and 6 are often encountered in the soil solution (Johnson-Maynard *et al.*, 1997, 1998; Pitman and Webber, 1998). Such pH values should cause degradation of ptaquiloside (Ojika *et al.*, 1987; Burkhalter *et al.*, 1996). However, the weakly sorbed ptaquiloside may quickly move to less acid soil horizons and deep sediments (aquifers), where conditions favour stabilization.

The aim of this study was to investigate the possibility that ptaquiloside leached from bracken stands and was stable enough to be found in the terrestrial

environment. As a part of the study the variation of the ptaquiloside content in the fronds and topsoil of different bracken stands was investigated to quantify which ptaquiloside levels could be encountered in Danish bracken populations. Multivariate data analysis was performed on different stand characters to investigate which variables determine the ptaquiloside content in fronds and soil material.

## Materials and Methods

Twenty sites in plantations or on heather situated all over Denmark were investigated at the end of the growth season, October 2000 (Fig. 38.1). The herbal-floor was dominated entirely by common bracken (var. *aquilinum*) at all sites.



**Fig. 38.1.** Map of Denmark showing study sites.

On each site, three homogeneous sub-sites were selected. Samples were taken for ptaquiloside analysis (fronds, litter (Oi/Oe-horizons) and epipedon samples (Oa/A-horizons)). The samples from each sub-site were pooled to get one sample. Fronds and soil material were brought back to the laboratory, dried at 50°C for 5 days, milled (fronds and O-horizons) or sieved (mineral soil samples) resulting in a particle size diameter less than 2 mm, and finally stored at 4°C until analysis. All epipedon samples were taken above rhizomes and roots in the upper 10 cm of soil. Care was taken not to include bracken material in the epipedon samples. The heights of the fronds (10 fronds site<sup>-1</sup>) were measured in addition to the frond density (3 measurements site<sup>-1</sup>, number of fronds m<sup>-2</sup>). Detailed descriptions of the sites were performed according to FAO (FAO, 1990). These descriptions were extended to include: size of population; turnover rate of the overstorey litter (Vesterdal, 1998); amount of bracken litter in the O-horizons; bracken population growth stage (new, young, mature, old (Watt, 1976)); and light conditions (shaded, small glade (< 200 m<sup>2</sup>), large glade (> 200 m<sup>2</sup>), open land). Ptaquiloside was extracted with water (1:10 soil:solution ratio) from all samples at room temperature for 60 min. The ptaquiloside content in fronds and Oi/Oe-horizons were determined from concentrations in the aqueous extracts after cleaning of the extract with a resin (Polyamide 6, Fluka) and conversion to pterosin B (Agnew and Lauren, 1991). The content in the extracts from the Oa/A-horizons were concentrated on XAD<sup>®</sup> 2 resin (Supelco) before analysis

(Rasmussen, unpublished data). This method results in measurements of both ptaquiloside and *iso*-ptaquiloside (Castillo *et al.*, 1997). Both ptaquilosides are, however, toxic (D.L. Lauren, Hamilton, 2000, personal communication). Pterosin B was determined at 214 nm on a Perkin Elmer Series 10 liquid chromatograph equipped with Merck LiChroCART® column (250 x 4 mm), LiChrospher® 100 RP-8, 4 µm. Measurements were performed using a water-methanol (45:55 vol-%) eluent, and a flow rate of 1.50 ml min<sup>-1</sup>. A 50 µl sample loop was used for frond extracts, while a 500 µl sample loop was used when analysing extracts of soil samples. Standards in the range of 0.0112-22.5000 µg ml<sup>-1</sup> ptaquiloside equivalents were used for quantification. All samples were measured in duplicate or triplicate. A standard of ptaquiloside was kindly obtained from Professor Ojika, Nagoya University, Japan.

Soil pH was measured in 1:2.5 or 1:10 soil:0.01 M CaCl<sub>2</sub> suspension in mineral soil or organic soil samples respectively. Organic carbon was determined by dry combustion using an Eltra CS 500 Carbon Sulfur Determinator (Nelson and Sommers, 1986). Two or three replicate measurements were performed.

Statistical analysis was performed in the UNSCRAMBLER VER. 7.6 SR-1 software package (Camo ASA). Multivariate data analysis (partial least squares regression, PLSR) was performed using all qualitative and quantitative measurements. Resulting models were validated and evaluated using site-segmented and full cross validation (Bro, 1996; Geladi *et al.*, 1999; Esbensen, 2000).

## Results and Discussion

The bracken populations studied are located throughout Denmark under different kinds of land use (Table 38.1). Most of the sites are situated in plantations on acid sandy soils. Some of the soils have rather high carbon contents compared to ordinary Danish forest soils (Sundberg *et al.*, 1999).

The content of ptaquiloside in the fronds ranged from 110 to 3800 µg g<sup>-1</sup>, which is in the range observed for common bracken (Table 38.2) (Smith *et al.*, 1994). Recalling that sampling was carried out towards the end of the growing season the content seemed high in some of the populations (Alonso-Amelot *et al.*, 1992, 1995, 2000). In the Oi/Oe-horizons, the ptaquiloside content ranged from 0.09 to 6.43 µg g<sup>-1</sup>, with an average content of 1.22 µg g<sup>-1</sup> (Table 38.2). These ptaquiloside levels are in accordance with previous investigations of the ptaquiloside content in Oi-horizons (Rasmussen *et al.*, 2001). The content in the epipedons ranged from 0.011 to 0.713 µg g<sup>-1</sup> (Table 38.2). This is

**Table 38.1.** Description of the sites.

Location	Light conditions	Precipitation (mm year <sup>-1</sup> )	Stand size (m <sup>2</sup> )	pH		Carbon (%)		Soil texture (%)		
				O <sup>a</sup>	Epi <sup>b</sup>	O	Epi	Clay	Silt	Sand
Als	Glade-S <sup>d</sup>	375	800	3.92	3.14	37.3	11.0	0.0	7.0	93.0
Brahe I <sup>c</sup>	Shaded	375	2,000	4.95	3.94	24.7	3.3	3.9	14.2	81.9
Brahe II <sup>c</sup>	Glade-L <sup>e</sup>	375	250	4.19	3.42	14.7	4.4	6.3	14.0	79.7
Brande	Glade-S	425	2,000	4.34	5.60	19.2	4.4	0.9	2.4	96.7
Halskov	Glade-S	325	1,500	3.96	2.96	42.9	7.8	3.5	9.5	86.9
Hannenov	Shaded	325	100	4.76	4.32	36.4	3.3	4.0	7.5	88.5
Hundshage	Shaded	375	2,000	3.95	2.81	45.6	32.5	0.0	1.0	99.0
Løvenholm	Glade-L	325	200	4.76	4.14	23.2	2.5	1.8	3.5	94.7
Møn	Glade-L	325	300	6.30	7.03	25.2	6.8	1.0	7.6	91.4
Præstø I	Open land	325	400,000	5.23	2.96	28.3	25.7	0.0	0.0	100.0
Præstø II	Glade-S	325	500	4.80	2.94	48.0	31.3	0.0	0.6	99.4
Salten I	Shaded	425	1,400	4.28	2.99	46.5	8.3	0.2	7.2	92.6
Salten II	Open land	425	20,000	5.14	3.53	45.6	4.5	2.5	10.8	86.8
Sorø	Glade-S	375	120	5.15	3.47	44.2	23.8	0.0	0.8	99.2
Søgård	Glade-S	475	500	3.76	2.91	33.3	7.7	4.5	9.4	86.1
Søholt I	Shaded	325	10,000	3.29	2.91	38.5	3.9	2.5	4.9	92.6
Søholt II	Shaded	325	10,000	3.61	2.75	43.0	21.9	0.0	1.0	99.0
Thejle	Shaded	375	500	5.73	3.88	34.1	4.0	1.8	3.2	95.0
Ulfshale	Shaded	325	1,000	4.38	2.81	45.2	43.6	0.0	0.0	100.0
Østbirk	Glade-S	375	150	4.48	3.72	15.3	2.5	4.3	13.3	82.4

<sup>a</sup>Oi/Oe-horizon; <sup>b</sup>epipedon; <sup>c</sup>Brahetrolleborg I and II; <sup>d</sup>small glade; <sup>e</sup>large glade.

**Table 38.2.** Ptaquiloside contents and height of bracken fronds.

Site	Pta <sup>a</sup> - frond	Pta-O <sup>b</sup>	Pta-epi <sup>c</sup>	Frond height <sup>d</sup> (cm)
	(µg g <sup>-1</sup> dry matter)			
Als	230	0.42	0.021	194 ± 21
Brahe I <sup>e</sup>	960	0.50	0.032	190 ± 30
Brahe II <sup>e</sup>	410	0.40	0.025	183 ± 21
Brande	310	0.23	0.023	127 ± 30
Halskov	230	0.80	0.074	175 ± 39
Hannenov	840	0.27	0.048	169 ± 15
Hundshage	370	0.62	0.029	130 ± 15
Løvenholm	460	0.09	0.020	178 ± 27
pMøn	220	5.95	0.092	209 ± 16
Præstø I	2490	6.43	0.021	23 ± 6
Præstø II	110	0.21	0.225	197 ± 41
Salten I	1430	1.00	0.014	162 ± 30
Salten II	3800	0.10	0.087	78 ± 16
Sorø	530	0.17	0.050	212 ± 25
Søgård	2140	5.24	0.007	92 ± 21
Søholt I	1590	0.37	0.052	184 ± 30
Søholt II	580	0.23	0.062	142 ± 21
Thejle	720	0.25	0.014	157 ± 19
Ulfshale	770	0.93	0.713	195 ± 22
Østbirk	150	0.21	0.011	173 ± 32

<sup>a</sup>Ptaquiloside in frond (CV<sub>AVG</sub> = 3.8%); <sup>b</sup>Oi/Oe-horizon (CV<sub>AVG</sub> = 10.9%); <sup>c</sup>epipedon (CV<sub>AVG</sub> = 16.2%); <sup>d</sup>standard deviation shown; <sup>e</sup>Brahetrolleborg.

the first time that the occurrence of ptaquiloside has been reported in the mineral soil environment.

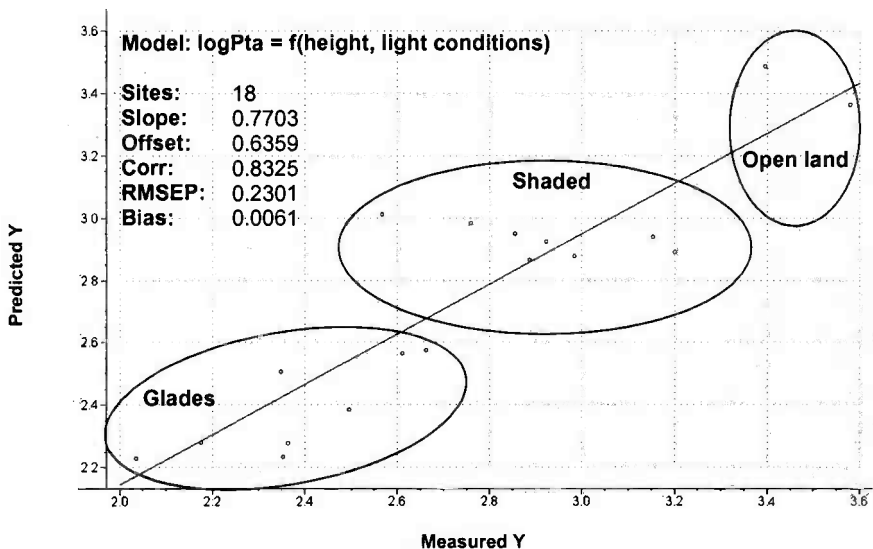
PLSR was initially performed using all variables, except the content of ptaquiloside in the soil, to obtain better understanding of the factors determining the ptaquiloside content in the fronds. Unimportant variables were removed to improve and simplify the model. The analysis resulted in the model depicted in Fig. 38.2. Sorø and Søgård were not included in the analysis. This final model is based on the height of the fronds, which had a negative impact on the content of ptaquiloside and the light conditions (open land, shaded, glades), which determined the general level of ptaquiloside in the ferns.

Brackens in open terrain had high ptaquiloside contents, while ferns growing in shaded areas under forest had medium ptaquiloside contents. Ferns growing in forest-glades had the lowest concentrations encountered. Further analysis of the three groups showed that the ptaquiloside-content in brackens growing in shade was positively correlated with the carbon-content in the epipedon and the turnover of the overstorey-litter, indicating a possible effect of plant available water and availability of nutrients. It was not possible to obtain a valid model for the ferns growing in glades (Rasmussen, unpublished data). These results indicate that the ptaquiloside content in Danish bracken fronds might be a

function of the edaphic stress the ferns are exposed to. The more stress, the higher ptaquiloside content. This hypothesis is supported by the fact that the ferns growing on open land might have been cut and are subjected to browsing from sheep and cattle.

The ptaquiloside content in Oi/Oe-horizons were found to be correlated in a rather complex way with the carbon content in the epipedon, the amount of bracken litter in the Oi/Oe-horizon, the precipitation, the turnover rate, and the size of the bracken population (Rasmussen, unpublished data).

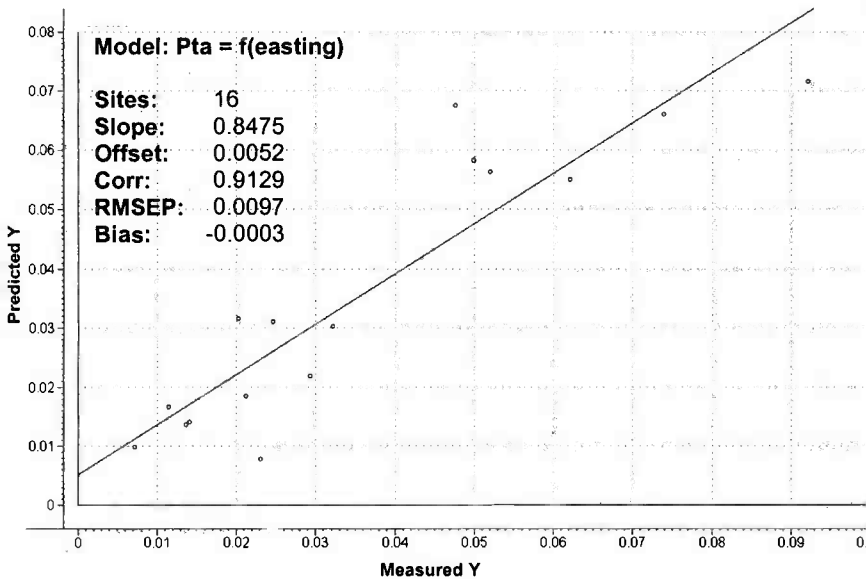
PLSR-analysis revealed four variables, which were highly correlated with the ptaquiloside content in the epipedons: The pH (epipedon), easting (geographical coordinate) and stand size had a positive impact on the content, while precipitation had a negative impact. The most prominent effect was precipitation and easting, i.e. more ptaquiloside is found in the soil in eastern parts of Denmark where precipitation is low. This indicates that when precipitation is high, leaching is also high and the content of ptaquiloside in the epipedon becomes low. The model resulting from using the geographical coordinate as the only variable is shown in Fig. 38.3.



**Fig. 38.2.** PLSR model for the ptaquiloside content in the fronds (3 PCs). The line is the resulting regression line between predicted and measured ptaquiloside content ( $\log \mu\text{g g}^{-1}$ ) using full cross validation. Ideally this line should have a slope of 1 and an offset of 0. The correlation coefficient is 0.83, which is considered as very good (Geladi *et al.*, 1999). RMSEP (Root Mean Square Error of Prediction) is based on cross validation, and can be interpreted as the average prediction error expressed in the same units as the original values. The frond height has negative influence on the ptaquiloside content, while the light conditions determine the general level of ptaquiloside in the fronds.

## Conclusion

This investigation revealed that ptaquiloside could be found in litter and in topsoil-material below bracken. It is the first time that the occurrence of ptaquiloside has been reported from a mineral soil environment. It indicates the possibility that ptaquiloside can leach from bracken fronds and enter the terrestrial environment. It was found that the ptaquiloside content in soil material is a function of climate, stand size, carbon content, amount of bracken litter, turnover rate and soil pH. The level of ptaquiloside in fronds was in accordance with other findings of the ptaquiloside content in common bracken. The variation seemed to be a function of the edaphic conditions: light, water and nutrients. Therefore ptaquiloside does seem to be produced as a response to environmental stress.



**Fig. 38.3.** PLSR model for the ptaquiloside content in the epipedon (1 PC). The line is the resulting regression line between predicted and measured ptaquiloside content in epipedons ( $\mu\text{g g}^{-1}$ ) using full cross validation. The correlation coefficient is 0.91, which is considered as very good (Geladi *et al.*, 1999). The model is based on the geographical coordinate easting, which has a positive influence on the ptaquiloside content.



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## Chapter 39

# Pathological Aspects of Cyanobacterial Toxicity

A.A. Seawright

*National Research Centre for Environmental Toxicology, 39 Kessels Road,  
Coopers Plains, Queensland 4108, Australia*

### Introduction

Cyanobacteria (blue-green algae) were first implicated in livestock toxicity at Lake Alexandrina in South Australia in 1878 (Francis, 1878). Since that time poisonings of livestock through consumption of drinking water infested with such blooms have been reported from all continents. A recent review of the organisms involved and the toxins they contain is provided by the WHO publication *Toxic Cyanobacteria in Water* (Chorus and Bartram, 1999).

There are three groups of toxins found, namely cyclic peptides, alkaloids and lipopolysaccharides. The cyclic peptides are microcystins and nodularins which cause liver damage. Over 60 different microcystins have been identified and are produced by the following genera of cyanobacteria: *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Hapalosiphon* and *Anabaenopsis*. Nodularins are found only in *Nodularia spumigena*. The toxic alkaloids found in cyanobacteria include anatoxin-a, anatoxin-a(S), aplysiatoxins, cylindrospermopsins, lyngbyatoxin-a and saxitoxins. Anatoxin-a(S) is found in *Anabaena* while anatoxin-a is also found in *Planktothrix* and *Aphanizomenon*. Saxitoxins (paralytic shell fish poisons) are found in *Anabaena*, *Aphanizomenon*, *Lyngbya* and *Cylindrospermopsis*. These are all rapidly acting neurotoxins and the intoxications are not characterized by definitive morbid anatomical changes. Aplysiatoxins and lyngbyatoxin-a are locally irritant to skin and mucous membranes and are found mostly in *Lyngbya*. Lipopolysaccharides are found in all cyanobacteria and may also cause local irritation. Cylindrospermopsins are also hepatotoxic and are found in *Cylindrospermopsis*, *Aphanizomenon* and *Umezakia*.

The cyanobacterial toxins of most relevance to this report are the hepatotoxins microcystins, nodularins and cylindrospermopsins. Each of the toxins causes characteristic pathological changes in the liver.

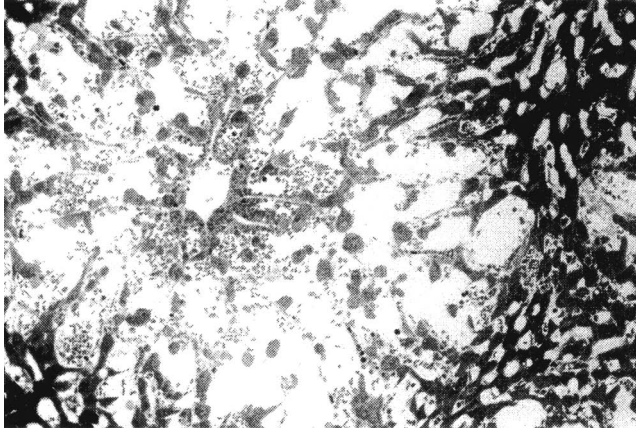
## Microcystins and Nodularins

Microcystins are cyclic heptapeptides with the general structure: cyclo-(D-alanine<sup>1</sup>-X<sup>2</sup>-D-MeAsp<sup>3</sup>-Z<sup>4</sup>Adda<sup>5</sup>-D-glutamate<sup>6</sup>-Mdha<sup>7</sup>). X and Z are variable L-amino acids, D-MeAsp is D-erythro- $\beta$ -methylaspartic acid, Mdha is N-methyldehydroalanine and Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.

Nodularins are cyclic pentapeptides with the general structure: cyclo-(D-MeAsp-L-arginine-Adda-D-glutamate-Mdhb). Mdhb is 2-(methylamino)-2-dehydrobutyric acid. The structural feature common to microcystins and nodularins is Adda-D-glutamate and both cyclopeptides cause precisely the same type of hepatotoxicity with the intraperitoneal LD50 in mice in the range of 50 to 300  $\mu\text{g kg}^{-1}$ .

These peptides are highly polar and, in order to elicit their toxic effects, uptake into cells occurs through membrane transporters, which are normally used to transport essential chemicals and nutrients. For these toxins the bile acid carrier is the transporter mechanism (Runnegar *et al.*, 1981, 1991) and this is located essentially on liver cell plasma membranes. Most bile acid transport occurs in the periportal zone and it has been suggested that the reason microcystin is able to target the periportal zone preferentially is that the carrier mechanism tends not to be saturated in this zone as it probably is in the periportal zone (Seawright *et al.*, 1996). Following access to the interior of the hepatocyte the Adda-D-glutamate part of the toxin molecule binds to the active centre of the protein phosphatases 1 and 2A, and the Mdha residue binds covalently to cysteine<sup>273</sup> of protein phosphatase 1 (MacKintosh *et al.*, 1995). This results in inhibition of these enzymes and hyperphosphorylation of proteins within the cell, particularly structural proteins such as actin. These filaments contract, causing the cells to round up and detach from one another. This effect is seen most strikingly in rodent livers, where following perfusion fixation after intoxication, only a thin rim of periportal hepatocytes remain, the affected hepatocytes having been washed out (Seawright *et al.*, 1996) (Fig. 39.1). This results in disorganization of the sinusoidal perfusion of blood within the liver causing extensive accumulation of blood within the organ, haemodynamic shock and death (Hooser *et al.*, 1990). Although the protein phosphatases 1 and 2A are cytosolic enzymes, recent studies have shown that microcystins also bind strongly in hepatocyte nuclei (Yoshida *et al.*, 1998). Although there was some doubt expressed initially that the cytotoxicity caused by these peptides was characterized by a form of apoptosis (Seawright *et al.*, 1996), more recent studies have demonstrated by means of *in situ* labelling of the DNA that the hepatocyte death is associated with such a process (Hooser, 2000). Tight covalent binding of microcystins in the liver can prevent these molecules being recovered in analysis of the organ in the toxicological diagnosis of this kind of poisoning.

Microcystins are, if anything, only very weak carcinogens but there is evidence that they can act as effective tumour promoters, particularly in the liver. Following



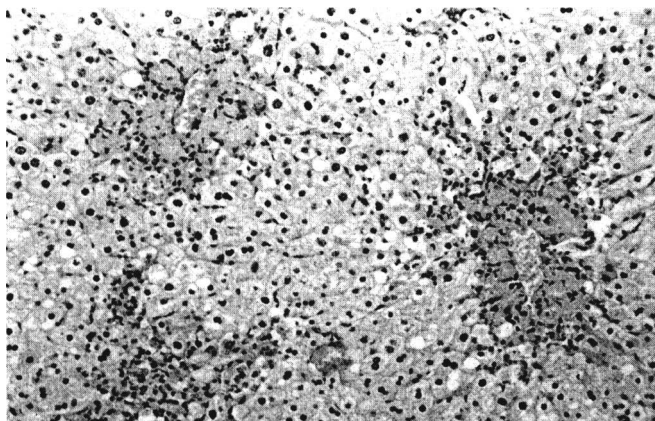
**Fig. 39.1.** The liver of a rat dosed intravenously with  $50 \mu\text{g kg}^{-1}$  of microcystin-LR showing the effects of perfusion fixation within 45 min of the dosing. Most of the affected periportal hepatocytes have been washed out (magnification  $\times 200$ ).

initiation with diethylnitrosamine, an intraperitoneal dose of  $10 \mu\text{g kg}^{-1}$  of microcystin twice weekly for 8 weeks was able to cause the formation of pre-neoplastic foci in the livers of rats, whereas dosing with the peptide alone was ineffective (Nishiwaki-Matsushima *et al.*, 1992).

### Cylindrospermopsins

*Cylindrospermopsis raciborskii* was first identified as a possibly toxic cyanobacterium following an outbreak of an enterohepatic disease in humans in North Queensland in 1979 (Bourke *et al.*, 1983). Extracts of this organism were subsequently shown to be hepatotoxic in mice. The liver lesion was characterized mainly by extensive fatty infiltration, periportal necrosis (Fig. 39.2) and parenchymal infarct-like lesions (Hawkins *et al.*, 1985). The toxin was subsequently characterized as a cyclic guanidino-uracil alkaloid and shown to have an intraperitoneal LD50 for mice of  $200 \mu\text{g kg}^{-1}$  (Ohtani *et al.*, 1992). By the oral route the LD50 is about  $6 \text{ mg kg}^{-1}$  in this species (Seawright *et al.*, 1999). While the mechanism of toxicity in the liver is not known, it has been shown that there is a marked inhibition of protein synthesis and this could account for the marked fatty change that occurs (Terao *et al.*, 1994).

Recent studies with  $^{14}\text{C}$ -cylindrospermopsin revealed that following intraperitoneal dosing, up to 14% of the label is found in the liver of which 50% is covalently bound (R.L. Norris, unpublished observations). There is some evidence



**Fig. 39.2.** The liver of a mouse 2 days after an intraperitoneal dose of  $200 \mu\text{g kg}^{-1}$  of cylindrospermopsin showing parenchymal fatty infiltration and periacinar coagulative necrosis (magnification  $\times 200$ ).

that cylindrospermopsin is metabolized to reactive metabolites by mixed-function oxygenation in the liver (Runnegar *et al.*, 1995), and this may account for the persistence of the radio label on the liver proteins. In addition, since mixed-function oxygenation in the liver is concentrated in the periacinar region, reactive metabolite formation in these hepatocytes may account for the periacinar necrosis that is commonly observed (Seawright *et al.*, 1999).

A particular feature of cylindrospermopsin toxicity in mice is the occurrence in up to 20% of animals of periorbital thrombosis and haemorrhage affecting one or both eyes, appearing first on about the third day of the intoxication (Fig. 39.3). Complete enucleation of the eyeball then usually occurs as the animals recover from the toxicity (Fig. 39.4). Thrombosis affecting blood vessels in the tail also commonly occurs followed by mummification and sloughing of the affected portion.

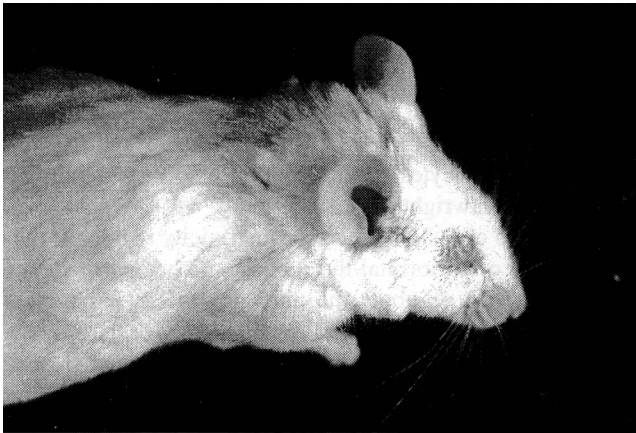
Since the potential toxicity of *C. raciborskii* in water bodies has been demonstrated, outbreaks of hepatotoxicity (previously undiagnosed) in cattle have been reported in tropical Australia (Thomas *et al.*, 1998). Such a toxicity has also been presumed to occur in humans in the past in Australia when drinking water had to be obtained from surface water bodies such as lakes and lagoons (Hayman, 1992). Definitive diagnosis of this toxicity is now possible by the demonstration of the presence of the alkaloid in the tissues of intoxicated animals.

### **Aplysiatoxins and Lyngbyatoxin**

These toxins differ from the microcystins, nodularins and cylindrospermopsins in



**Fig. 39.3.** A mouse 3 days after a toxic dose of cylindrospermopsin showing periocular haemorrhage affecting left eye (Agriculture NSW photo).



**Fig. 39.4.** A mouse 10 days after a toxic dose of cylindrospermopsin showing loss of right eye (R.L. Norris photo).

that they are found in marine cyanobacteria, mainly *Lyngbya majuscula*. The toxins are activators of protein kinase C and as such are extremely irritant, causing severe dermatitis and oral and gastrointestinal inflammation. Intoxications tend to occur in swimmers and fishermen exposed to water heavily infested with these blooms in tropical and subtropical seas in hot weather (Moore *et al.*, 1993).

## Conclusion

Pathological aspects of cyanobacterial toxicity have greatest relevance to veterinary pathological diagnosis. Intoxications caused by hepatotoxic cyanobacteria containing microcystins or nodularins are clearly distinguishable from those caused by organisms containing cylindrospermopsin. The former is a haemorrhagic necrosis of the liver while the latter is fatty liver with periacinar coagulative necrosis. It has to be remembered however, that mixed blooms of toxic cyanobacterial species also occur and that an outbreak of toxicity in livestock may be complicated by the presence of these two forms of hepatotoxicity as well as an alkaloid-induced neurotoxicity.

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## Chapter 40

# Effect of *Senna occidentalis* Seed in Laying Hens: Evaluation of the Toxicity and Egg Production

M. Haraguchi<sup>1</sup>, P.C. Raspantini<sup>2</sup>, M.L.Z. Dagli<sup>2</sup> and S.L. Górnaiak<sup>2</sup>

<sup>1</sup>Centre of Animal Sanitation, Biological Institute of São Paulo, Av. Conselheiro Rodrigues Alves 1252, CEP 04014-002, São Paulo, Brazil; <sup>2</sup>Research Centre for Veterinary Toxicology (CEPTOX), Department of Pathology, Faculty of Veterinary Medicine and Zootechnology, University of São Paulo, São Paulo, SP, Brazil

### Introduction

The leguminous plant *Senna occidentalis* (Syn. *Cassia occidentalis* - So) is a common contaminant weed in maize, soybean and other cereal crops. It is found throughout the tropical and subtropical regions of the world (Lal and Gupta, 1973), causing intoxication in animals that feed on it (Brocq-Rosseau and Bruere, 1925; Henson *et al.*, 1965; Torres *et al.*, 1971; Martins *et al.*, 1986; Barros *et al.*, 1990). Although maize and sorghum are cleaned mechanically before being processed for animal rations, they may be contaminated with *Senna* seeds that are similar in size and density to the crop grains. The toxic principles of this seed have been identified as dianthrone (Haraguchi *et al.*, 1996).

Independently of the animal species, the ingestion of large amounts of the plant produces incoordination, recumbency, reluctance to move, anorexia, muscle weakness, ataxia, diarrhoea, muscle tremors, stubbing, bodyweight loss and death (O'Hara *et al.*, 1969; Torres *et al.*, 1971; Martins *et al.*, 1986).

Skeletal muscle degeneration is the predominant lesion found in the majority of animals intoxicated with So seeds (Henson *et al.*, 1965; Pierce and O'Hara, 1967; O'Hara *et al.*, 1969; Barros *et al.*, 1990; Calore *et al.*, 1997; Haraguchi *et al.*, 1998a, b); however, other lesions have been described, such as degenerative myopathy of myocardial muscle (Tasaka *et al.*, 2000), congestion and pulmonary oedema (Irigoyen *et al.*, 1991), hepatic cell hypertrophy and necrosis (Suliman *et al.*, 1982; El Sayed *et al.*, 1983; Tasaka *et al.*, 2000).

Spontaneous cases of intoxication with So seeds were reported in livestock when animals were fed a heavily contaminated ration. Similarly, experimental studies conducted on different animal species have shown the occurrence of sickness and death with moderate to high levels of the seed in the ration (2% up to 20%) during a short or prolonged period of time (Suliman *et al.*, 1982; Colvin *et al.*, 1986; Barros *et al.*, 1990; Irigoyen *et al.*, 1991; Cavalieri *et al.*, 1997;

Haraguchi *et al.*, 1998b; Calore *et al.*, 2000; Tasaka *et al.*, 2000). On the other hand, very little information is available about whether the chronic ingestion of lower concentrations of the plant could be toxic to animals since this kind of intoxication has been poorly documented. Thus, the present study was conducted to verify the influence of 0.2% of So parts in the ration on production performance of laying hens throughout the life cycle.

## Materials and Methods

Ripe *S. occidentalis* (So) seeds collected from a culture at the Biological Institute of São Paulo, São Paulo State, were humidified with water overnight in a refrigerator and dissected to obtain the external/internal tegument (ET/IT) and cotyledons (CD). Each material, dried and ground, was mixed with commercial ration at a dose of 0.2%.

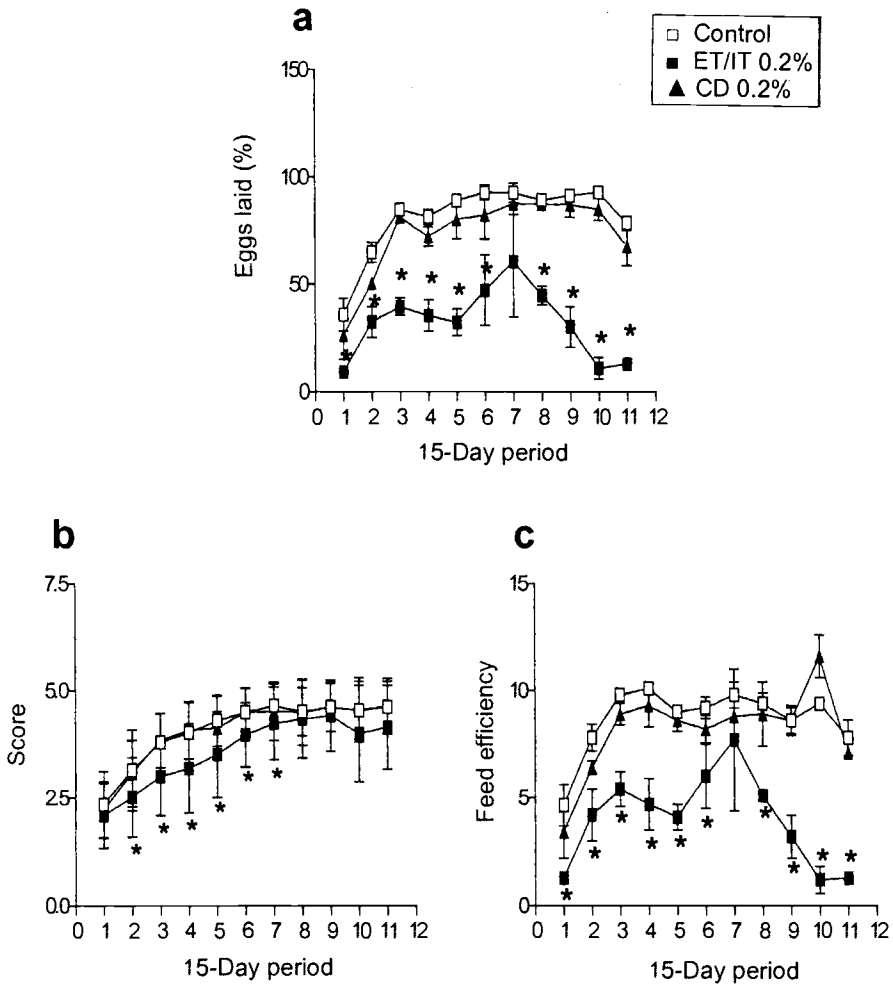
Fifty-six one-day-old White ISA layers from the Faculty of Veterinary Medicine and Zootechny, University of São Paulo, were housed in colony cages (40 x 30 cm), two birds to a cage. The hen-house was provided with programmable lighting and adequate ventilation. The birds were randomly distributed into three groups of 16, 16 and 24 animals receiving 100–120 g day<sup>-1</sup> per hen laying hen diet which contained 0.2% ET/IT; 0.2% CD and the no seed supplementation, respectively, throughout their life cycle (42 weeks). Bodyweight and feed consumption were recorded weekly and mortality was recorded if it occurred.

After 20 weeks, the production and quality of eggs were recorded daily and the data were pooled into twelve 15-day periods. Each egg was classified for size as follows: jumbo (E), extra-large (A), medium (B), small (C), peewee (D) and undergrade (LD), corresponding to scores of 6, 5, 4, 3, 2 and 1, respectively.

## Results

The laying hens fed on a diet containing 0.2% ground ET/IT of So seeds throughout their life cycle (42 weeks) presented a significant decrease in feed consumption during some weeks (22nd, 33rd, 37th, 42nd). On the other hand, the bodyweight of the experimental groups did not differ significantly from that of the control group (data not shown).

While no deaths occurred in any group, the mean percentage of eggs laid by the ET/IT group was markedly reduced throughout the experiment (Fig. 40.1a). A significant reduction in size was also observed in eggs laid by hens fed ET/IT of So seeds from the second to the seventh 15-day period compared to the CD group (Fig. 40.1b). Besides, treatment with ET/IT of So seeds caused lower feed efficiency (eggs kg<sup>-1</sup> of feed consumed) throughout the experimental period (Fig. 40.1c).



**Fig. 40.1.** (a) Production, (b) quality of eggs and (c) feed efficiency (eggs kg<sup>-1</sup> of feed consumed) of laying hens fed with ration containing 0.2% external/internal tegument (ET/IT) and 0.2% cotyledons (CD) *Senna occidentalis* during all life ( $P < 0.05$ , ANOVA followed by the Tukey-Kramer test).

## Discussion

Many studies are available in the literature about the identification of the toxic principles of *S. occidentalis*. Earlier work recorded the occurrence of a toxic albumin in So seeds (Moussu, 1925) and has also proposed that a volatile toxic alkaloid (Puleo, 1966) or oxymethylanthraquinone (O'Hara *et al.*, 1969) was the substance responsible for *Senna* toxicity. However, Hebert *et al.* (1983) and Graziano *et al.* (1983), working together, suggested that there were two toxic principles in So seeds: one heat stable and the other heat labile. Recently, we identified dianthrone, anthraquinone-derived compounds, of So seeds (Haraguchi *et al.*, 1996) that proved to cause the characteristic mitochondrial myopathy verified in So toxicosis (Haraguchi *et al.*, 1998a); in fact, similar lesions were found by Lewis and Shibamoto (1989) when they administered anthraquinones from a *Senna obtusifolia* seed extract. However, we had not shown in which part of So seed the toxic principles were located. The results obtained here permit us to conclude that most, if not all, dianthrone are localized in the ET/IT of So seeds.

Little importance has been attributed to the toxic effects of chronic administration of So seed in the ration of chickens, in contrast to the large number of studies showing the toxicity of this plant when given during short or prolonged periods of time (Simpson *et al.*, 1971; Torres *et al.*, 1971; Calore *et al.*, 1998; Haraguchi *et al.*, 1998a, b). In addition, to our knowledge, no studies have been performed relating the effects of low concentration of So seeds administered during the entire life cycle of hens. The results obtained in the present study show a decrease in the quality and quantity of eggs laid by hens chronically fed ET/IT of So seeds. Besides, a previous experiment conducted in our laboratory demonstrated by histochemistry that the skeletal muscle of these hens exhibited characteristic features of mitochondrial myopathy (Calore *et al.*, 1997).

Thus, considering these results as a whole, it is feasible to assume that the constant presence of low concentrations of So seeds in the ration represents a major adverse influence on egg production to the laying hen industry.

## Acknowledgements

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## Chapter 41

# **Intoxication by *Crotalaria retusa* in Ruminants and Equidae in the State of Paraíba, Northeastern Brazil**

V.M.T. Nobre<sup>1</sup>, F. Riet-Correa<sup>2</sup>, A.F.M. Dantas<sup>3</sup>, I.M. Tabosa<sup>1</sup>, R.M.T. Medeiros<sup>1</sup> and J.M. Barbosa Filho<sup>4</sup>

<sup>1</sup>Faculdade de Veterinária, Universidade Federal da Paraíba, Patos, PB, 58700-000, Brazil; <sup>2</sup>Laboratório Regional de Diagnóstico, Faculdade de Veterinária, UFPel, 96010-900, Pelotas, RS; <sup>3</sup>Universidade de Cuiabá, Av. Beira Rio 3100, 78015-480, Cuiabá; <sup>4</sup>Laboratório de Tecnologia Farmacéutica, Faculdade de Farmácia, UFPB, 58051-970, João Pessoa, Brazil

In Brazil there are more than 40 species of *Crotalaria*. The spontaneous intoxication by *Crotalaria* spp. has been reported in horses, caused by seeds of *Crotalaria juncea* in the state of Minas Gerais (Nobre *et al.*, 1994), and in cattle caused by *Crotalaria* spp. in the state of Mato Grosso do Sul and Minas Gerais (Lemos and Barros, 1998). Chronic liver disease was produced in cattle by the experimental intoxication by *Crotalaria anagyroides*, but the authors stated that no case histories were obtained incriminating this species as a cause of spontaneous disease (Tokarnia and Dobereiner, 1983). The experimental intoxication by *Crotalaria mucronata* in cattle caused an acute disease, but not liver lesions (Tokarnia and Dobereiner, 1982). The intoxication by *Crotalaria spectabilis* seeds was produced experimentally in pigs (Souza *et al.*, 1997; Torres *et al.*, 1997). Recently the intoxication was diagnosed in the state of Paraíba in horses (Nobre *et al.*, 1997), sheep (Dantas *et al.*, 1999) and cattle. The species causing those outbreaks was identified as *Crotalaria retusa* (common name: guizo de cascavel, cascaveleira, feijão de guizos). The objective of this paper is to review the intoxication by *C. retusa* in the semiarid region of the state of Paraíba, Northeastern Brazil.

### **Naturally Occurring Intoxication in Horses**

From 1996 to 1999, intoxication was observed in horses from 15 farms. In eight of those farms the intoxication occurred in 1997 during a severe drought period. Horses three to ten years old were affected. Most farms had only one affected horse out of three to four horses. In one farm, two out of three horses were



affected. One outbreak occurred in February, two in March, two in April, four in May, one in July, one in September, one in October and three in November.

Clinical signs were anorexia, weight loss, depression, jaundice and nervous signs. Many farmers mentioned a progressive weight loss for 3-4 months. Nervous signs included dullness, hyperexcitability, head pressing, compulsive walking, convulsions and terminal coma. Some horses showed frenzy and violent uncontrollable galloping. Nervous signs were observed for periods of 7-15 days. The disease was frequently diagnosed erroneously as rabies or equine encephalomyelitis. The livers were hard, with increased lobular pattern or nutmeg appearance. In one horse numerous whitish small nodules were observed on the cut surface. Oedema and moderate congestion was observed on the lungs. Mild jaundice and ascites were also present. Histological lesions of the liver were characterized by fibrosis, mainly periportal, megalocytosis and bile duct cell proliferation. Focal areas of haemorrhagic necrosis were also observed in most animals (Nobre *et al.*, 1997). Hepatic encephalopathy characterized by the presence of single or small groups of Alzheimer type II astrocytes in the cerebral cortex and basal nuclei was also observed. Large amounts of *C. retusa* were found in the farms where the disease occurred.

## **Experimental Intoxication in Donkeys and Horses**

The intoxication was produced experimentally in donkeys following the recommendations of the ethical control committee for animal experiments. The dry whole *C. retusa* was mixed with grass and given to three experimental donkeys. Experimental doses were 10 g kg<sup>-1</sup> bodyweight daily for 30 days, 5 g kg<sup>-1</sup> bodyweight daily for 90 days, and 2.5 g kg<sup>-1</sup> bodyweight daily for 120 days. The donkey treated with 5 g kg<sup>-1</sup> died 48 days after the start of the experiment and the other two were killed at 120 days. Depression, jaundice and nervous signs were observed in the donkeys treated with 5 and 10 g kg<sup>-1</sup> daily. Oedema of the mesentery, hard livers with increased lobular pattern, and mild haemorrhages in the cranial lobes of the lung were observed at necropsies. Histological lesions of the liver were characterized by fibrosis, mainly periportal, megalocytosis and bile duct cell proliferation. Focal areas of haemorrhagic necrosis were also observed in both animals (Nobre *et al.*, 1999). Hepatic encephalopathy characterized by the presence of single or small groups of Alzheimer type II astrocytes in the cerebral cortex and basal nuclei was observed in the donkey that died spontaneously. Intoxication was also produced experimentally in a horse by the daily administration of 100 g of seeds for 25 days. Anorexia, depression and mild jaundice were observed 15 days after the start of the experiment and nervous signs appeared on day 35. The horse died on day 48. Gross and histological lesions were similar to those observed in the donkeys (Morato *et al.*, 1997).

## Naturally Occurring Intoxication in Cattle

Two out of four cattle were affected in one farm. One case was observed in June and the other in December 1997. The farmer reported similar cases in previous years. One cow had severe photosensitization for nearly 30 days followed by incoordination, recumbency and death. The other had weight loss, anorexia, incoordination and recumbency, and died after a clinical manifestation period of 48 hours. The livers were hard with accentuation of the lobular pattern. The liver from the animal with photosensitization had a diffuse slight green colour. Oedema of the wall of the gall bladder was also observed. Diffuse fibrosis with megalocytosis and bile duct cell proliferation was observed in the cow that had photosensitization. The other had centrilobular liver necrosis suggesting an acute intoxication. The pastures were severely infected by *C. retusa*.

Another case of liver disease associated with the ingestion of *C. retusa* was observed in February 2001, during the dry period. An adult cow out of six had photosensitization for nearly 3 months. After this period the animal was blind and had submandibular oedema. Serum levels of gamma-glutamyltransferase (GGT) were increased. It was killed and at necropsy had a hard whitish liver and nodules up to 2 mm in the mucosa of the gall bladder. The main histological lesions of the liver were severe periportal fibrosis, bile duct cell proliferation, pericholangiolitis, mild megalocytosis, and hepatocytic degeneration. The gall bladder had an adenomatous epithelial proliferation forming focal cystic cavities surrounded by epithelium. Connective tissue proliferation and infiltration by inflammatory cells were observed in the submucosa. Large amounts of seeding and dry *C. retusa* were observed in the farm.

## Naturally Occurring Intoxication in Sheep

An outbreak of pyrrolizidine alkaloid intoxication occurred during the dry season, in a flock of 80 sheep. Some sheep of the flock were occasionally introduced into an area severely invaded by *C. retusa* in seeding stage and with very little forage available. Sixteen sheep died in a period of 3 months (July, August and September 1997). Anorexia, severe depression, mild jaundice, incoordination and recumbency were observed. The animals died approximately 12 hours after the observation of clinical signs. The liver had an increased lobular pattern and fibrin was present on the surface. The gall bladder was enlarged. Haemorrhages were observed in the endocardium and pericardium. Mild jaundice, hydropericardium, hydrothorax and ascites were also present. Sheep that died at the start of the outbreak had histological lesions of the liver characterized by fibrosis, megalocytosis and bile duct cell proliferation. Some sheep that died at the end of the outbreak, when the only forage available was *C. retusa*, had centrilobular necrosis (Dantas *et al.*, 1999). Seeds of *C. retusa* were found in large amounts in the rumen of the dead animals.

Another outbreak occurred in December 1997, affecting a flock of 20 adult sheep. Two sheep had depression, anorexia and weight loss followed by recumbency and death in 2-3 days. The animals were fed with *Pennisetum purpureum* mixed with *C. retusa*, because the grass had been cut in an area severely invaded by the weed. Subcutaneous oedema, ascites and hydrothorax were observed at necropsies. The livers were hard, with increased lobular pattern. The gall bladder was enlarged. Diffuse fibrosis, megalocytosis and mild proliferation of bile duct cells were observed in the liver.

## Chemical Studies

Samples of seeds collected during the dry season in 1998 contained 0.3% of monocrotaline (Medeiros *et al.*, 2000). A sample of the whole plant collected in the rainy season in 2000 contained 0.5% monocrotaline (Azevedo Jr *et al.*, 2000).

## Discussion

Intoxication by *C. retusa* is very important in horses and probably in cattle in the semiarid region of the state of Paraíba. Probably many cases of intoxication in cattle have been confused with rabies, because, in most cases suggestive of rabies, which is very important in the region, practitioners used to send only the head of the dead animal for laboratory diagnosis. Intoxication by *C. retusa* also occurs in sheep in areas invaded by the plant and with severe shortage of forage. Eight outbreaks in horses and outbreaks in cattle and sheep were observed during a very dry period in 1997, suggesting that the shortage of forage is an important condition for the occurrence of the disease. In the semiarid region of Paraíba it is very common to find, during the dry period, large amounts of green *C. retusa*, mainly in the bed of dry rivers and dams. The observation of centrilobular haemorrhagic necrosis in the liver of some horses, cattle and sheep suggest that when *C. retusa* is ingested in large amounts acute intoxication occurs.

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## Chapter 42

# Pathology of Experimental *Stemodia kingii* Intoxication in the Mouse

M.F. Raisbeck<sup>1</sup>, J.G. Allen<sup>2</sup>, S.M. Colegate<sup>3</sup> and A.A. Mitchell<sup>4</sup>

<sup>1</sup>Department of Veterinary Sciences, University of Wyoming, Laramie, Wyoming 82070, USA; <sup>2</sup>Agriculture Western Australia, 3 Baron-Hay Court, South Perth, Western Australia 6151; <sup>3</sup>Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Victoria 3220, Australia; <sup>4</sup>Northern Australia Quarantine Strategy, Australian Quarantine Inspection Service, C/O NT DPIF, PO Box 990, Darwin, NT 0801, Australia

### Introduction

*Stemodia kingii* is a dicotyledonous shrub of the family Scrophulariaceae found exclusively in the Pilbara region of Western Australia. The plant is a short-lived perennial that competes poorly with grasses under normal conditions, but may dominate pastures under conditions of overgrazing. Anecdotal reports link *Stemodia* to poisoning in sheep under field conditions (S. Housen, Mulga Downs Station, personal communication, 2000). The toxicity of *Stemodia* to sheep was confirmed experimentally by Allen and Mitchell (1998) and *Stemodia florulenta* was reported to be toxic to sheep with clinical signs similar to those reported by Allen and Mitchell (1998) (Hurst, 1942). Other species within the genus *Stemodia* contain biologically active compounds (Sweeney *et al.*, 2001; Dantas *et al.*, 2001) but little else is known about the toxicity of these species.

The failure of initial attempts to isolate and identify the active toxin(s) of *Stemodia* with a non-mammalian (brine shrimp) bioassay re-emphasized the need for a model representative of the target species, sheep. Laboratory mice were subsequently investigated as a model and used to successfully guide isolation and identification of two novel toxins (Allen *et al.*, Chapter 78 this volume). This paper details the clinical signs and lesions produced in laboratory mice by various extracts of *S. kingii* and validates the mouse as a model of *Stemodia* intoxication.

### Materials and Methods

Plants were collected from Mulga Downs station near Wittenoom, Western Australia, air-dried and ground as described previously (Allen and Mitchell, 1998). Ground plant material was stored dry at  $-20^{\circ}\text{C}$  until used.

## Materials and Methods

Plants were collected from Mulga Downs station near Wittenoom, Western Australia, air-dried and ground as described previously (Allen and Mitchell, 1998). Ground plant material was stored dry at  $-20^{\circ}\text{C}$  until used.

Thirteen BALB/C mice were obtained from the Animal Resources Centre (Murdoch, WA) and housed on wood shavings in plastic shoe box cages in accordance with an animal use protocol approved by the CSIRO Division of Animal Health Animal Care and Use committee. Feed (Barastoc GR2 rat and mouse breeder cubes, Ridley Agriproducts) and tap water were provided *ad libitum*.

An initial experiment was conducted to determine whether mice were susceptible to *S. kingii*. Plant material was ground together with rodent chow to constitute 12.5%, 25% and 50% of the total ration and fed *ad libitum* to pairs of mice as the sole feed source. Consumption of the treated ration was calculated by weighing feed remaining in the feeder and spill tray daily. In subsequent experiments putative *Stemodia* toxins were administered to individual mice via a 22 gauge gavage needle as pH-neutral, aqueous solutions at a constant rate of  $10\text{ ml kg}^{-1}$  bodyweight (BW). Materials that were insufficiently water soluble to administer as solutions were given as aqueous suspensions immediately after treatment with an ultrasonic water bath for 10 min. Although mice were given different extracts of plant material, all doses were held constant in terms of plant material ( $10\text{ g kg}^{-1}$ ) and, with two exceptions, each extract contained the same four groups of compounds.

Mice were observed every 2 hours for the first 8–10 hours after dosing, then every 8–10 hours for the subsequent 2 days. Any mouse that became moribund was humanely terminated by cervical dislocation. Dead mice were subjected to complete post-mortem examinations and representative samples collected into neutral buffered formalin, where appropriate, for histopathology.

## Results

### Clinical signs

Mice ate approximately 10 (12.5% ration) or 20 (25% and 50% rations) g *Stemodia*  $\text{kg}^{-1}$  BW  $\text{day}^{-1}$  for 3 days without signs of toxicity, nor did they show any clinical signs during the subsequent week. There was some indication, however, in the group fed the diet containing 50% *Stemodia* that mixed ration had been scattered beyond the spill tray of the feeder, thus the consumption value for this group may not be completely accurate. Two mice given 0.4 ml (200 mg plant material) of an aqueous extract of ground plant material remained asymptomatic for 48 hours.

In a second experiment, ground *Stemodia* was slurried with tap water in a polytron. One of two mice given a single dose of slurried plant material at  $0.5 \text{ g kg}^{-1}$  BW appeared to have a rough hair coat after 24 hours. The other mouse, and mice similarly given  $0.25$  and  $0.125 \text{ g kg}^{-1}$  BW were unaffected throughout the subsequent 5 day observation period.

Mice, given slurried plant material by gavage twice daily, began to show clinical signs after their fifth dose. Three of four treated mice appeared lethargic and remained in one corner of their cage unless stimulated to move. Dosing was discontinued at a cumulative dose of  $6.25 \text{ g kg}^{-1}$  BW. Affected mice exhibited a hunched posture, poor capillary refill, cold extremities and piloerection. Two mice exhibited watery diarrhoea and one was moribund by the following morning. Both affected mice were euthanized and subjected to post-mortem examination. In a second, similar, experiment a cumulative dose of  $3.12 \text{ g kg}^{-1}$  had no effect.

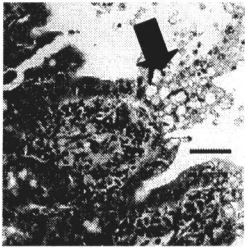
Although all mice received the same general compounds, some extracts were richer in certain groups than others. Signs tended to occur sooner after extracted toxins than slurried plant material and the course of the disease was more rapid. Diarrhoea was more common and more severe in mice given relatively polar extracts as compared to mice given slightly less polar fractions. Cardiac signs (cold extremities, poor refill, sudden death) also appeared to predominate with less polar fractions.

### Post-mortem lesions

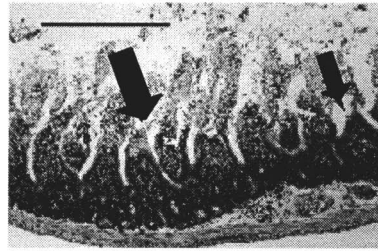
At post-mortem examination there was considerable evidence of antemortem diarrhoea in most mice. The perineum was wet and there was often pasty, pale coloured faeces adhering to the underside of the tail. The large bowel was usually distended with gas, but there was little, if any, grossly apparent inflammation or necrosis. In two mice the lungs appeared to be wet and heavy upon visual examination and in a few, especially those given slurried plant material, there appeared to be haemorrhages in the myocardium. The liver of a single mouse contained what appeared to be massive haemorrhages in all lobes.

Histologically, there was extensive damage in the jejunum and colon. The earliest change was epithelial vacuolation near the tips of the villi in the jejunum (Fig. 42.1). The process in these cells appeared to progress to pyknosis, necrosis, and eventually sloughing of the epithelium to expose the underlying lamina propria. In more advanced lesions the villi became clubbed, resulting in a loss of absorptive surface (Fig. 42.2). The colonic mucosa was flattened as a result of sloughing of the epithelium (Fig. 42.3). Damage in the colon was generally more chronologically advanced than in the jejunum of the same mouse and the lumen of many tissue sections contained massive numbers of gram positive bacteria.

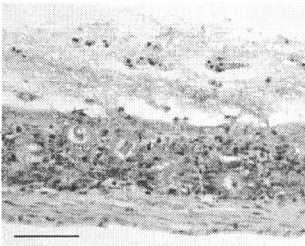
Livers from three mice, which received methanol extracts or methanol eluates from  $C_{18}$  columns (i.e. containing significant amounts of all four groups of compounds), had foci of hepatocytes with cytoplasmic vesicles containing



**Fig. 42.1.** Jejunum of mouse given *Stemodia* extract. Note vacuolation near tip of villus (arrow). Bar equals 5  $\mu$ m.



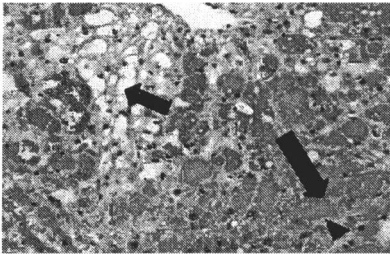
**Fig. 42.2.** Jejunum from mouse given *Stemodia* extract. Note necrotic villi (large arrow) and new epithelium (small arrow). Bar equals 50  $\mu$ m.



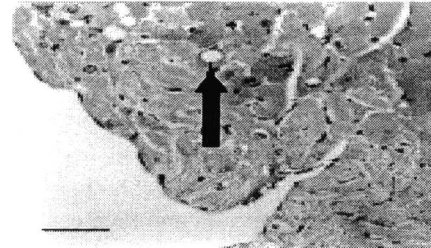
**Fig. 42.3.** Colon from mouse given methanolic extract of *Stemodia*. Note flattened mucosa, sloughed epithelium and bacteria in lumen. Bar equals 50  $\mu$ m.



**Fig. 42.4.** Hepatic lesion in three mice given methanolic extracts. Note phagocytized debris (small arrow) and erythrocyte (large arrow). Bar equals 10  $\mu$ m.



**Fig. 42.5.** Liver from mouse given hydrolysed purified B4 toxin. Note necrosis and haemorrhage (large arrow) at periphery of lesion. In many areas, centrilobular hepatocytes have completely disappeared (small arrow). Bar equals 10  $\mu$ m.



**Fig. 42.6.** Myocardial lesion in mice given *Stemodia*. Note degenerate myocardial cells, intracytoplasmic vacuolates (arrow). Bar equals 5  $\mu$ m.



amorphous pink to hyaline material. Some vesicles contained nuclear debris suggestive of phagocytosis of adjacent, apoptotic cells. A very few contained what appeared to be erythrocytes (Fig. 42.4). Liver from the mouse with grossly visible haemorrhage contained these changes in addition to more severe damage. In this mouse there were extensive areas of acute necrosis and haemorrhage in the peri-acinar and midzonal region of most lobules and considerable congestion of the surviving midzonal sinusoids (Fig. 42.5).

The myocardium of three mice contained lesions similar to those reported by Allen and Mitchell (1998). These consisted of foci of acute myocardial degeneration and necrosis. Many of the cardiac myocytes within or near these degenerative foci also contained vacuolated cytoplasm (Fig. 42.6). These foci were widely scattered throughout the myocardium and not nearly as extensive as reported previously in the sheep. A final, mild lesion attributable to *Stemodia* was single cell necrosis in germinal centres of the spleen and Peyer's patches. There were no histological lesions attributable to *Stemodia* in brain, lung or kidney.

## Discussion

The predominant clinical signs of *Stemodia* poisoning in sheep are watery diarrhoea, polyuria and hypotension resulting in shock and death (Allen and Mitchell, 1998). Mice dosed with either plant material or simple extracts of *Stemodia* exhibited diarrhoea and hypotension as reflected in poor capillary refill and cold extremities. Since the mice were housed in cages with bedding, it was not possible to determine if they were polyuric, but most exhibited a wet perineum and their behaviour was clinically consistent with hypovolemic shock.

Post-mortem lesions were also similar between the mouse model and sheep. The most dramatic and consistent post-mortem lesions in both sheep and mice occurred in the GI tract. In sheep there was hyperemia, vacuolation and necrosis of the ruminal papillae and the large and small intestinal epithelium. This change was especially pronounced in the ileum (Allen and Mitchell, 1998). In mice, the lesion was confined to the small and large intestine but was again most pronounced in the ileum. Single cell necrosis (apoptosis) of lymphocytes in germinal centres is probably not causally related to acute death reported in previous experimental and field cases, but might have long-term health effects in animals grazed on sub-lethal amounts of *Stemodia*.

Myocardial necrosis and vacuolation occurred in both the mouse and the sheep. The lesion was not as extensive in this study as in sheep. This may be due to the fact that the mice in this study died or were euthanized within a few ( $\ll$  24) hours of dosing, whereas sheep in the original report survived for 1–4 days and thus had more time for lesions to become histologically evident. The latter hypothesis is supported by the fact that those mice that did exhibit myocardial lesions received relatively impure (i.e. less concentrated) extracts and survived longer than mice that received more highly purified toxins.

Mice included in this study were treated with various extracts of plant material during the early stages of an effort to isolate and identify the active toxin(s) of *S. kingii* (see Allen *et al.*, Chapter 78). Although all of the extracts used in this chapter contained the same four groups of compounds, the latter, more highly refined preparations were richer in the highly toxic 'B' group and contained much less of the other, less acutely lethal groups (A, C and D): It is thus interesting to note that the mild liver lesions described above occurred with relatively crude extracts that contained an abundance of all four groups, or in semi-purified fractions that were richer in the less-polar groups of compounds. The exception to the rule of simple extracts were first the mouse with severe hepatic necrosis and second a mouse which had relatively well developed GI and myocardial lesions, but no liver lesions. The former received highly purified B4 toxin, which had been hydrolysed to produce compounds with chromatographic properties similar to the 'A' group, the latter purified B4 toxin. This suggests that there is more than one toxic principle involved in *Stemodia* intoxication and that further work is needed to identify the additional toxin(s) and determine their relative contributions to *Stemodia* intoxication.

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## Chapter 43

# A Procedure for the Estimation of the Daily Intake of Saponins from Pasture by Sheep

J.I. Loader<sup>1</sup>, E. Ryste<sup>2</sup>, A. Flåøyen<sup>3,4</sup>, K. Hove<sup>2</sup> and A.L. Wilkins<sup>1</sup>

<sup>1</sup>Chemistry Department, University of Waikato, Private Bag 3105, Hamilton, New Zealand; <sup>2</sup>Department of Animal Science, Agricultural University of Norway, Box 5025, 1432 Ås, Norway; <sup>3</sup>National Veterinary Institute, PO Box 8156 Dep., 0033 Oslo, Norway; <sup>4</sup>Department of Large Animal Clinical Sciences, Norwegian School of Veterinary Science, PO Box 8146 Dep., 0033 Oslo, Norway

### Introduction

*Nartheccium ossifragum* (Eng.: Bog asphodel), a member of the lily family, is known to cause alveld (literally: elf-fire), a hepatogenous photosensitization of lambs in Norway, the British Isles and the Faroe Islands (Flåøyen, 1999). Steroidal saponins of the plant have been suggested to cause the liver lesions resulting in retention of the photosensitizing agent phylloerythrin (Flåøyen, 1999). Attempts to reproduce alveld and related diseases experimentally have been hampered by a lack of knowledge concerning the level of steroidal saponins ingested by animals during natural toxicity outbreaks (Flåøyen *et al.*, 1991). Hitherto we have proposed that it might be possible to determine the level of steroidal saponins ingested by dosing sheep once daily with a known amount of isotopically labelled sapogenins, and determine the ratio of deuterated to natural sapogenins in faecal material (Flåøyen *et al.*, 2001).

An attempt to exploit this approach using 20,23,23-D<sub>3</sub>-sarsasapogenin failed due to loss of deuterium during ruminal metabolism of the dosed sapogenin, and/or the extraction, derivation and analytical procedures (Flåøyen *et al.*, 2001). An important observation during this trial was that about 96 h after the commencement of the dosing experiment in which a sheep was dosed once daily with 20,23,23-D<sub>3</sub>-sarsasapogenin, and three times daily with *N. ossifragum* plant material, essentially steady state levels of sapogenins were present in faeces collected at varying times during the remaining trial period (Flåøyen *et al.*, 2001). This finding supported our hypothesis that, provided a suitable dosing substrate could be identified (i.e. one not prone to deuterium exchange under ruminal and analytical conditions), it would be possible to dose a sheep once daily with a

known amount of an isotopically labelled saponin and determine natural saponin intake by comparing the ratio of deuterated to natural genins present in faecal material.

In this chapter we report evaluations of the suitability of 2,2,4,4-D<sub>4</sub>-sarsasapogenone and a c. 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin as isotopically labelled dosing substrates.

## Materials and Methods

### Synthesis of deuterated saponins

2,2,4,4-D<sub>4</sub>-sarsasapogenone was prepared by refluxing sarsasapogenone (10 g) for 24 h in a mixture of dioxane (300 ml) and D<sub>2</sub>O (25 ml) to which Na (0.35 g) had been cautiously added. Two cycles of deuterium exchange afforded material that consisted predominantly (> 96%) of 2,2,4,4-D<sub>4</sub>-sarsasapogenone. Reduction of 2,2,4,4-D<sub>4</sub>-sarsasapogenone (10 g) in ethanol:methanol (1:1, 400 ml) with sodium borohydride (2 g) afforded a c. 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin. A more detailed account of the synthesis of these substrates and their spectroscopic characterization will be reported elsewhere.

### Preliminary trial (deuterium retention assessment)

A 2-year-old male Dala sheep was caged and administered a single intraruminal dose of 2,2,4,4-D<sub>4</sub>-sarsasapogenone (1 g) suspended in 100 ml of water:ethanol (80:20) and washed down with 50 ml water. Eight days later, the same sheep was administered a single dose of a c. 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin (600 mg) suspended in 100 ml of water:ethanol (80:20) and washed down with 50 ml water. Hay was offered *ad libitum* during each of the experimental periods and 24 h faeces and urine samples were collected during the two dosing periods. Sub-samples of the faeces samples were freeze dried and analysed for free and conjugated saponins.

### Validation trial (plant intake calibration)

Three 2-year-old male Dala sheep were caged and on each of days 0 to 10 were offered 300 g wet weight (= 72 g dry matter (DM)) of *N. ossifragum* plant material, and also administered a single 70 mg intraruminal dose of a c. 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin suspended in 100 ml of water:ethanol (80:20) and washed down with 50 ml water. Hay was offered *ad libitum* during the experimental period. Faeces and urine samples were collected once daily during the trial period. Sub-samples of the faeces samples were freeze dried and analysed for total saponins.

### Sapogenin extraction and GC/MS analyses

*Nartheceium ossifragum* and faecal samples were freeze dried, and either sequentially extracted with dichloromethane (free sapogenin extracts) and methanol (conjugated sapogenin extracts) (plant samples and preliminary trial samples), or with methanol alone (total sapogenin extracts) (calibration trial samples). Conjugated sapogenin and total sapogenin extracts were hydrolysed with 0.5 M HCl. All extracts were spiked with sarsasapogenin propionate as internal standard, acetylated with pyridine/acetic anhydride and analysed by selected ion mode (SIM) GC/MS as described previously (Wilkins *et al.*, 1994), other than that a 25 m x 0.25 mm id HP-5 (Hewlett Packard, USA) column was used and  $m/z$  139,  $m/z$  315-320 (genin acetates) and  $m/z$  271-275 (sarsasapogenone) ions were monitored. The ratio of natural (non-deuterated) and deuterated genin acetates were determined using the sums of the  $m/z$  315 + 316 ( $^{13}\text{C}$  isotope of the  $m/z$  315 ion) and  $m/z$  317-320 ( $\text{D}_2, \text{D}_3, \text{D}_4 + ^{13}\text{C}$  isotope of the  $m/z$  320 ion) ion currents respectively. Plausible structures for the  $m/z$  139 and 315 (319) ions of genin acetates are given in Fig. 43.1. Urine samples were extracted and analysed as reported previously (Flåøyen and Wilkins, 1997).

### Results

The dosing experiments in which a sheep was administered a single dose of 2,2,4,4- $\text{D}_4$ -sarsasapogenone on day 0, and 8 days later a *c.* 1:4 mixture of 2,2,4,4- $\text{D}_4$ -sarsasapogenin and 2,2,4,4- $\text{D}_4$ -episarsasapogenin showed that maximum levels of free and conjugated sapogenins were, in each case, present in faecal material collected on days 2 and 3 (Tables 43.1 and 43.2). Sapogenin concentrations were determined as previously reported using integrated  $m/z$  139 ion contributions (Wilkins *et al.*, 1994). The percentage deuterated sapogenins were determined by comparing the peak areas of the  $m/z$  317+320 (deuterated sapogenin acetates) and  $m/z$  315-320 ion currents (total sapogenin acetates).

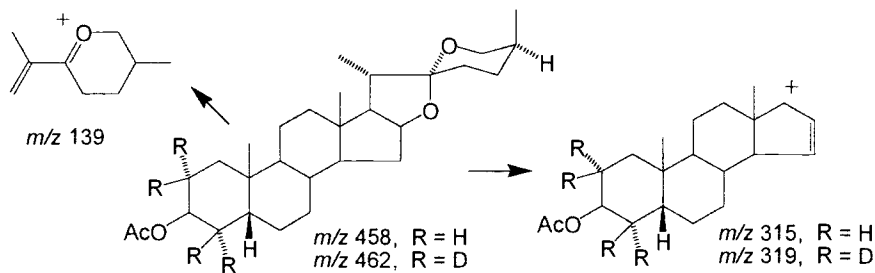


Fig. 43.1. Plausible structures for the  $m/z$  139 and 315 (319) ions of genin acetates.

**Table 43.1.** Free and conjugated saponin levels (mg kg<sup>-1</sup> DM) found in faecal samples after administration of a single dose of 2,2,4,4-D<sub>4</sub>-sarsasapogenone.

Day	Extract	Sar-CO <sup>a</sup>	Sarsa <sup>b</sup>	Episar <sup>c</sup>	Total <sup>d</sup>	% D <sup>e</sup>
0	Free	—	—	—	—	
	Conjugated	—	—	—	—	
1	Free	136	25	67	228	
	Conjugated	5.7	7.1	20	33	
2	Free	459	110	353	923	95.2
	Conjugated	21	33	106	159	96.1
3	Free	152	95	346	592	93.4
	Conjugated	8.4	19	74	102	92.8
4	Free	40	37	125	202	
	Conjugated	6.4	9.1	32	48	
5	Free	9	13	34	56	
	Conjugated	1.5	2.5	5.4	9.4	
6	Free	—	11	26	37	
	Conjugated	—	2.0	3.6	5.6	
7	Free	—	—	—	—	
	Conjugated	—	—	—	—	

<sup>a</sup>sar-CO = sarsasapogenone; <sup>b</sup>sarsa = sarsasapogenin; <sup>c</sup>episar = episarsasapogenin; <sup>d</sup>total = total genins sar-CO, sarsa and episar levels; <sup>e</sup>% D = % deuterium retained in episarsasapogenin.

**Table 43.2.** Free and conjugated saponin levels (mg kg<sup>-1</sup> DM) found in faecal samples after administration of a single dose of a c. 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin.

Day	Extract	Sar-CO <sup>a</sup>	Sarsa <sup>b</sup>	Episar <sup>c</sup>	Total <sup>d</sup>	% D <sup>e</sup>
0	Free	—	—	—	—	
	Conjugated	—	—	—	—	
1	Free	3.5	10	53	66	
	Conjugated	—	1.0	2.3	3.3	
2	Free	27	69	421	516	96.7
	Conjugated	6.6	12	54	73	95.6
3	Free	20	54	220	294	92.4
	Conjugated	4.6	10	35	49	90.4
4	Free	9.2	27	85	120	
	Conjugated	2.9	6.1	19	28	
5	Free	—	12	35	47	
	Conjugated	—	0.7	2.0	2.7	
6	Free	—	—	—	—	
	Conjugated	—	—	—	—	

<sup>a</sup>sar-CO = sarsasapogenone; <sup>b</sup>sarsa = sarsasapogenin; <sup>c</sup>episar = episarsasapogenin; <sup>d</sup>total = total genins sar-CO, sarsa and episar levels; <sup>e</sup>% D = % deuterium retained in episarsasapogenin.

## Validation Trial

The total levels of deuterated and natural saponin detected in faeces recovered from one of the three sheep that consumed 72 g day<sup>-1</sup> DM of *N. ossifragum*, and which were also dosed once daily for 11 days with a c. 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin are presented in Table 43.3. The percentage deuterated saponin content of the samples is presented in Table 43.3. A mean level of 3,655 (standard deviation 705) mg kg<sup>-1</sup> DM was found in randomly selected *N. ossifragum* samples. Duplicate analyses of plant sub-samples agreed to within 0.5–2.8%. In accord with other dosing trial results, episarsasapogenin was the dominant genin constituent detected in faecal material (Flåøyen and Wilkins, 1997; Flåøyen *et al.*, 2001). No saponins were detected in urine samples.

Since 70 mg of deuterated saponins were dosed daily and mean percentage D level faecal material was of 18.5% (Table 43.3) the calculated average daily intake of saponins was 378 mg day<sup>-1</sup>, of which 308 mg day<sup>-1</sup> could be attributed to the ingestion of *rome* saponins. Furthermore, since the average saponin level of the dosed plant material was found to be 3655 (standard deviation 705) mg kg<sup>-1</sup> DM, the calculated mean daily intake of *N. ossifragum* was 84 g DM, compared to the 72 g DM (= 300 g wet weight) that was dosed. Generally similar results were obtained for sheep 2 and 3 (calculated mean plant intakes of 64 and 69 mg kg<sup>-1</sup> DM respectively). Given variations in the saponin level of the dosed plant material, and some lack of homogeneity in the distribution of natural and

**Table 43.3.** Percentage deuterated saponins (% D), calculated total saponin levels in faecal samples collected from a sheep consuming 72 g DM *N. ossifragum* daily and dosed once daily with a c. 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin, and calculated daily consumption of *N. ossifragum* (g DM day<sup>-1</sup>).

Day	% D	Calc. total saponins (mg kg <sup>-1</sup> DM day <sup>-1</sup> )	Calc. plant saponins (mg kg <sup>-1</sup> DM day <sup>-1</sup> )	Calc. plant (g DM day <sup>-1</sup> )
3	19.1	366	296	81
4	17.9	391	321	88
5	18.4	380	310	85
6	17.3	405	335	92
7	17.3	405	335	92
8	19.3	363	293	80
9	16.0	438	368	101
10	20.4	273	343	75
11	21.2	330	260	71
Mean	18.5	378	308	84
Std dev	1.6	48	32	9

deuterated saponin in faecal material, agreement to within 1 to 1.5 standard deviations was considered to be acceptable.

## Conclusions

It is apparent from the results presented in Table 43.2 that the *c.* 1:4 mixture of 2,2,4,4-D<sub>4</sub>-sarsasapogenin and 2,2,4,4-D<sub>4</sub>-episarsasapogenin afforded by sodium borohydride reduction of 2,2,4,4-D<sub>4</sub>-sarsasapogenone (*c.* 1:4 is the ratio at which these saponins are typically present in faeces samples from animals consuming *N. ossifragum*), can be utilized as an isotopically labelled dosing substrate for determining the intake of *N. ossifragum* saponins under normal grazing conditions.

Calculated intakes of 64–84 g DM day<sup>-1</sup> of *N. ossifragum* (89–117% of actual intakes) were considered satisfactory for future use of this isotope dilution technique in measuring intake of *N. ossifragum* under natural conditions.

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## Chapter 44

# Exposure of Native Australian Parrots to Pyrrolizidine Alkaloids

J. Starks<sup>1</sup>, S.M. Colegate<sup>2\*</sup> and P. Hooper<sup>2</sup>

<sup>1</sup>*Birds Australia, 415 Riversdale Road, Hawthorn East, Victoria 3123, Australia;*

<sup>2</sup>*CSIRO Livestock Industries, Plant Toxins Research Group, Private Bag 24, Geelong, Victoria 3220, Australia*

\*Author to whom correspondence should be addressed.

The Orange-bellied Parrot (*Neophema chrysogaster*) is one of Australia's rarest and most endangered species, with its total wild population numbering about 180 mature individuals (Garnett and Crowley, 2000). Captive breeding populations are maintained in Hobart and Melbourne to allow for release of birds into the environment.

In Australia, *Heliotropium europaeum* (heliotrope, potato weed, common heliotrope) is an introduced plant and a consequent weed of agricultural areas. Since heliotrope contains hepatotoxic pyrrolizidine alkaloids (PAs), the impact of the inclusion of heliotrope seeds in the diet of the parrots was investigated for potential implications to the captive breeding/release programme.

## Toxic Effects of Pyrrolizidine Alkaloids

Hepatic metabolism of PAs possessing particular structural requirements (Culvenor *et al.*, 1976) leads to the *in vivo* production of the didehydro-PAs ('pyrroles') that are potent alkylating agents. Reaction of the pyrrolic metabolites with tissue components such as proteins and DNA, leads to effects including parenchymal megalocytosis (enlarged cells), centrilobular necrosis, hepatic fibrosis leading to cirrhosis and hepatic failure, pulmonary hypertension and cancers (Stegelmeier *et al.*, 1999).

Animal susceptibility to PAs is species and, sometimes, PA specific (Cheeke, 1998). Avian species adversely affected by oral ingestion of PA-containing plants or seeds include chickens, ducks, turkey poults and geese. Japanese quail are resistant to adverse effects of dietary PAs and eggs from quail hens exposed to PAs in the diet were fertile and produced normal chicks (Cheeke, 1998).

Age and gender can also influence the susceptibility of animals to the toxic effects of PAs. Thus, young, neonatal and nursing animals have developed fatal hepatic disease while their lactating dams were unaffected by the PAs

(Stegelmeier *et al.*, 1999). The transfer of PAs into the eggs of chickens has been shown (Edgar and Smith, 2000) and, utilizing a chick embryotoxicity screening system, Peterka *et al.* (1994) have demonstrated an embryotoxicity range of between 3 and 30 µg of senecionine per egg.

How the acute or chronic toxic effects of PAs, the increased susceptibility of young animals or the *in ovo* presence of PAs impacts upon the regeneration of the Orange-bellied Parrot population remains to be determined.

## Diagnosis of Exposure to Pyrrolizidine Alkaloids

Since toxic effects of PA consumption can occur many months after the exposure to the PAs, it is not always easy to associate the toxic insult with intake of the plant.

Clinical signs of poisoning can include signs of liver failure such as anorexia, depression, icterus, visceral oedema and ascites. Chronically poisoned animals often show no overt clinical signs but liver damage can be ongoing resulting in increased hepatocyte death leading ultimately to cirrhosis (Stegelmeier *et al.*, 1999).

## Histopathology

Histopathologically, PA intoxication can be divided into acute and chronic lesions. The first cellular indication of PA intoxication is a dose dependent swelling of hepatocytes. Consequent necrosis of hepatocytes ensues if cellular degeneration continues to loss of cellular homeostasis.

High doses of PAs, resulting in acute panlobular, hepatocellular damage is manifested as extensive necrosis with haemorrhage and minimal inflammation (Stegelmeier *et al.*, 1999). These changes are non-specific and can be caused by other hepatotoxic chemicals, viruses or immunological diseases.

Chronic intoxication-related changes include focal hepatocyte necrosis, peribiliary fibrosis and bile duct proliferation. Because of the anti-mitotic effect of the pyrrolic PA metabolites, the damaged hepatocytes can develop into abnormally large cells called megalocytes.

## Physico-chemical detection

The sulphur-bound, pyrrolic metabolites of the toxic PAs can be oxidatively released from tissues and trapped as alkyl ethers of the pyrrolic PA metabolites (Mattocks and Jukes, 1992; Schoch *et al.*, 2000).

Thus, samples of tissue from animals suspected of succumbing to PA-intoxication, after extensive washing with ethanol, acetone, diethyl ether and finally ethanol again, are treated with an acidified solution of silver nitrate in ethanol. The resultant diethyl ether of the pyrrolic metabolite (1-ethoxymethyl-7-

ethoxy-6,7-dihydro-5H-pyrrolizine) has been detected by thin layer chromatography after visualization with Ehrlich's reagent, or by gas chromatography/mass spectrometry (Mattocks and Jukes, 1992; Schoch *et al.*, 2000).

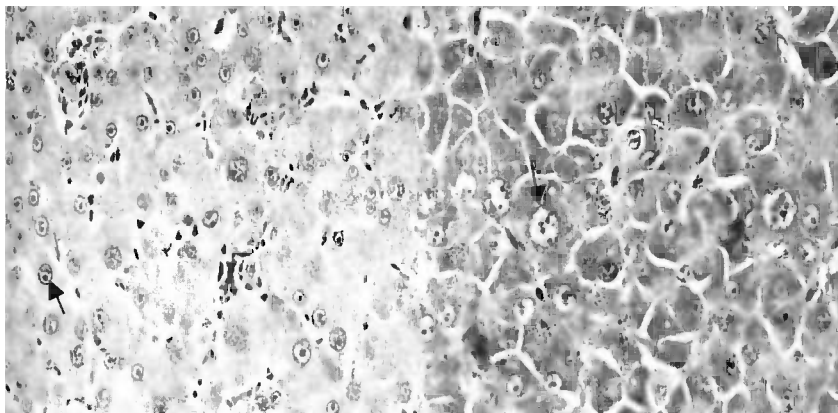
## Investigations on Australian Parrots

An Adelaide birdwatcher reported finding an Orange-bellied Parrot accompanying Blue-winged Parrots (*Neophema chrysostoma*) in farmland south of Robe, South Australia, in June 1997. A search of this area in August of that year revealed two Orange-bellied Parrots and 128 Blue-winged Parrots foraging together in sheep paddocks where *H. europaeum* was growing. On the same day, over 200 Blue-winged Parrots were found apparently foraging on *H. europaeum* growing under rows of grapevines in a newly established vineyard in the same area. Galahs (*Cacatua roseicapilla*), Red-rumped Parrots (*Psephotus haematonotus*), Goldfinches (*Carduelis carduelis*) and Red-browed Finches (*Neochmia temporalis*) were also seen foraging amongst the heliotrope in the vineyard. Heliotrope has not been previously recorded as a food plant for these native birds.

Permits were obtained from the South Australian Department of Environment and Natural Resources to collect some Blue-winged Parrots for tissue examination. Six adult males and two juvenile males were collected from the vineyard in September. Samples of various organs, including the livers, kidneys and hearts from each were collected into formalin solution for histological and, since literature reports indicated that storage in formalin did not adversely affect the detection of pyrrolic PA metabolites (Mattocks and Jukes, 1992), chemical examination. The crops from two birds were also collected for examination of the contents.

## Histopathological results

There was no gross or histopathological evidence for acute PA poisoning or advanced chronic intoxication in the form of fibrosis or cirrhosis of the livers. The histopathological parameter for assessment of the tissues was that of megalocytosis. The observations (Table 44.1) indicated very mild to advanced megalocytosis in the livers of seven of the eight birds examined (Fig. 44.1). Very mild to moderate megalocytosis was observed in the kidneys, pancreas (Fig. 44.2) and/or proventriculus of four of those also showing signs in the liver.



**Fig. 44.1.** High power photomicrograph of a liver section showing numerous megalocytes (example arrowed).

**Fig. 44.2.** High power photomicrograph of a pancreatic section showing moderate megalocytosis (example arrowed).

Bird 6 was far more severely affected than any other bird. However, the significance of the large cells seen in the proventriculus of birds 5 and 8 was hard to assess as these were the only proventriculi seen in these samples.

### Physico-chemical results

The livers of the birds were all treated as described by Mattocks and Jukes (1992) including extensive washing with water to remove the formaldehyde fixative. Analysis of the silver-nitrate-treated liver extracts using GCMS failed to detect any pyrrolic ethers that would have confirmed the exposure to PAs.

The crop contents of two birds were homogenized in dilute acid and the filtered extract treated as usual to isolate the PAs, both those actually present as PAs and those derived from zinc/acid reduction of the respective PA N-oxides present. Chemical ( $\text{CH}_4$ ) ionization GCMS/MS examination of the derivatized (methyl boronation and acetylation) extract of the crop contents revealed the presence of lasiocarpine as its methyl boronate derivative ( $m/z$  436,  $M^+ + H$ ; 336,  $M^+ + H$  - angelic acid; 220,  $M^+ + H$  - lasiocarpic acid; 120,  $M^+ + H$  - lasiocarpic acid-angelic acid) and heliotrine as its acetyl derivative ( $m/z$ , 356,  $M^+ + H$ ; 198,  $M^+ + H$  -  $\text{C}_8\text{H}_{14}\text{O}_3$ ; 180,  $M^+ + H$  - heliotric acid; 120,  $M^+ + H$  - heliotric acid-acetic acid) supporting the ingestion of heliotrope and demonstrating exposure to the PAs.

**Table 44.1.** Observations of megalocytosis in Blue-winged Parrots.

1 = some very mild megalocytosis with an average of more than two megalocytes per high power field; 2 = large cells common, some evident reduction in the numbers of cells visible, even at low power; 3 = advanced megalocytosis, numerous megalocytes, many quite large and bizarre; 4 = advanced hepatic disease with other lesions secondary to the megalocytosis.

Bird number	Age	Liver	Kidney	Other
1	Adult	-	-	-
2	Adult	1	-	-
3	1st year juvenile	1	-	-
4	1st year juvenile	2	1	-
5	Adult	2	1	Proventriculus - 2
6	Adult	3	2	Pancreas - 1
7	Adult	2	-	-
8	Adult	1	-	Proventriculus - 1

## Conclusions

Megalocytosis is a fairly common finding in the livers of caged birds (Rod Reece, NSW Agriculture, personal communication). This may reflect long-term consumption of a variety of seeds, some of which may contain toxins such as PAs and some mycotoxins. The inability to detect any pyrrolic metabolites does not unequivocally eliminate chronic exposure to PAs. It has been shown in other studies (Stegelmeier *et al.*, 1999) that although megalocytosis and pyrrole ether detection can both be observed in cases of PA exposure, either indicator of exposure can be observed without the other. In addition, it has been shown (Schoch *et al.*, 2000) that the levels of pyrrolic ethers recoverable decreases dramatically if a tissue is fixed in formalin rather than treated fresh or frozen.

However, a combination of the observation of the birds feeding in the vicinity of *H. europaeum*, the hepatic megalocytosis and the detection of heliotrine and lasiocarpine in the crop contents of some birds support a finding that these birds, and presumably the endangered Orange-bellied Parrot as well as the other species in the area, are utilizing seeds of *H. europaeum* in their diets.

The possible implications to the success of the captive breeding/release programme of this inclusion need to be considered. Will exposure to PAs adversely affect reproduction in the wild? Are females more adversely affected than males? Will chicks be assaulted *in ovo* by transferred PAs? When should birds be released in order to avoid at least one season of exposure to PAs?

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## Chapter 45

# Poisonous Sedges: the Galegine Content of *Schoenus rigens* at Various Growth Stages

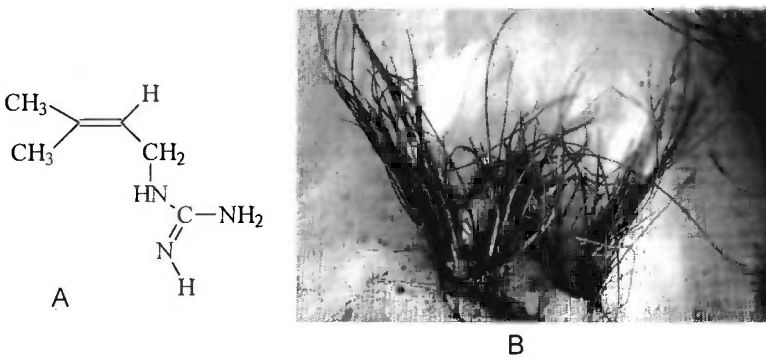
P.R. Dorling<sup>1</sup>, S.M. Colegate<sup>2\*</sup> and C.R. Huxtable<sup>3</sup>

<sup>1</sup>Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch WA 6150, Australia; <sup>2</sup>Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Victoria 3220, Australia; <sup>3</sup>Department of Biomedical Science, S2-110A, College of Veterinary Medicine, Cornell University, Ithaca NY, USA

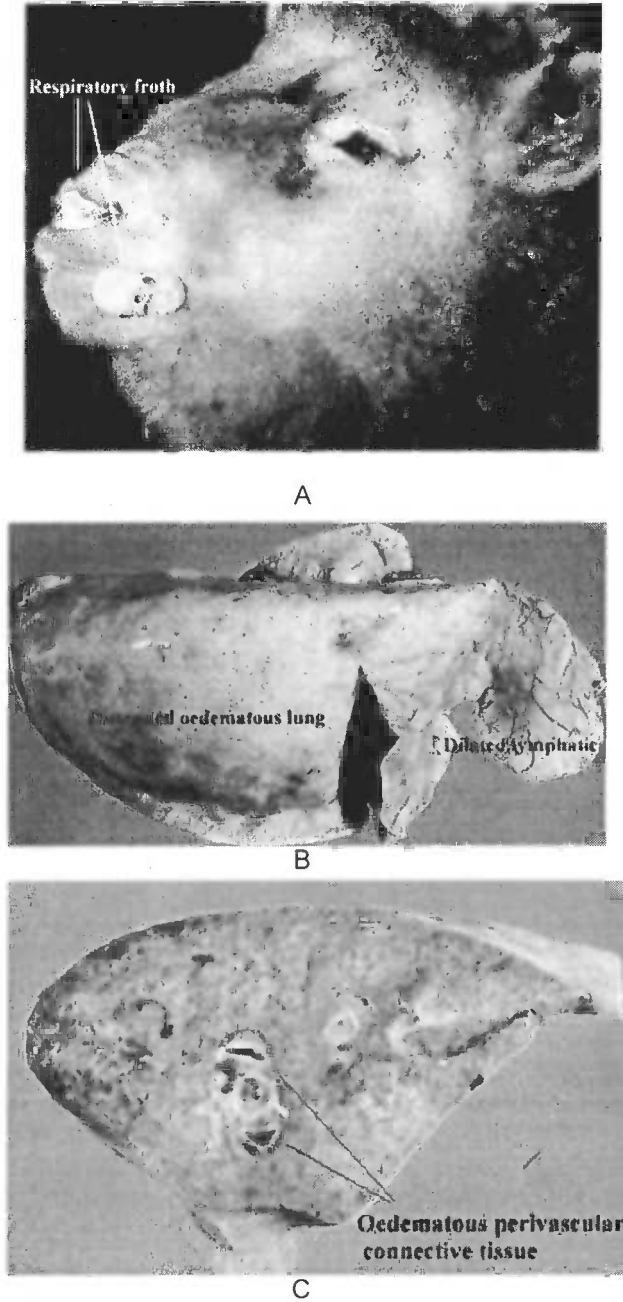
\* Author to whom correspondence should be addressed.

The sedge *Schoenus asperocarpus* (Cyperaceae) has long been recognized as the possible cause of a fatal intoxication of sheep in Western Australia (Everist, 1981). The toxic principle was shown to be the guanidinoisoprenoid, galegine (Fig. 45.1) (Huxtable *et al.*, 1993; Colegate *et al.*, 1994).

Intra-peritoneal administration of saline solutions of synthetic galegine sulphate to sheep reproduced the field syndrome causing a fatal pulmonary oedema and associated proteinaceous exudate in the respiratory tract and plural cavity (Fig. 45.2) (Huxtable *et al.*, 1993). This exudate contained sufficient fibrinogen to promote clotting on exposure to air.



**Fig. 45.1.** Structure of galegine (A), the toxic principle of *Schoenus asperocarpus* and *Schoenus rigens* (B).



**Fig. 45.2.** (A) Fatal intoxication of sheep showing stable fibrin-containing respiratory froth, (B) oedematous lung showing dilated lymphatics, and (C) cross-section of lung showing oedematous perivascular connective tissue.



Large-scale outbreaks of sedge-like sudden death in sheep on grazing land apparently devoid of *S. asperocarpus* were attributed by farmers to another sedge identified as *S. rigens*. Samples of various sedges were collected at the site of previous intoxications and analysed for galegine content using HPLC separation with fluorimetric detection of a benzoin derivative of galegine. In this way, of 18 Western Australian sedges analysed only *S. asperocarpus* and *S. rigens* showed the presence of galegine (Colegate *et al.*, 1994). This supported the anecdotal evidence from farmers that *S. rigens* was responsible for the sudden death in sheep.

To improve the analytical procedure and further investigate the anecdotal claims of farmers that tended to implicate the young, newly shooting *S. rigens* in the fatal intoxications, a liquid chromatography/mass spectrometric (LC-MS) method of galegine analysis was developed. This was applied to the analysis of *S. rigens* (young plant, mature plant and seeds) collected on farms where farmers have reported stock losses. Analysis was also conducted on *S. asperocarpus* (mature plant) collected at a site in the Scott River district on the south coast of Western Australia at the time of a significant poisoning of sheep in the 1970s. This latter plant collection was used to experimentally induce intoxication.

## Results and Discussion

### Plant samples: collection and extraction

Samples of *S. rigens* from farm paddocks in sand plain/limestone country about 200 km north of Perth in Western Australia, were collected at young and mature stages of growth. Sudden death in sheep, similar to the death of 1800 head of sheep overnight whilst grazing on a similar pasture several kilometres to the south, had been observed at this site of collection. Voucher specimens were lodged with the Western Australian Herbarium (RJC 8738).

An accurately weighed (approximately 2 g) sample of dried, ground plant was stirred in methanol (30 ml) overnight. The methanolic extract was filtered and the methanol evaporated to dryness. The green coloured, residual gum was partitioned between diethyl ether and water. Subsequent evaporation of the aqueous fraction, under reduced pressure, yielded a solid residue that was itself extracted with methanol/acetone (50/50, 2 ml). Filtration separated the methanolic acetone-soluble material from the insoluble inorganic salts. Evaporation of the methanolic acetone under a flow of nitrogen and reconstitution of the residue in water containing 1% of glacial acetic acid (1.5 ml) provided the analytical sample.

### High pressure liquid chromatography/mass spectrometric analysis

An aliquot of the sample solution was injected on to a reverse phase C8 silica column (Alltech, Alltima 250 x 4.6 mm) and eluted with a gradient flow (0.5 ml

$\text{min}^{-1}$ ) of aqueous acetonitrile containing 0.01% of trifluoroacetic acid (30% to 70% acetonitrile over 15 min). The column effluent was directed to the atmospheric pressure, electrospray ionization chamber of a Finnigan LCQ Ion Trap Mass Spectrometer.

Calibration of the response for galegine was accomplished using solutions of synthetic galegine sulphate in 1% aqueous acetic acid (Fig. 45.3).

The galegine content of dried, mature *S. asperocarpus* collected in the 1970s was estimated at  $60 \mu\text{g g}^{-1}$  DW (dry weight of plant). It is of interest that this plant collection is still potentially toxic after almost 30 years storage at room temperature.

In contrast to the mature *S. asperocarpus*, the mature *S. rigens* was virtually devoid of galegine with observed levels between 0 and  $0.2 \mu\text{g g}^{-1}$  DW. Surprisingly, since seeds are usually a repository of alkaloids, basic amino acids and other secondary metabolites, the seeds from *S. rigens* were also devoid of galegine. However, young, newly shooting *S. rigens* was found to have galegine in excess of  $200 \mu\text{g g}^{-1}$  DW (Fig. 45.4).

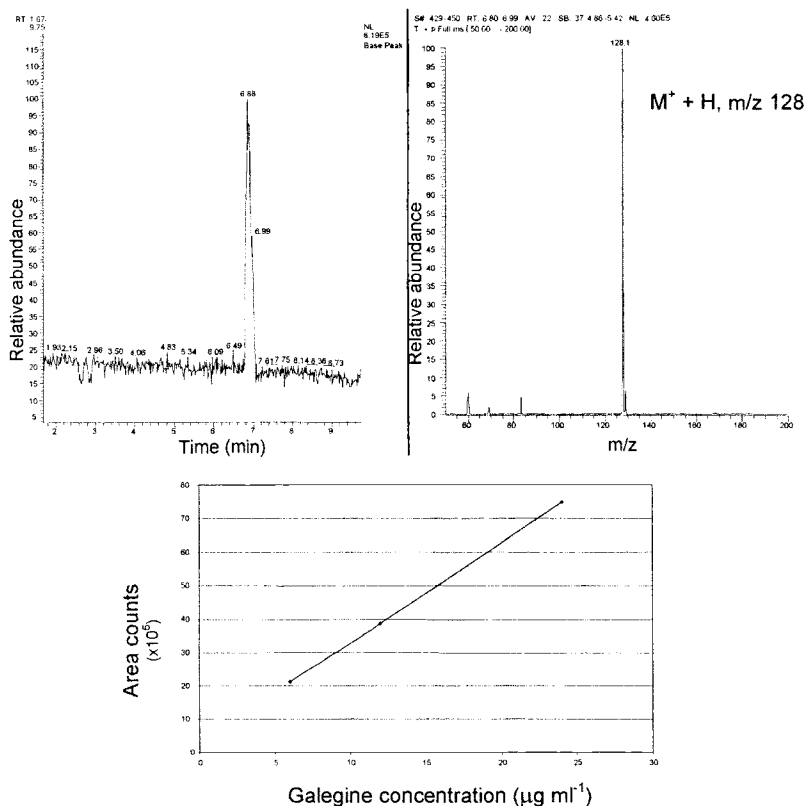
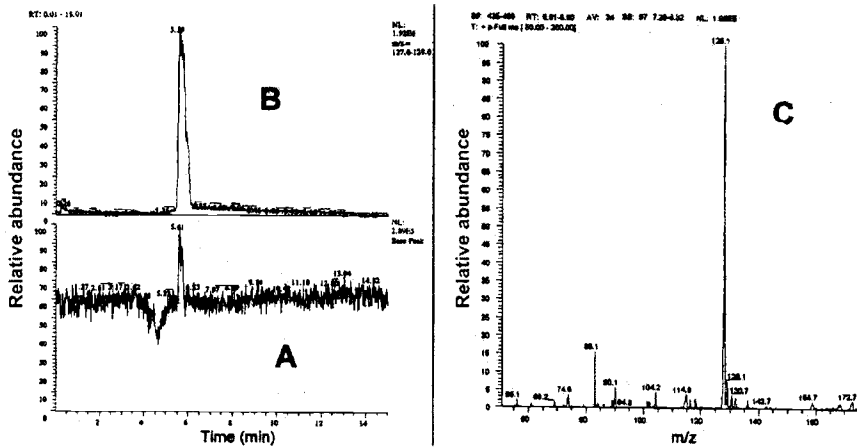


Fig. 45.3. Chromatogram and mass spectrum of authenticated galegine ( $m/z$  128,  $M^+ + H$ ) and calibration curve over the range of  $6\text{--}24 \mu\text{g ml}^{-1}$ .



**Fig. 45.4.** Base ion (A) and  $m/z$  127-129 (B) chromatograms and corresponding mass spectrum (C) for extract of *Schoenus rigens*.

## Conclusions

In accordance with the anecdotal evidence for *S. rigens*, the LC-MS analysis clearly showed very high levels of galegine in young plants, very low to none detected in mature plants and none detected in seeds. In contrast to mature *S. rigens* the mature *S. asperocarpus* contained significant amounts of galegine and was toxic to stock.

The results support farmer observations that sheep should be kept away from the young *S. rigens* at the break of season when there is little other feed for the grazing sheep, especially for the first few seasons on newly cleared land when this plant can be the dominant regrowth species.

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## Chapter 46

# Lupin Non-starch Polysaccharides and their Effects on Chickens

A.J. Cowieson and T. Acamovic

*Avian Science Research Centre, SAC, Ayr, KA6 5HW, Scotland, UK*

*Lupinus* species seeds are a source of vegetable protein for poultry. Historically, the quinolizidine alkaloids have been the major toxic problem in using lupins in animal diets (0.2–19 g kg<sup>-1</sup>). In ‘sweet’ lupin varieties the alkaloid content has been reduced by as much as a factor of 100 to less than 10 mg kg<sup>-1</sup> (Abdel-Halim *et al.*, 1999). Indigestible soluble carbohydrates can cause adverse effects in poultry and are now considered to be the major constraint in the use of lupin seeds in diets and can reduce feed conversion with significant economic impact. *Lupinus albus* and *Lupinus luteus* are two of the most abundantly cultivated lupins worldwide and have protein, oil and non-starch polysaccharide (NSP) contents of around 400, 100 and 400 g kg<sup>-1</sup>, respectively (Evans *et al.*, 1993; Van Barneveld and Hughes, 1994).

The NSPs are the major carbohydrates in lupins and are primarily pectin-based galacturonides and  $\alpha$ -galactosides (Mohamed and Rayas-Duarte, 1995). The anti-nutritive effects of these NSPs are not fully understood but they are known to increase chyme viscosity, impair nutrient uptake and interact with the microflora of the distal ileum and caecum (Bedford, 1996; Apajalahti and Bedford, 2000). This can increase endogenous losses and compromise bird health, significantly reducing performance, particularly in young chicks. Endogenous losses are extremely expensive, nutritionally, for animals. The anti-nutritive effects of lupin NSPs may be reduced by addition of exogenous enzymes or by heat treatment. Thus the effects of lupin NSPs and enzyme treatment on nutrient digestibility and endogenous losses were investigated using a modified tube feeding study.

### Materials and Methods

The animal studies were conducted with the approval of the SAC Animal Ethics Committee.

NSPs extracted from *L. albus* (Frauenhofer, Freising, Germany) had a dry matter content of 500 g kg<sup>-1</sup> and, on a dry matter basis, contained 170 g kg<sup>-1</sup> crude

protein, 730 g kg<sup>-1</sup> NSPs, 100 g kg<sup>-1</sup> ash and a gross energy (GE) of 14.5 MJ kg<sup>-1</sup>. The NSP fraction was composed of raffinose oligosaccharides (raffinose, stachyose and verbascose), the main oligosaccharide being stachyose-based. The NSP extract was treated with a range of enzymes including protease, xylanase, cellulase, phytase,  $\alpha$ -galactosidase, pectinase and  $\beta$ -glucanase at typical commercial dietary inclusion levels. Heat treatment was administered by autoclaving at 120°C for 15 min and enzymes were added to the heat-treated NSP extract thereafter. Viscosity was determined using a Brookfield digital viscometer maintained at 40°C with a CP-40 cone connected to a water bath. Sialic acid was determined (Jourdain *et al.*, 1971), which measures total, free and glycosidically bound sialic acid by a periodate-resorcinol reaction.

A total of 112 Ross broilers (~3 kg) were used in the 14 treatment, 8 replicate precision feeding trial (Ferraz de Oliveira *et al.*, 1994). Excreta were quantitatively collected, freeze-dried and milled (1 mm). Nitrogen, GE and sialic acid (N-acetylneuraminic acid) were determined. Statistical analysis was done using Minitab, release 12.0 and significance detected using ANOVA and Tukey's LSD.

## Results

All birds remained healthy and survived the experiment. Excreta from birds fed the NSP extract treated with enzymes were generally less sticky and wet than birds fed the untreated NSP extract.

### **Influence of enzyme and heat treatments on the viscosity of *L. albus* NSPs**

The viscosity of the untreated NSP extract was 22.8 cP. The most effective treatment was with cellulase, which reduced the viscosity of the NSP extract from 22.8 to 13.3 cP ( $P < 0.01$ ). Heat treatment had a large effect on the viscosity, with heat treatment +  $\alpha$ -galactosidase reducing viscosity to 9.4 cP ( $P < 0.01$ ) while  $\alpha$ -galactosidase without heat treatment reduced viscosity to only 18.8 cP. The combination of heat and enzyme supplementation was most effective in reducing the viscosity of the NSP extract.

### **Effect of enzyme and heat treatment on the coefficients of nutrient digestibility of *L. albus* NSPs**

The coefficient of true dry matter digestibility (TDMD) was improved by both xylanase and pectinase addition, from 0.59 to 0.88 and 0.89 respectively ( $P < 0.01$ ). The other enzymes or enzyme + heat combinations were unsuccessful in improving TDMD values of the NSP extract ( $P > 0.05$ ). The true coefficient of nitrogen retention (CNR) was improved only by pectinase addition (from -0.01

xylanase, protease, pectinase and cellulase addition ( $P < 0.05$ ), although the greatest improvement in TMEn was seen with pectinase. Pectinase improved the TMEn from 4.7 MJ kg<sup>-1</sup> DM in the unsupplemented NSP extract to 10.6 MJ kg<sup>-1</sup> DM with pectinase addition. Heat treatment failed to improve the TMEn of the NSP extract ( $P > 0.05$ ). The same trends for TMEn/GE (metabolizability of energy) were seen as with TMEn.

### **Effect of enzyme and heat treatment of lupin NSPs on endogenous losses**

There was no effect of treatment on the concentration of sialic acid in the excreta ( $P > 0.05$ ). Also, due to relatively high variation across treatment groups, which may be indicative of the inimical nature of the material fed, only a small reduction ( $P = 0.08$ ) in total sialic acid excretion was seen with enzyme + heat treatment. When pooled enzyme treatments were compared to untreated NSP extract there was a reduction in the excretion of sialic acid ( $P < 0.05$ ).

## **Discussion and Conclusions**

Dietary fibre (DF) has been defined as a heterogeneous mixture of structural and non-structural polysaccharides and lignin, which cannot be digested by endogenous secretions (Souffrant, 2001). Feeding diets high in fibre to monogastrics has negative effects on performance that can only be partially attributed to nutrient dilution. In lupins, DF is a combination of soluble and insoluble polysaccharides, and though insoluble carbohydrates such as cellulose may act simply as fibrous diluents, the soluble carbohydrates have potentially much greater effects. The water-holding capacity of these sugars can be substantial, resulting in large, viscous gums being formed in the gastrointestinal tract (GIT), which can impair nutrient uptake, reduce feed consumption, and damage the epithelial tissues of the villi (Iji *et al.*, 2001). The rate of glucose absorption from the intestinal lumen has been demonstrated to be more than halved in the presence of soluble NSPs but only marginally reduced by insoluble, non-viscous DF sources (Bach Knudsen, 2001). Both soluble and insoluble NSPs also interact with the microflora in the distal ileum, caecum and colon and create an environment that can favour pathogenic rather than fermentative bacteria (Apajalahti and Bedford, 2000). Gas production can also be a problem, especially in diets rich in raffinose-based oligosaccharides. The consequence is that birds fed diets rich in NSPs have poorer feed conversion ratios, lower growth rates and are more susceptible to bacterial infection, as passage time, pH, aeration of the GIT and the microfloral balance are sub-optimal. These adverse effects can have profound financial consequences on poultry producers.

Improvements in TDMD with enzyme addition are likely to be due to the hydrolysis of the polysaccharides and reduced steric hindrance allowing endogenous enzymes greater access to their substrates. The direct effect of the

enzymes on the NSPs and the subsequent absorption of the resultant mono- and disaccharides are likely to contribute towards the higher digestibility coefficients. A reduction in endogenous losses may also be a contributor to the improvements observed.

Coefficients of N retention were improved only by addition of pectinase. Improvements in CNR associated with pectinase are not surprising, as the NSPs in lupin seeds are uronic-acid-based. Hydrolysis of the uronic-acid-based carbohydrates reduces viscosity and improves N retention. The failure of heat treatment to improve CNR could be due to the production of Maillard compounds. A dark brown colour was noted after the solutions were autoclaved. The presence of heat (during autoclaving), moisture, protein and sugar in the NSP extract would favour such reactions and the melanoidins produced would render unrecoverable, some amino acids (Van Barneveld, 1993).

The improvements in TMEn and TMEn/GE associated with enzyme treatment may be due to an increased availability of nutrients and/or a decrease in endogenous loss. Enzyme treatment may also have reduced the affinity of the NSPs for mucopolysaccharides and proteins within the gastrointestinal tract, thus reducing endogenous losses. Supplementation with exogenous enzymes also reduces bacterial numbers by substrate limitation, mediated through improved nutrient uptake in the upper GIT (Bedford, 2000).

Sialic acid (N-acetylneuraminic acid) is found widely in nature as components of the oligosaccharides units of mucins and glycoproteins (Jourdian *et al.*, 1971). With the exception of a few microbial sources where sialic acids are involved in cytoadherence (Bonhila *et al.*, 1995), the sialic acids are only produced by animal cells, of all species of vertebrates (Nakano *et al.*, 1994). Thus, they can be used as an estimation of the quantity of endogenous material being produced by the bird. Loss of sialic acid from erythrocytes has been linked with cellular senescence, bacterial infection and certain pathological conditions (Vajreswari and Narayanareddy, 1992). The reduction in endogenous secretions with enzyme treatment could be due to a number of factors. It has been shown that NSPs can lead to hypertrophy and hyperplasia of the ileum and increase pancreatic enzyme production. An increase in DF content of a diet for pigs from 50 to 180 g kg<sup>-1</sup> resulted in a doubling of gastric, salivary and pancreatic secretions, increasing the metabolic demand on the animal (Wenk, 2001). Supplementation with enzymes may cause a reduction in the requirement of the animal to produce endogenous enzymes, with a consequential reduction in metabolic cost and protein turnover, which may partially explain the reduced endogenous secretion. Enzyme supplementation also increases the amount of digestion in the duodenum and jejunum (Bedford, 2000), causing less nutrients to be available in the lower GIT for microbial fermentation. This improved nutrient utilization, reduction in size of the ileum and caecum and a lower microbial load will also reduce endogenous losses. Reduction in the abundance of bacteria in the lower GIT will also reduce the likelihood of bacterial infection, which is a known source of sialic acid excretion. In the present study, the reduction in viscosity with both enzyme and



of the ileum and caecum and a lower microbial load will also reduce endogenous losses. Reduction in the abundance of bacteria in the lower GIT will also reduce the likelihood of bacterial infection, which is a known source of sialic acid excretion. In the present study, the reduction in viscosity with both enzyme and heat treatment may explain the reduction in the excretion of sialic acid. Increased chyme viscosity is known to stimulate an increase in sialic acid loss from cells through an increase in mucoprotein secretion (Larsen *et al.*, 1993).

Thus, NSPs do not simply act as fibrous diluents but can have potent anti-nutritive effects. Economic implications associated with feeding diets containing even low levels (< 50 g kg<sup>-1</sup>) of NSPs to poultry are large. Improved knowledge of lupin NSPs will lead to more specific targeting of diets with exogenous enzymes. This will encourage the use of lupin seeds as a source of protein in poultry diets, reduce pollution, and lead to savings for growers of chickens, especially in a commercial environment.

## Acknowledgements

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## Chapter 47

# Lectin Histochemical Study of Lipopigments Present in the Cerebellum of *Solanum fastigiatum* var. *fastigiatum* Intoxicated Cattle

F.B. Paulovich<sup>1</sup>, E.L. Portiansky<sup>1</sup>, E.J. Gimeno<sup>1</sup>, A.L. Schild<sup>2</sup>, M. del C. Méndez<sup>2</sup> and F. Riet-Correa<sup>2</sup>

<sup>1</sup>*School of Veterinary Sciences, PO Box 296, 1900 La Plata, Argentina;*

<sup>2</sup>*Regional Diagnostic Laboratory, Veterinary Faculty, Pelotas, Brazil*

### Introduction

*Solanum fastigiatum* var. *fastigiatum* intoxication affects bovine livestock causing a cerebellar dysfunction characterized by transient nervous signs with locomotion incoordination, nystagmus, opisthotonus and a falling in dorsal or lateral decubitus with muscular tremors (Riet-Correa *et al.*, 1983).

The initial lesions are characterized by vacuolization of Purkinje cells and the presence of axonal spheroids in the granular layer and cerebellar white matter. Later, the Purkinje cells disappear and are replaced by astroglial cells. Electron microscopy studies showed that the vacuoles found at the perycarion of Purkinje cells correspond to lipidic inclusions, which are similar to those found in hereditary neuropilidosis. These inclusions seem to derive from the endoplasmic reticulum (Barros *et al.*, 1987).

Many studies performed on neuropilidosis have demonstrated that, in addition to the lipid storage, there is an abnormal accumulation of oligosaccharides (Tsay and Dawson, 1976; Barker *et al.*, 1985; Jolly and Walkley, 1997). Although the PAS (periodic acid-Schiff) staining technique can identify the presence of carbohydrates, the use of lectins allows the characterization of sugars in the histological slides with high specificity (Alroy *et al.*, 1986, 1988; De Maria *et al.*, 1998).

Lectins are carbohydrate-binding proteins and glycoproteins which can be used to identify and localize specific carbohydrate residues. Their histochemical value resides in their ability to locate, identify and distinguish tissue carbohydrates with high sensitivity and specificity (Leathem, 1986; Damjanov, 1987). This method has been used to identify accumulated carbohydrate residues in hereditary and acquired glycoprotein storage diseases (Alroy *et al.*, 1984, 1985; Driemeier *et al.*, 2000). Knowing that *S. fastigiatum* intoxication induces abnormal lipid storage in cerebellar Purkinje cells, we tried to identify

carbohydrates associated with them, as well as their specificity by using lectin histochemical techniques.

## Materials and Methods

For the experimental reproduction of the illness, *S. fastigiatum* var. *fastigiatum* was gathered directly from farms where the intoxication occurs. The dried ground plant was administered to two 1-year-old cattle by a ruminal cannula. The animals received 5 g kg<sup>-1</sup> bodyweight daily, 5 days a week, during periods of 107 and 140 days, respectively. Weekly control tests were carried out to determine cerebellar signs by the head raising test (Pienaar *et al.*, 1976). At the end of the study, unconsciousness was produced by physical stunning before the animals were bled to death while simultaneously perfusing the central nervous system (CNS) with physiologic solution and buffered formaldehyde 10%. The head was then immersed in 10% formalin during 48 hours when the CNS was extracted. Sampling was also conducted in one normal animal and tissues processed as controls. For the histological study, transverse sections of the cerebellum were performed. Gross sections were embedded in paraffin, cut at 6 µm and stained with haematoxylin-eosin (Zambrano *et al.*, 1985).

Tissue slides were deparaffinized with xylol and hydrated with graded ethanol. The lectin histochemical procedure has been previously described (Driemeier *et al.*, 2000). Table 47.1 lists the lectins used in this study, their acronyms and their major sugar specificity. The lectins were commercially obtained (Vector, Burlingame, CA, USA).

**Table 47.1.** Lectins as used in the histochemical studies.

Lectins	Acronym	Specificity <sup>a</sup>
<i>Concanavalia ensiformis</i>	Con-A	α-D-Man; α-D-Glc
<i>Glycine max</i>	SBA	α-D-GalNAc; β-D- GalNAc; α and β-Gal
<i>Dolichos biflorus</i>	DBA	α-D-GalNAc
<i>Ulex europaeus-l</i>	UEA-1	α-L-Fuc
<i>Triticum vulgare</i>	WGA	β-D-GlcNac >> NeuNac
Succinyl-WGA	sWGA	(β-(1-4)-D- GlcNac) <sup>2</sup>
<i>Arachis hypogaea</i>	PNA	β-D- Gal(1-3) GalNAc
<i>Ricinus communis-l</i>	RCA-I	β-D- Gal > α-D-Gal
<i>Bandeirea simplicifolia</i>	BS-I	α-D-Gal

<sup>a</sup>Goldstein and Hayes (1978). Fuc, Fucose; Gal, Galactose; GalNAc, N-acetyl-galactosamine; Glc, Glucose; GlcNac, N-acetyl-glucosamine; Man, Mannose; NeuNac, N-acetyl-neuraminic acid (sialic acid).

## Results

Histological studies revealed vacuolization of the Purkinje cells irregularly distributed in the treated animals. At higher magnification, a fine granular material was observed in the vacuoles. The distribution of these vacuoles varied from cell to cell.

Purkinje cells showed high affinity for Con-A and sWGA. Staining was also evident for WGA, RCA-I; irregular for PNA and SBA where some cells were well stained whilst others were weakly stained. Poor to null reactivity was registered for DBA, UEA-I, BS-I (Table 47.2). Control sections were negative.

**Table 47.2.** Lectin binding intensity to Purkinje cells in poisoned and normal calves.

sWGA	WGA	UEA-I	PNA	RCA-I	SBA	DBA	Con-A	BS-I
3 (0) <sup>a</sup>	2-3 (3)	0-1 (0)	0-3 (0-2)	2-3 (0)	0-3 (0)	0-1 (0-1)	3 (0)	0 (0)

<sup>a</sup>Numbers indicate intensity of staining in a scale of 0 (no reaction) up to 3 (strong reaction). Values of the control animals are given within parentheses.

## Discussion

The specific classification for glycolipid storage diseases is impossible by means of morphological methods. The histochemical methods allow the differentiation between glycoproteins and glycolipids (Alroy *et al.*, 1985) and, in the best cases, to distinguish the lipid class (Dietzmann, 1989). By lectin histochemistry, the glycoprotein storage diseases can be classified in three groups:  $\alpha$ -mannosidosis,  $\alpha$ -fucosidosis and sialidosis (Alroy *et al.*, 1984, 1986; Driemeier *et al.*, 2000). A major difference between the lectin staining patterns of glycolipid and glycoprotein storage diseases is that in the latter, only a few lectins stained the abnormal stored oligosaccharides (Alroy *et al.*, 1984, 1985). In the glycoprotein storage diseases, the specific sugar residues at the end of accumulated oligosaccharides are known and therefore the lectin binding pattern is easily understood. In glycolipid storage diseases, a greater variety of lectin binding sites are present (Alroy *et al.*, 1985).

In the *S. fastigiatum* intoxication together with other *Solanum* spp. plant poisoning, the clinical signs and lesions are quite similar. The histological studies reveal a progressive vacuolization of Purkinje cells, followed by lysis and cellular loss. These cells are lately replaced by astroglial cells (Pienaar *et al.*, 1976; Riet-Correa *et al.*, 1983; Summers *et al.*, 1995; Tokarnia *et al.*, 2000).

Membranous and laminar bodies can be identified in Purkinje cells by electron microscopy. The syndrome in bovines is reminiscent of a lysosomal storage disease and the observed membranous bodies are similar to those found in the gangliosidosis (Barros *et al.*, 1987).

The use of lectins allowed the detection of sugars that strongly react with Con-A, sWGA, WGA and RCA-I. This would seem to indicate that there is a deficiency of the enzymes that act specifically upon the  $\alpha$ -D-mannose,  $\alpha$ -D-glucose,  $\beta$ -D-N-acetyl-glucosamine, N-acetyl-neuraminic acid,  $\beta$ -(1-4)-D-N-acetyl-glucosamine,  $\beta$ -D-galactose and  $\alpha$ -D-galactose, allowing their metabolites to remain bound to lipids by their terminal ends.

Correlating our results with those obtained by Alroy *et al.* (1988) in the infantile gangliosidosis GM1 of canines, it has been possible to verify that the specific reactivity of the aforementioned lectins coincides with their homologous counterparts in dog gangliosidosis. The lectin-binding pattern recently described by De Maria *et al.* (1998) in a new form of feline GM1-gangliosidosis is also coincident with our results. However, the binding profile observed in inborn bovine GM1 gangliosidosis (Alroy *et al.*, 1986) was different from our data. It is well known that variations exist in the reactivity to the lectins among different species, a reason why Alroy *et al.* (1986) express that it is difficult and inappropriate to apply the human classification of GM1-gangliosidosis (infantile, juvenile and mature forms) to animal models.

The molecules having the sugar residues reactive to Con-A, sWGA, WGA and RCA-I are unknown. These molecules could be undegraded substrates, such as N-linked oligosaccharides that accumulate due to deficient activity of the particular lysosomal hydrolase (Alroy *et al.*, 1988). However, ultrastructural evidence (Barros *et al.*, 1987) may indicate that the lipidic inclusions could be the result of the formation of complex lipids resistant to the metabolism rather than an enzymatic defect. Thus, the underlying molecular mechanism could be similar to the so-called drug-induced lipidosis (Drenckhahn and Lullmann-Rauch, 1979; Robison *et al.*, 1985; Halliwell, 1997). As far as we know, no lectin binding studies have been conducted in this kind of lipidosis. The material produced by modifications of endoplasmic reticulum could become later on included in lysosomes as occurred in drug induced lipidosis (Drenckhahn and Lullmann-Rauch, 1979; Robison *et al.*, 1985; Barros *et al.*, 1987). Such a relationship has already been established in cases of Niemann-Pick disease (Wallace *et al.*, 1966). Lectin binding on that disease has been reported only in cats (Kamiya *et al.*, 1991). Our results are partially coincident with this study.

The stored material present in *S. fastigiatum* intoxicated animals reacted with four different lectins with a broad range of specificity. This feature seems to confirm that this toxic plant causes a glycolipid storage disease. Further studies would be necessary to fully characterize the disease.

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## Chapter 48

# ***Ipomoea carnea* Induced Enhanced Macrophage Activity (Phagocytosis and Peroxide Production) in Immunocompetent Rats**

I.M. Hueza<sup>1</sup>, E.S.M. Fonseca<sup>1</sup>, C.A. Paulino<sup>2</sup>, M. Haraguchi<sup>3</sup> and S.L. Gómiak<sup>1</sup>

<sup>1</sup>Research Center for Veterinary Toxicology (CEPTOX), Department of Pathology, School of Veterinary Medicine, University of São Paulo, Pirassununga, Brazil; <sup>2</sup>University Bandeirante of São Paulo, São Paulo, Brazil; <sup>3</sup>Biological Institute of São Paulo, São Paulo, Brazil

### **Introduction**

*Ipomoea carnea* (formerly *Ipomoea fistulosa*) is a plant found throughout most regions of Brazil (Hoehne, 1939) and other tropical countries. This plant is an evergreen that blooms throughout the year. Natural intoxication occurs when different animal species such as cattle, sheep and mainly goats chronically ingest the plant (Hoehne, 1939). Such intoxication is clinically characterized by inappetence, soft faeces and weight loss, and by central nervous system signs such as head shaking, hyperesthesia and death (De Balogh *et al.*, 1999).

The toxic principles have been recently identified by De Balogh *et al.* (1999) as three alkaloids, calystegines B2 and C1 and swainsonine (SW). Calystegines B2 and C1 are powerful glycosidase inhibitors affecting  $\beta$ -glucosidase and  $\alpha$ - and  $\beta$ -galactosidase. Inhibition of these glucosidases may produce phenocopies of human genetic lysosomal storage diseases (Dorling, 1984). SW is a potent inhibitor of lysosomal  $\alpha$ -mannosidase that promotes lysosomal accumulation of incompletely processed oligosaccharides, loss of cellular function and ultimately cell death (Tulsiani *et al.*, 1988). SW also inhibits Golgi mannosidase II, which is involved in N-linked glycoprotein processing (Elbein, 1989). The resulting alteration in the synthesis, processing and transport of glycoproteins causes alteration and dysfunction of cell adhesion molecules, circulating hormones and various membrane receptors. This effect is seen clinically as abnormal embryogenesis, endocrine and gastrointestinal function, and alteration of the immune system.

The modification resulting from glycoprotein metabolism has received much attention because of its potential immunomodulatory properties. Indeed, in

studies involving field observation SW was reported to cause immunodeficiency in animals exposed to plants containing this compound (Stegelmeier *et al.*, 1998) and susceptibility to the occurrence of pneumonia, foot rot or 'pink eye' (Sharma *et al.*, 1984), experimental studies conducted *in vitro* showed that SW has potential immunomodulatory properties such as increasing murine splenic natural-killer (NK) activity (Humphries *et al.*, 1988), enhancing the generation of lymphokine-activated-killer (LAK)-cell activity (Bowlin and Sunkara, 1988), increasing human large granular lymphocytes cytotoxicity against NK-resident colon carcinoma cells (Yagita and Saksela, 1990) and activating resident tissue macrophages (Das *et al.*, 1995).

Thus, the purpose of the present study was to assess *in vivo* the effect of *I. carnea* on macrophage activity of rats, specifically macrophage spreading, phagocytosis and H<sub>2</sub>O<sub>2</sub> production by peritoneal cells after lipopolysaccharides (LPS) activation.

## Materials and Methods

*Ipomoea carnea* leaves collected from the Research Centre for Veterinary Toxicology (CEPTOX), University of São Paulo (USP) – Pirassununga, Brazil, in May 2000 were submitted to maceration to obtain a 96% ethanol extract. After total solvent evaporation under reduced pressure at 50° C, a dark green fraction was obtained, which was suspended in water to remove the waxy residue and consecutively fractionated with n-butanol saturated with water. The remaining aqueous solution was lyophilized to give the aqueous fraction (alc), which, by previous assay, revealed the presence of the active principles.

### Macrophage activity

Forty-two Wistar rats, from our own colony, weighing 150 to 200 g, about 60 days of age, were divided at random into six groups: two control groups (C1 and C2) and four experimental groups (A1, B1, A2 and B2) of seven animals each. The animals were housed in temperature-controlled (24-26° C) and artificially lit rooms on a 12-h light/12-h dark cycle (lights on at 7:00 a.m.) with free access to food and water, and used in accordance with the guidelines of the National Research Council, USA.

Animals in the experimental groups were treated orally with alc diluted in drinking water in order to obtain the target daily doses of dry leaves of 3.0 g kg<sup>-1</sup> bodyweight (A1 and A2 groups) and 15.0 g kg<sup>-1</sup> bodyweight (B1 and B2 groups) administered for 14 days (A1 and B1 groups) and 21 days (A2 and B2 groups). Control groups received only tap water during the same period. The drinking water of all animals was prepared daily and its consumption was also measured. Twenty-four hours before sacrifice, the rats received an intraperitoneal (i.p.) injection of LPS (1.0 mg ml<sup>-1</sup>) in order to promote peritoneal macrophage

activation. The macrophage activity was evaluated using the protocols described below.

### *Spreading and phagocytosis*

The methods used to study macrophage spreading and phagocytosis were based on those described by Rabinovitch and De Stefano (1973) with modifications introduced by Paseti (1993). Briefly, a total of 200 cells per slide were counted for each rat and the macrophage spreading index (SI) and phagocytosis index (PI) were calculated as follows: SI or PI = number of spreading macrophages or phagocytic activity  $\times$  100  $\div$  200 adherent cells counted, i.e. SI = percentage of spreading macrophages and PI = percentage of macrophages with phagocytized zymosan particles.

The mean of four counts obtained from the two slides of each rat was used to express the SI or PI.

### *H<sub>2</sub>O<sub>2</sub> release*

Spontaneous and phorbol myristate-acetate solution (PMA)-induced H<sub>2</sub>O<sub>2</sub> release by macrophages was measured by the method of Russo *et al.* (1989). H<sub>2</sub>O<sub>2</sub> concentration was calculated from absorbance measurements, as described by Pick and Mizel (1981).

Spontaneous and PMA-induced H<sub>2</sub>O<sub>2</sub> production experiments were repeated four times for each rat in each group and the mean value of the four counts was used to determine H<sub>2</sub>O<sub>2</sub> concentration.

All data are reported as mean  $\pm$  SEM. Experimental and control data were analysed statistically by analysis of variance (ANOVA) and by the Tukey-Kramer test, with the level of significance set at  $P < 0.05$ .

## **Results**

### **Daily consumption of alc**

The mean consumption of alc by animals of the experimental groups A1 and B1 was  $2.38 \pm 0.20$  and  $7.65 \pm 0.99$  g kg<sup>-1</sup> bodyweight daily, respectively, and the mean consumption of alc by animals of the experimental groups A2 and B2 was  $1.90 \pm 0.27$  and  $10.2 \pm 1.64$  g kg<sup>-1</sup> bodyweight daily, respectively.

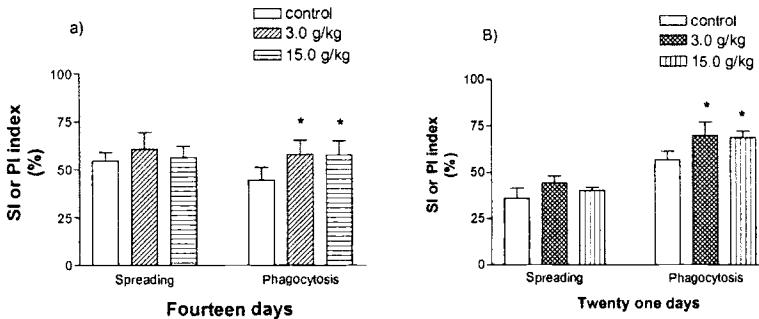
### **Effect of alc administration on macrophage spreading and phagocytosis activity**

The spreading activity of peritoneal macrophages from animals of the experimental groups was not affected by the toxic principles of *I. carnea* when compared with that of control animals ( $P > 0.05$ ). On the other hand, alc

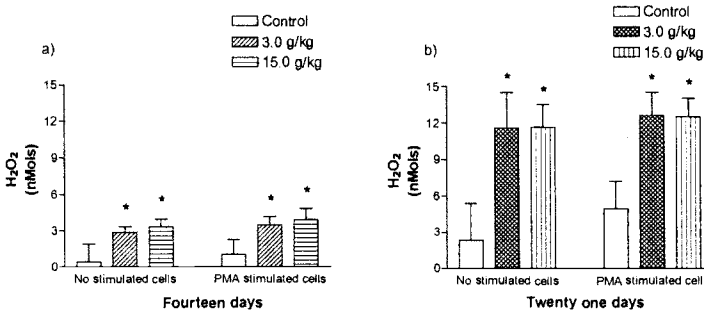
administration increased macrophage phagocytosis of all animals of the different experimental groups ( $P < 0.05$ ) compared with control (Fig. 48.1).

### Effect of alc administration on spontaneous and induced $H_2O_2$ release by intraperitoneal macrophages

Administration of aqueous fraction of *I. carnea* (alc) for 14 and 21 days increased the spontaneous  $H_2O_2$  release by intraperitoneal macrophages in all experimental groups ( $P < 0.05$ ) when compared with untreated controls, the same was observed when the intraperitoneal macrophages were induced to release  $H_2O_2$  by PMA ( $P < 0.05$ ) (Fig. 48.2).



**Fig. 48.1.** Effect of administration of aqueous fraction of *I. carnea* (alc) on macrophage spreading and phagocytosis activity of rats treated with the target doses of 3.0 (A1) and 15.0 (B1)  $g\ kg^{-1}$  bodyweight daily for 14 days (a); and treated with the target doses of 3.0 (A2) and 15.0 (B2)  $g\ kg^{-1}$  bodyweight daily for 21 days (b). Results represent means  $\pm$  SEM. \*Significance from control ( $P < 0.05$ ).



**Fig. 48.2.** Effect of administration of the aqueous fraction of *I. carnea* (alc) on spontaneous and induced  $H_2O_2$  release by intraperitoneal macrophages of rats treated with the target doses of 3.0 (A1) and 15.0 (B1)  $g\ kg^{-1}$  bodyweight daily for 14 days (a); and treated with the target doses of 3.0 (A2) and 15.0 (B2)  $g\ kg^{-1}$  bodyweight daily for 21 days (b). Results represent means  $\pm$  SEM. \*Significance from control ( $P < 0.05$ ).

## Discussion

The present study clearly shows for the first time in an *in vivo* experiment that the *I. carnea* aqueous fraction (alc) had stimulatory effects on peritoneal macrophage activity in rats, with the occurrence of enhanced phagocytosis and peroxide production with or without PMA stimulation.

It is known that the immune responses are largely regulated by cell-surface and secreted glycoproteins. In addition, interaction between sugars and lectins might be functionally involved in immune recognition and activation, including those receptors involved in phagocytosis (Linehan *et al.*, 2000). Thus, one explanation for the enhanced phagocytosis activity of macrophages observed here could be the alteration of the synthesis and processing of a hybrid type of oligosaccharide caused by SW. Further supporting this hypothesis was the observation that peritoneal macrophages of rats treated with *I. carnea* showed enhanced peroxide production of the same intensity as that observed when PMA was added. It is known that PMA increases peroxide production acting directly on protein kinase C (PKC). PKC participation can be involved in the transduction of phagocytic signals generated by various receptors (Kwiatkowska and Sobota, 1999). Since Breton *et al.* (1990) suggested that SW indirectly mediated the same event as that induced by PMA in the modulation of PKC activity, we may propose that receptor alterations promoted by SW can be responsible for the enhanced activation of these effector cells.

Thus, the results obtained here show that *in vivo* treatment with *I. carnea* enhances peritoneal macrophage activity, in agreement with previous *in vitro* studies. However, future experiments are needed to better understand the discrepancies between laboratory and field data.

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## Chapter 49

# Advanced Multidimensional NMR Experiments as Tools for Structure Determination of Amaryllidaceae Alkaloids

P. Forgo<sup>1</sup>, J. Hohmann<sup>2</sup>, G. Dombi<sup>3</sup> and I. Máthé<sup>2</sup>

<sup>1</sup>Department of Organic Chemistry, University of Szeged, Hungary; <sup>2</sup>Department of Pharmacognosy, University of Szeged, Hungary; <sup>3</sup>Department of Pharmaceutical Analysis, University of Szeged, Hungary

### Introduction

Plants of the family Amaryllidaceae are known to produce structurally unique, tyrosine-derived alkaloids with high physiological effects. Many alkaloids are relatively toxic and accumulate mainly in the bulbs of the plants. Amaryllidaceae species are often grown for their ornamental qualities in parks and gardens. Accidental human poisoning has occurred as a result of confusion between the bulbs of edible Liliaceae (onion, shallots, etc.) and those of the garden Amaryllidaceae (daffodil, snowdrop, amaryllis).

The present study focused on *Ismene festalis* and *Sprekelia formosissima*, both species are native to Middle and South America and have been cultivated in Europe as ornamental plants. Previously, the chemical constituents of these species have not been investigated in detail, thus we aimed to isolate and structurally characterize their alkaloids. The structure determination was carried out by extensive NMR studies using advanced experiments. Scalar and dipolar interactions were monitored between spins by the application of homonuclear  $z$ -gradient enhanced two-dimensional NMR experiments (PFG-COSY, PFG-TOCSY and NOESY). Heteronuclear gradient enhanced inverse detected two-dimensional experiments (PFG-HSQC, PFG-HMBC) were applied to establish unambiguous assignment of the  $^{13}\text{C}$  spectra. The isolated alkaloids belong to a moderate sized class of natural compounds incorporating only one nitrogen atom. Heteronuclear  $^{15}\text{N}$  spectroscopy provides useful data about the solution structure, because the  $^{15}\text{N}$  chemical shift is very sensitive to the chemical environment. Moreover, long range  $^1\text{H}$ - $^{15}\text{N}$  coupling constants offer valuable information about the conformation of the dissolved molecule. Since the amount of available sample was limited to a few milligrams, direct  $^{15}\text{N}$  detection was not possible. The  $^{15}\text{N}$  chemical shifts were extracted from inverse detected two-dimensional gradient enhanced HSQC experiments.

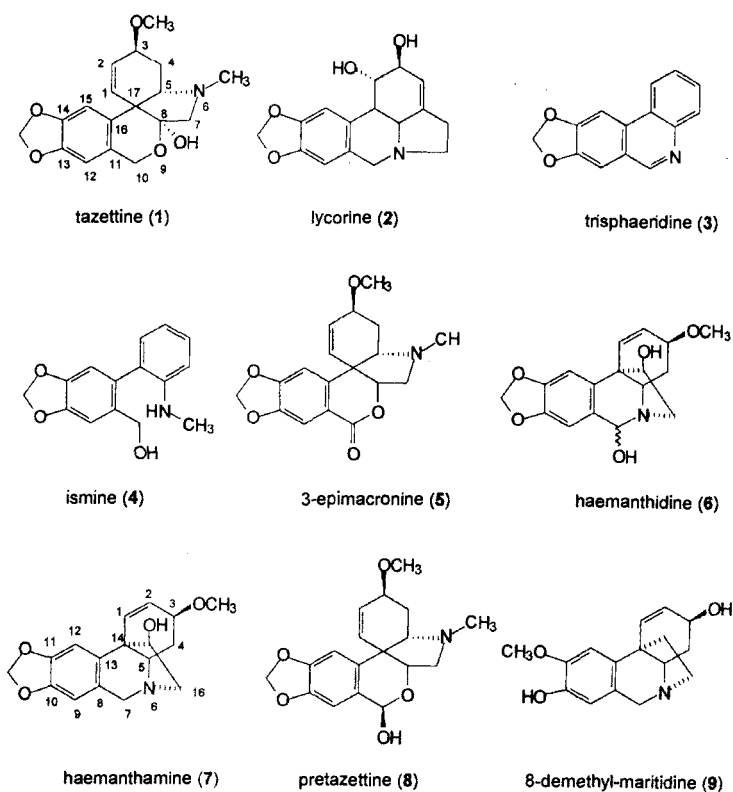


Fig. 49.1. Alkaloids isolated from *Ismene festalis* (1–5) and from *Sprekelia formosissima* (1, 6–9).

## Isolation

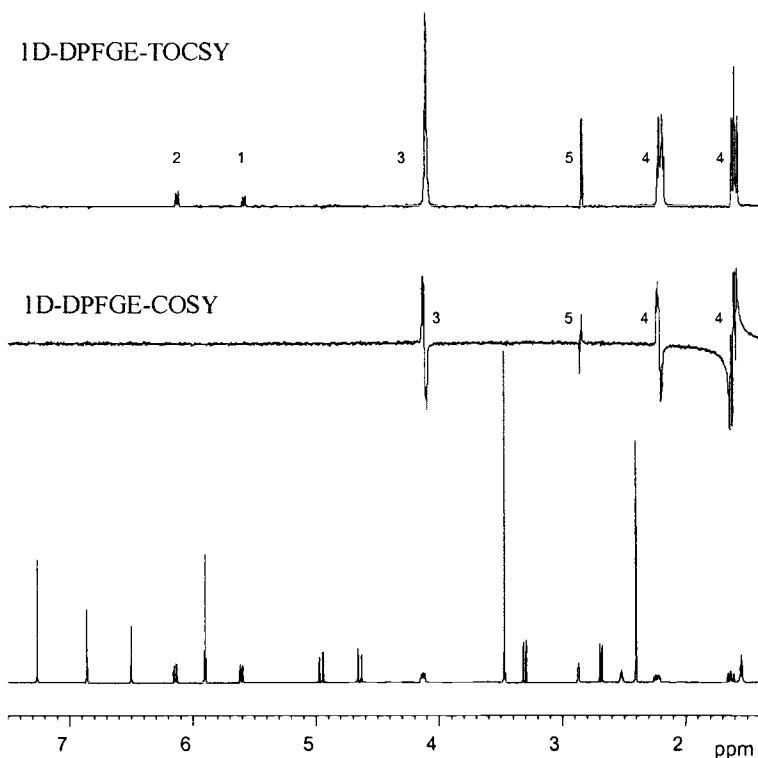
The plant materials, which were grown in floriculture in Szeged, were extracted with methanol. The alkaloid-containing fraction, obtained with standard procedure of alkaloid isolation, was chromatographed on silica gel column and then on preparative TLC plates yielding five pure compounds from *I. festalis* (1–5) and five from *S. formosissima* (1, 6–9) (Fig. 49.1). The isolation process allowed an efficient and simple extraction of pharmacologically interesting alkaloids.



## Structure Determination

Since the isolated alkaloids belong to the class of moderate sized compounds, their NMR spectra exhibit well-resolved signals (bottom on Fig. 49.2). These isolated multiplets are ideal targets in selective experiments providing scalar or dipolar coupling information only for the selected spin. A double pulsed field gradient spin-echo (DPFGSE) sequence (Stott *et al.*, 1995) was used in one-dimensional COSY (Hurd, 1990; Berger, 1997) and TOCSY (Kövéér *et al.*, 1998) experiments to identify these interactions (middle and top on Fig. 49.2).

Multiplicity edited PFG-HSQC experiments (Willker *et al.*, 1993) were used to assign the  $^{13}\text{C}$  chemical shifts in the model compounds (Fig. 49.3). This experiment provided information of the identity of the functional group besides the chemical shift information. The methylene signals have a  $180^\circ$  phase shift relative to  $^{13}\text{C}$  resonances in CH and  $\text{CH}_3$  groups.



**Fig. 49.2.**  $^1\text{H}$ -NMR spectrum (bottom), 1D-COSY (middle) and 1D-TOCSY (top) of 1.

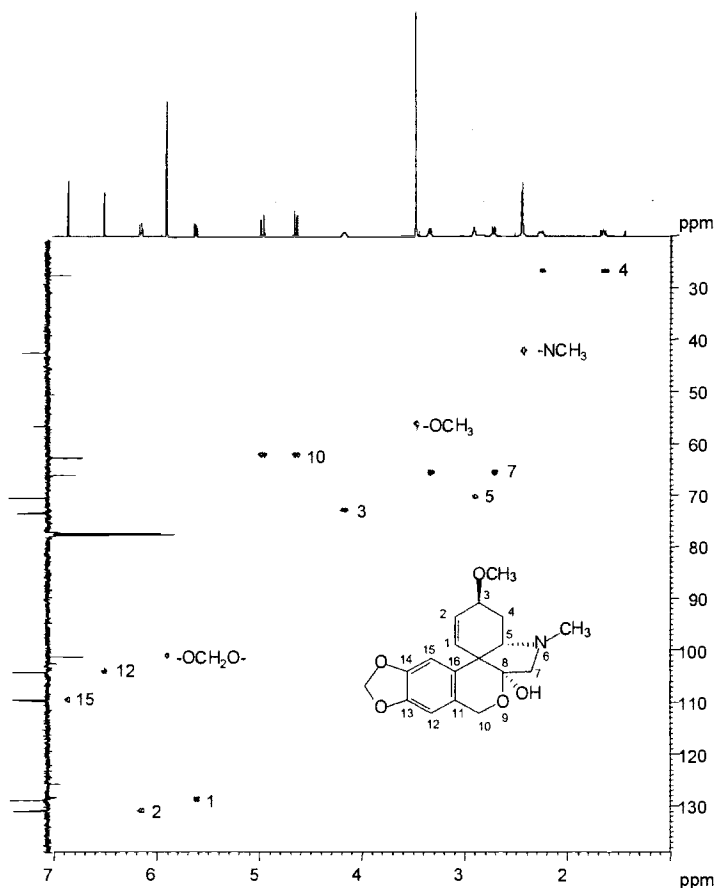


Fig. 49.3. Multiplicity edited PFG-HSQC spectrum of 1.

The isolated alkaloids contain only one nitrogen atom, the chemical shift of the  $^{15}\text{N}$  cannot be obtained from direct detection of the  $^{15}\text{N}$  spectrum, mainly because of the limited amount of the samples available. However, inverse ( $^1\text{H}$ ) detection provides an excellent solution through its high sensitivity. A recently developed two-dimensional experiment (PFG-HSQMBC) (Williamson *et al.*, 2000) was used to obtain  $^{15}\text{N}$  chemical shifts of the model compounds (Fig. 49.4). This experiment uses the modulation of heteronuclear single-quantum coherence during the incremented delay providing pure absorptive anti-phase signals in the spectrum with respect to the active heteronuclear coupling. Using the method described above  $^{15}\text{N}$  chemical shifts were extracted for 1, 2, 6, 7 and 8. The applied NMR methods provided the complete spectral assignments of the isolated model compounds. Tabulated chemical shift values for two representative compounds are given in Table 49.1.

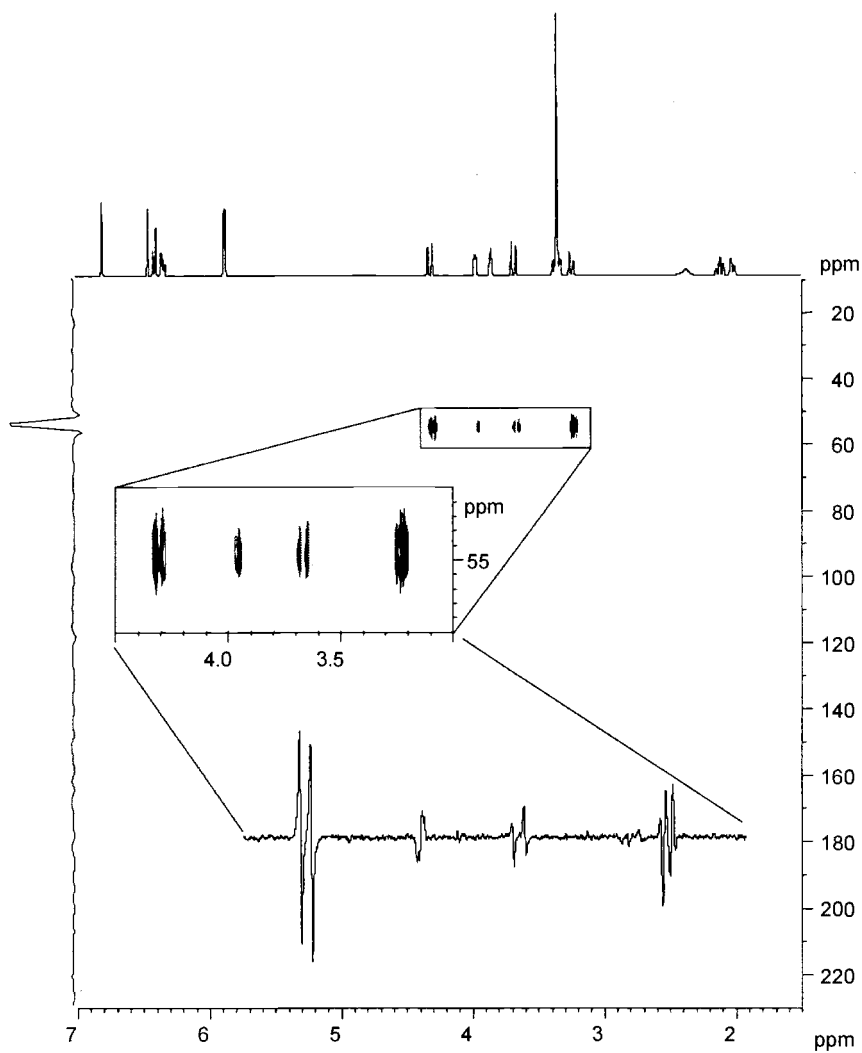


Fig. 49.4.  $^1\text{H}$ - $^{15}\text{N}$  PFG-HSQMBC spectrum of 1.

**Table 49.1.**  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  chemical shifts for compounds 1 and 7.

	1		7	
	$^1\text{H}$ (ppm)	$^{13}\text{C}/^{15}\text{N}$ (ppm)	$^1\text{H}$ (ppm)	$^{13}\text{C}/^{15}\text{N}$ (ppm)
1	5.6	128.7	6.42	127.3
2	6.14	130.7	6.36	132.1
3	4.12	72.9	3.86	72.8
4	2.23; 1.62	26.8	2.11; 2.01	28.2
5	2.86	70.0	3.34	62.7
6	-	31.3	-	55.1
7	3.30; 2.68	65.7	4.32; 3.68	63.2
8	-	102.2	-	126.9
9	-	-	6.46	106.8
10	4.95; 4.63	62.1	-	146.2
11	-	125.5	-	146.5
12	6.50	104.0	6.82	103.3
13	-	146.0	-	135.4
14	-	146.7	-	50.2
15	6.86	109.4	3.98	80.2
16	-	128.1	3.37; 3.24	61.4
17	-	50.1	-	-
-OMe	3.46	56.1	3.36	56.5
-OCH <sub>2</sub> O-	5.90	101.0	5.88	100.8
-NMe	2.40	41.9	-	-

## Acknowledgements

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## Chapter 50

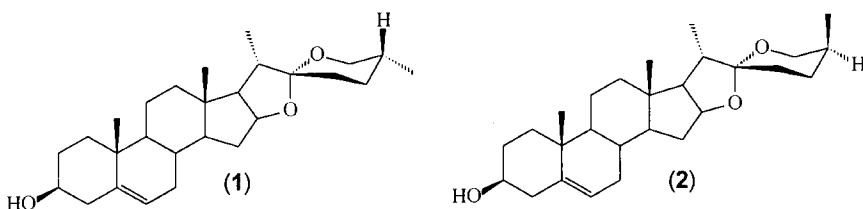
# Characterization of Steroidal Sapogenins in *Panicum virgatum* L. (Switchgrass)

S.T. Lee<sup>1</sup>, B.L. Stegelmeier<sup>1</sup>, D.R. Gardner<sup>1</sup> and K.P. Vogel<sup>2</sup>

<sup>1</sup>Poisonous Plant Research Laboratory, ARS, USDA, Logan, UT, USA; <sup>2</sup>Wheat, Sorghum and Forage Research Unit, ARS, USDA, Lincoln, NE, USA

Many plants of the *Panicum* genus have been reported to cause hepatogenous photosensitization in animals throughout the world (Flåøyen, 2000).

Glycosidic steroidal saponins have been found in some species of the *Panicum* genus and these compounds have been suggested as one of the primary agents causing hepatogenous photosensitization in animals grazing these grasses (Patamalai *et al.*, 1990; Holland *et al.*, 1991; Miles *et al.*, 1992). The hydrolysis of the saponins isolated from plant material yields sapogenins. The sapogenin diosgenin (1) has been identified in *Panicum dichotomiflorum* and in *Panicum schinzii* (Holland *et al.*, 1991; Miles *et al.*, 1992), while diosgenin (1) and an isomer, yamogenin (2), have been isolated from *Panicum coloratum* (Patamalai *et al.*, 1990) (Fig. 50.1).



**Fig. 50.1** Chemical structures of diosgenin (1) and yamogenin (2).

In 1992, Puoli *et al.* (1992) reported hepatogenous photosensitization in lambs grazing switchgrass (*Panicum virgatum* L. cv. Cave-in-Rock). In the summer of 2000, poisoning of horses grazing a pasture of switchgrass (*Panicum virgatum* L.) in eastern Nebraska was reported to our laboratory. The purpose of this study was to determine if saponins and their hydrolysis products, sapogenins, were present in the Cave-in-Rock cultivar switchgrass that was reported to cause photosensitization in lambs and in the switchgrass from the pasture where the horses were poisoned. We also analysed a sample of Kleingrass 75 (*P. coloratum* L.), previously implicated in a photosensitization case.

## Materials and Methods

### Materials

Switchgrass sample 1 (*P. virgatum* L. cv. Cave-in-Rock) was harvested at the seed head stage by Kenneth P. Vogel from research fields at the University of Nebraska, Agricultural Research and Development Center, located about 50 km west of Omaha. Switchgrass sample 2 was provided by Ted Forke, Lincoln, Nebraska and was harvested at the seed head stage from a pasture in Lancaster County, Nebraska where the liver problems with horses occurred. It was identified as *P. virgatum* L. by the Intermountain Herbarium (voucher #230944), Utah State University, Logan, UT. Kleingrass 75 (*P. coloratum* L.) was provided by Byron L. Burson (USDA, ARS, Southern Crops Research Laboratory, College Station, Texas).

Diosgenin was purchased from Steraloids Inc. (Newport, RI). *N,O*-bis(Trimethylsilyl)trifluoroacetamide silylation reagent was purchased from Pierce Chemical Co. (Rockford, IL).

### Saponin isolation and hydrolysis

All grass samples were dried and ground. Saponins were extracted and hydrolysed to sapogenins using the methods of Patamalai *et al.* (1990) and Wall *et al.* (1952).

### Gas chromatography-mass spectrometry

Sapogenin residues and the diosgenin standard were derivatized with *N,O*-bis(Trimethylsilyl)trifluoroacetamide silylation reagent using standard silylation methods.

Gas chromatography-mass spectrometry was performed on a Finnigan GCQ system (Finnigan, Austin, TX). The gas chromatography column was a J&W Scientific (Folsom, CA) DB-5MS Capillary Column (30 m, 0.25 mm I.D., 0.25  $\mu$ m film). Manual injections of 2.0  $\mu$ l were made. The GC temperature for analysis was 100°C for 1 min, then 40°C min<sup>-1</sup> to 250°C followed by 10°C min<sup>-1</sup> to 320°C, and held at 320°C for 5 min for a total analysis time of 16.75 min. Under these conditions the TMS derivative of a diosgenin (1) standard eluted at 13.02 min.

Diosgenin-TMS: EI-MS (70 eV), *m/z* (%) 486 (0.07, [M]<sup>+</sup>), 414 (0.79), 372 (8.26), 343 (9.01), 282 (100), 267 (33.16), 253 (22.25), 187 (18.78).

### Flow injection mass spectrometry

Flow injection atmospheric pressure chemical ionization (APCI) mass spectral data were acquired on a Finnigan LCQ Mass Spectrometer from Finnigan Corporation (San Jose, CA). Samples were loop injected (20  $\mu$ l) into the APCI

source in a 50:50 methanol:20 mM ammonium acetate solution at a flow rate of 0.5 ml min<sup>-1</sup>.

Ionization was achieved using an APCI source vaporizer temperature of 450°C and corona discharge current of 5 mamps. The inlet capillary temperature and voltage were 200°C and 16V, respectively. Full scan mass data were collected for a mass range of 100–2000 amu. MS<sup>n</sup> product ion spectra were collected after isolation of the target ion ( $\pm 5$  amu) and the relative collision energy manually adjusted to observe significant fragmentation of the selected ion.

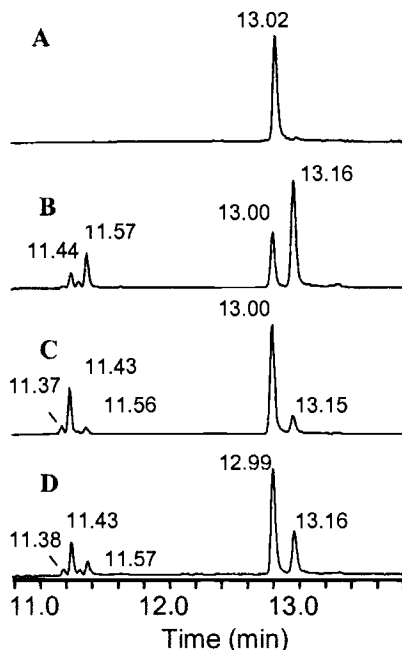
## Results and Discussion

The trimethylsilylated sapogenin residues and the diosgenin standard were analysed by the capillary gas chromatography-mass spectrometry (GC-MS) method described. Figure 50.2 shows the reconstructed ion chromatograms for the diosgenin standard, kleingrass sapogenin residue and switchgrass sapogenin residues. The kleingrass and switchgrass samples all contained several peaks that eluted in two regions (11.37–11.57 min and 12.99–13.16 min). The peaks eluting in these regions all had essentially the same EI-MS fragmentation patterns to the standard diosgenin (Materials and Methods). Diosgenin (1) was identified as the major sapogenin in both switchgrass samples. We speculate that the major peak in the kleingrass sample, eluting at 13.16 min, is yamogenin (2) based on the earlier report (Patamalai *et al.*, 1990) that yamogenin (2) and diosgenin (1) were isolated from kleingrass. Diosgenin (1) and yamogenin (2) appear to be the major sapogenin components in both switchgrass and kleingrass. The chromatograms of the sapogenin residues indicate that all grass samples contain as many as four spirostadiene side products from acid catalysed dehydration reactions, with a molecular mass of 396 that elute in the retention time of 11.39–11.57 min (Bedour *et al.*, 1964; Fazli and Hardman, 1971; A.L. Wilkins, personal communication).

The non-hydrolysed saponin material was also analysed by APCI-MS in order to gain further understanding of the attached glycosidic residues. The tandem mass product ion spectrum (MS<sup>2</sup>) resulting from collision-induced dissociation of the protonated molecular ion MH<sup>+</sup> ( $m/z = 1177$ ) resulted in a detailed fragmentation pattern. The mass difference between the protonated molecular ion ( $m/z = 1177$ ) and the aglycone fragment ( $m/z = 415$ ) is 762 amu and can only be accounted for by attachment of two hexose (e.g. glucose) and three deoxyhexose (e.g. rhamnose) units.

Sequential losses of the glycosidic units were also observed (Fig. 50.3A). MS<sup>2</sup> fragment ions at  $m/z = 1031$  and 1015 indicate losses of a terminal deoxyhexose (MH<sup>+</sup> - 142) and a terminal hexose (MH<sup>+</sup> - 162) unit. A hexose (162 amu) must be added at the alpha position to the aglycone to account

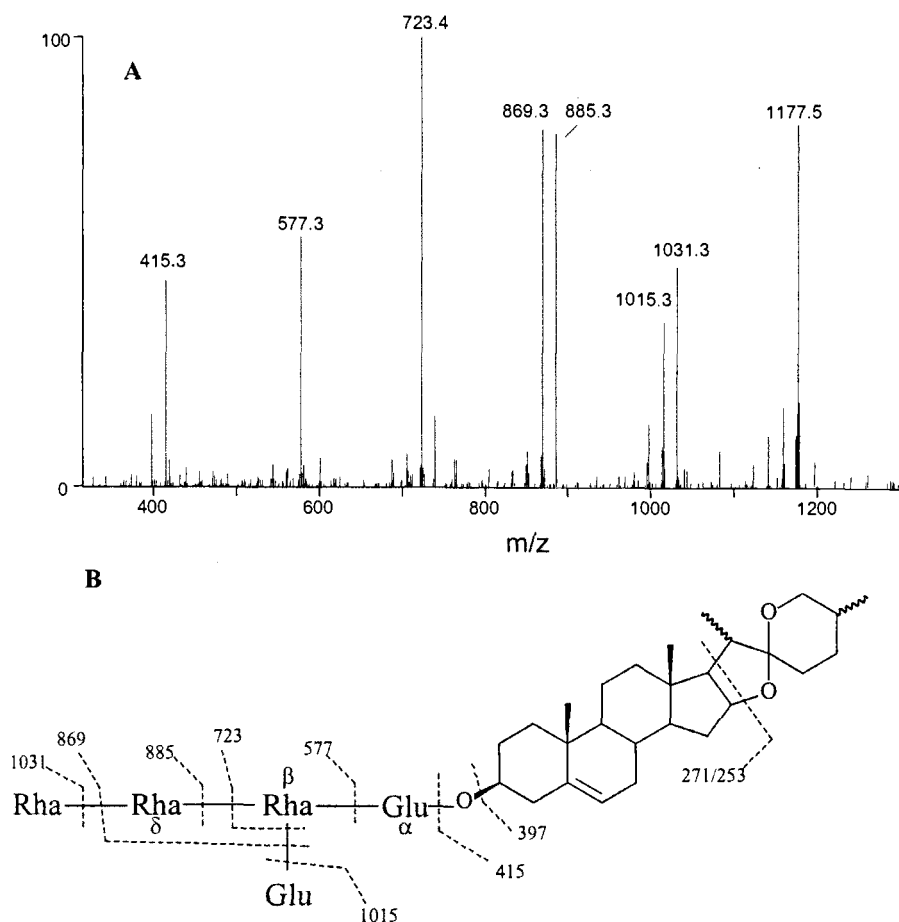




**Fig. 50.2.** Reconstructed ion chromatograms ( $m/z = 282.3$ ) for sapogenin samples. (A) Diosgenin standard. (B) Kleingrass 75 (*P. coloratum* L.). (C) Switchgrass sample 1 (*P. virgatum* L. cv Cave-in-Rock). (D) Switchgrass sample 2 (*P. virgatum* L.).

for the fragment ion at  $m/z = 577$  (diosgenin-hexose). A second deoxyhexose unit is added at the beta position accounting for the ion at  $m/z = 723$  (diosgenin-hexose-deoxyhexose). The glycosidic sequence either branches after this point or is a mixed sequence to account for the ions at  $m/z = 869$  (diosgenin-hexose-deoxyhexose-deoxyhexose) and  $m/z = 885$  (aglycone-hexose-deoxyhexose-hexose). The two remaining glycosidic moieties could then be added in an unknown sequence (Fig. 50.3B). Direct APCI-MS of the intact saponin material is useful for determining the molecular weight of the major saponin fraction, and some useful structural information can be deduced from the fragmentation pattern. However the APCI-MS data cannot differentiate between the possible anomeric glycoside residues or their exact linkage (Fang *et al.*, 1999).

The kleingrass and both switchgrass samples have similar APCI mass spectra for the non-hydrolysed saponin material. However, the differences between the kleingrass and switchgrass samples result from the relative ratios of the isomeric steroidal aglycone compounds and not necessarily in the arrangement of the hexose and deoxyhexose sugar moieties.



**Fig. 50.3.** (A) APCI-MS<sup>2</sup> product ion spectrum ( $MH^+ = 1177$ ) of isolated saponin material from switchgrass sample 2 (*P. virgatum* L). (B) Proposed structure of major saponin from switchgrass and kleingrass plant material and plausible APCI ion fragments.

In this study we showed the presence of steroidal saponins in two samples of switchgrass that have been implicated in hepatotoxicosis in sheep and horses. After hydrolysis of the glycosides, diosgenin (1) was determined to be the major sapogenin in both switchgrass samples. We confirmed the presence of diosgenin in a kleingrass sample after acid hydrolysis of the glycosides as well as an additional steroidal isomer which we speculate is yamogenin (2). The non-hydrolysed saponin components were shown to be glycosides of diosgenin,

yamogenin, and other isomeric steroidal aglycones with the glycoside portion being composed of two hexose and three deoxyhexose sugar units.

## Acknowledgements

The authors thank Lillian A. Buhler for technical assistance. We thank Ted Forke, Lincoln, NE, for the switchgrass sample 2. We also thank Byron L. Burson, USDA, ARS, Southern Crops Research Laboratory, College Station, TX for the kleingrass sample.

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## Chapter 51

# Common Natural and Experimental Plant Intoxications in Animals Reported in Cuba

E. Marrero, M. Aparicio, M.A. Figueredo, C. Bulnes, L.M. Sánchez, I. Palenzuela and R. Durand

*Centro Nacional de Sanidad Agropecuaria (CENSA), Apto 10, San José de las Lajas, La Habana, Cuba*

### Introduction

Plant toxicosis is one of the most important accidents to consider in commercial animal exploitation in the tropics where there exists an exuberance of flora and grazing areas invaded by toxic plant species. In Cuba 388 poisonous plant species have been reported, 13 of which produce serious losses in cattle and other animal species (Roig y Mesa, 1974; Alfonso *et al.*, 1998). The present work includes some of the main plants that produce serious intoxication in animals and these have also been studied from the experimental point of view.

### *Urechites lutea* L. Britton

*Urechites lutea* L. Britton belongs to the Apocynaceae family and is commonly known to farmers as 'curamaguey and bejuco marrullero'. It is monotypical in Cuba and has a low representation in other areas of tropical America (Roig y Mesa, 1974). The plant contains three cardiotoxic steroidal glycosides characterized by an unsaturated lactone ring at C17 and a hydroxyl group (OH) at C3. This compound is structurally related to cardiotonics of the digitalis family. Aglycones identified in the plant extract were gitoxigenin (PM = 390), oleandrogenin (PM = 432) and gitoxigenin monoanhydride (PM = 372). Biosynthesis of these products by the plant is highest from December to May, which is in agreement with the occurrence of disease. Consumption of *U. lutea* results in two different clinical forms of the disease: one acute, characterized by sudden death that appears in cattle on pasture under a system of intensive exploitation management; and the other a haemorrhagic syndrome episode, called by the farmers haemorrhagic diarrhoea, that occurs in forage-fed stabled animals (Marrero, 1996).

The haemorrhagic diarrhoea starts with depression, dyspnea and mucosal diarrhoea and, after some hours, the animals become anorexic with irregular arterial pulses and bradycardia. Bovines also show bloody faeces, prostration and petechiation of mucosal surfaces. There are no prodromal signs with the sudden death syndrome. The animals are apparently healthy, but when they are submitted to stress, for example 'tick bath', they fall down and die some minutes later.

In an experimental study the action of glycosides on heart activity was investigated by electrocardiographic monitoring of six cross-breed Holstein calves, 6 months of age, that received between 0.50-0.30 mg of total glycosides  $\text{kg}^{-1}$  bodyweight intravenously. All animals died following severe heart electrical conduction disturbances that ended with ventricular fibrillation (Marrero *et al.*, 1984). Once the aetiology of this intoxication was identified, elimination of the toxic plant from forage areas was recommended to farmers. Reports of these toxic accidents have diminished, but when they occur veterinarians are able to prevent further cases by controlling access to *U. lutea*.

### ***Ageratum houstonianum* Mill, *Lantana camara* L. and *Crotalaria* spp.**

These plant species result in an important hepatogenous photodermatitis in cattle and also produce intoxication in other animal species.

Hepatogenous photodermatitis cases in cattle caused by *Lantana camara* L. were first reported in Cuba in 1967 (Alfonso *et al.*, 1982).

*Ageratum houstonianum* Mill produced haemorrhagic disorders and photodermatitis in cattle when it was offered to the animals mixed with the forage (Alfonso *et al.*, 1989). The plant has blue or violet coloured flowers and it is distributed all over the island with preference in the occidental region. Intoxication is more frequent during spring and early summer. The seeds of the plant are propagated in the soil having been spread by birds, water, agricultural equipment and directly by animals (Roig y Mesa, 1974; Sánchez *et al.*, 1993).

The phytochemical study of *A. houstonianum* leaf organic extracts showed: hydrocarbons of high molecular mass; fitoesterols; a mix of acids; ethylic esthers and lipids (Aparicio, 2000), while the alcoholic extracts showed coumarinic and triterpene compounds (Sánchez *et al.*, 1993). In the histological examination of the liver from natural and experimentally intoxicated bovines, intense pycnosis of hepatocyte nucleus with evolution to karryorhexis and karryolysis including cytoplasmolysis was found, which is indicative of primary hepatic lesions caused by the natural toxin.

*Crotalaria incana* and *Crotalaria retusa* were both responsible for serious intoxication cases in different animal species (Alarcón *et al.*, 1984; Alfonso *et al.*, 1986; Figueredo, 2000). It is known that this plant species contains pyrrolizidine alkaloids that have been responsible for large scale losses of livestock (Seawright, 2000).

### ***Cynodon nlenfuensis* and *Manihot sculenta* Crantz**

These plants accumulate cyanogenic glycosides. Those from *Cynodon nlenfuensis* have been responsible for acute intoxication accidents in cattle while those from *Manihot sculenta* produced acute accidents in pigs, although this has not been important from an economical point of view.

Bovine toxicosis cases associated with *C. nlenfuensis* have been studied when this pasture was introduced in Cuba (Aguilera *et al.*, 1985a, b, 1986). In one experiment, the cyanide content of leaves and stems of this plant cultivated with three different levels of nitrogen fertilization (0, 200 and 600 kg ha<sup>-1</sup> year<sup>-1</sup>) was determined. Samples were obtained from the third to the seventh week. It was concluded that cyanide accumulation takes place preferentially in the leaves with increasing concentration according to the higher fertilization levels (Aguilera *et al.*, 1985a). Another example was referred to in an experimental study carried out over 47 weeks, in three different *C. nlenfuensis* grazing areas to establish the possible relationship between rainfall and the grass cyanide content. Seven of 169 determinations gave cyanide concentrations higher than those referred to as lethal, 200 µg kg<sup>-1</sup> green pasture ( $P < 0.05$ ). During the dry season only one sample was higher than the lethal doses, and it corresponded with a period of days of intense rainfall. In general, *C. nlenfuensis* toxicosis cases were only presented after bad weather periods, for example tropical hurricanes.

### ***Cestrum diurnum***

This plant produces calcinosis, which is a pathology that affects domestic animals, most frequently cattle, provoking a high morbidity with moderate mortality. Several aetiological agents including *Cestrum diurnum* cause this pathology. In Cuba, natural intoxication by *C. diurnum* has been observed mainly in bovines (Durand *et al.*, 1999). The active principles are steroids with a function similar to that of 1,25 dihydroxycalciferol Vit D 3, which influence all the bio-productive parameters and induce irreversible lesions caused by the mineralization of the soft tissue. In one case, 26 Holstein calves of 3 to 4 months old had a history of progressive weight loss and high mortality. Clinical inspection of the animals reflected slenderness and paleness of mucosae. Eighty per cent of the grazing areas were invaded with undesirable plants, where *C. diurnum* prevailed. Pulmonary reinforcement with a weak aortic tone was noted in 12 calves. It was interpreted as an auricle-ventricular failure, which could be attributed to a stenosis of the aorta, as well as to generalized valvular disorders corresponding in the most serious cases, with an occurrence of a negative venous pulse of the jugular. Necropsy findings indicated the presence of an abnormal deposit of substances affecting all layers of some vessels, particularly the aorta, pulmonary and renal arteries. These deposits had a brilliant whitish colour, were firm in consistency and formed an irregular surface on the above-mentioned vessels forming atheromatose plaques. This alteration was also observed in the

spleen capsule of some animals and was also observed in other cases. Microscopic examination showed severe mineralization of the internal and middle parts of the aorta and pulmonary arteries. It is well known that vitamin D causes bone demobilization. Calcium determinations in bone ash samples from the hip joint tuberosity of the animals revealed a mean value of 36.84%.

Experimental intoxication in six New Zealand rabbits supplemented with 0.25, 0.50 and 1 g each of dry and milled leaves of *C. diurnum* mixed with concentrate during 14 days showed interesting results. The calcification observed in the aorta and pulmonary arteries, heart and external part of the trachea resembled natural bovine intoxication.

### ***Cassia occidentalis (Senna occidentalis)***

A 31% morbidity occurred in a group of heifers on a cattle farm in a western region of the country (Marrero *et al.*, 1998). The most prominent clinical symptoms were decreased muscle tone, weakness and slow gait that progressed to prostration in a few days, the animals being unable to rise again. Most of the affected animals became recumbent and developed dark urine suggestive of myoglobinuria. Chemical study of the toxic principles in *Cassia occidentalis* plants obtained from the grazing areas showed the presence of triterpenes and alkaloids, which were also present in the liver and muscles of the affected animals. Thin layer chromatography of plant, liver and muscle samples revealed two spots with R<sub>f</sub>s of 0.13 and 0.84. Post-mortem examinations led to the conclusion that a toxic metabolic problem was present due to characteristic ill-defined pallor of much of the muscle mass. The important histological lesion was destruction of the skeletal muscle fibres of the affected extremities. A diffuse hyalinisation of the muscular fibres and proliferation of reticuloendothelial cells was observed.

The above mentioned plant intoxication cases were studied by carrying out multidisciplinary studies to evaluate them from epidemiological, botanical, chemical, clinical and morphopathological points of view.

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*for Humans and Animals.* National Centre for Animal and Plant Health, CENSA, Havana, Cuba.

## Chapter 52

# Intoxication by *Prosopis juliflora* Pods (Mesquite Beans) in Cattle and Goats in Northeastern Brazil

I.M. Tabosa<sup>1</sup>, F. Riet-Correa<sup>2</sup>, S.S.D. Simões<sup>1</sup>, R.M.T. Medeiros<sup>1</sup> and V.M.T. Nobre<sup>1</sup>

<sup>1</sup>Faculdade de Veterinária, Universidade Federal da Paraíba, Patos, PB, 58700-000; <sup>2</sup>Laboratório Regional de Diagnóstico, Faculdade de Veterinária, UFPel, 96010-900, Pelotas, RS

*Prosopis juliflora* (Leguminosae, Mimosaceae) was introduced to the states of Pernambuco and Rio Grande do Norte, northeastern Brazil, in 1942 and 1948, respectively, with seeds from Peru and the Sudan (Secretaria de Produção Animal, 1989). Currently it is estimated that there are approximately 150,000 ha cultivated with *P. juliflora* in northeastern Brazil, mainly in the semiarid region. It is a xerophilous tree with rapid growth, up to 8-12 m high, that produces fruits within its second or third year (Gomes, 1987). Due to their palatability and nutritional value the pods of mesquite beans or its bran are largely used for feeding dairy cattle (Nobre, 1981), beef cattle (Silva, 1981; Talpada *et al.*, 1982), sheep (Buzo *et al.*, 1972), goats (Bitú *et al.*, 1986), swine (Silva *et al.*, 1989a, b), chickens (Brandão *et al.*, 1989) and rabbits (Espíndola *et al.*, 1985) with good nutritional and economical results. It has been used also for human consumption as bread, biscuits, jellies, sweets and spirits (Souza and Tenório, 1982; Mendes, 1986). The object of this chapter is to review the intoxication by *P. juliflora* pods in ruminants in Brazil.

### Epidemiology

The intoxication by *P. juliflora* pods in cattle was reported in the USA, in the states of Hawaii (Hendershot, 1946; Adler, 1949), Texas, New Mexico and Oklahoma (Dollahite and Anthony, 1957), in Peru (Baca *et al.*, 1966) and in Brazil (Dantas, 1996; Figueiredo *et al.*, 1996).

In Brazil the disease was reported in cattle in the semiarid regions of the states of Paraíba in 1981, and Rio Grande do Norte in 1986 (Dantas, 1996; Figueiredo *et al.*, 1996). The intoxication occurs also in the state of Pernambuco. From 1992 the number of outbreaks increased, with 50% of cattle affected on some farms (Dantas, 1996).

The disease also occurs spontaneously in goats in Peru (Baca *et al.*, 1966). In Brazil the intoxication was produced experimentally in goats, but had not been observed spontaneously in this species, suggesting that cattle are more susceptible than goats to the intoxication. This was confirmed by the fact that goats need to ingest the pods for a longer period than cattle to become intoxicated (Tabosa *et al.*, 2000).

## Clinical Signs and Experimental Intoxication

Clinical signs are characteristic of a cranial nerve impairment, affecting mainly the trigeminal nuclei. Probably other cranial nerve nuclei are also involved (Tabosa *et al.*, 2000). In cattle, clinical signs, more prominent during rumination, are characterized by masseter atrophy, tongue protrusion, mandible slackening, tilting of the head during chewing, involuntary movements of the tongue and continuous licking of the nostrils, profuse salivation, yawning, and swallowing impairment. Because most affected animals hold their head to one side during chewing the disease was named in Portuguese as 'cara torta', which means twisted face. Continuous chewing, nervousness, dysphagia, ruminal atony, anemia, submandibular oedema and gradual emaciation are also observed (Dollahite and Anthony, 1957; Dantas, 1996; Figueiredo *et al.*, 1996). Some cattle, when they are not chewing, stay with the mouth slightly opened and the tongue protruded 2-3 cm; the saliva hangs continuously from the mouth in long strings.

Clinical signs in cattle are observed 4-5 months after starting the consumption of pods (Dantas, 1996). In the USA clinical signs were observed after the administration of the pods *ad libitum* for 2-10 months. In cattle ingesting only pods typical clinical signs appeared 4-10 months after the start of the experiment, but some cattle ingesting pods and hay had clinical signs after 67 days of ingestion. Suggestive signs of intoxication appeared 30 days after the ingestion of pods and hay and 60-90 days after the ingestion of pods as the only food (Dollahite and Anthony, 1957). In Brazil, the disease was produced experimentally in cattle ingesting food containing 50% and 100% of pods. All animals had clinical signs 3 months after the beginning of feeding. Cattle ingesting 100% of pods died after 6-10 months of ingestion. Cattle ingesting 50% of pods, which showed less severe clinical signs than those ingesting 100%, were fully recovered 12 months after the end of the administration (Menezes, 1998). If the animals have been affected for no more than 60 days they fully recover after the withdrawal of the pods; but in animals affected for more time the clinical signs are not reversible (Dollahite, 1964). In severely affected cattle, the denervation atrophy of the masseter and other muscles is irreversible and clinical signs remain after the withdrawal of the plant.

Clinical signs in goats are characterized by twitching of the lips, head tremors, salivation and emaciation (Baca *et al.*, 1966). Experimentally, clinical signs were observed 9-11 months after the ingestion, *ad libitum*, of a concentrate containing 80% of pods, plus chopped maize stalks and leaves. Another group of goats that

received mesquite bean pods *ad libitum* and small amounts of dried whole maize plants died of emaciation after 43-102 days of ingestion, without showing other clinical signs (Baca *et al.*, 1966). In Brazil, three groups of six goats each were fed with a ration containing 30%, 60% and 90%, on a dry matter base, of *P. juliflora* pods. Two hundred and ten days after the start of the experiment three goats that ingested 60% of pods in the food and four that ingested 90% showed mandibular tremors, mainly during chewing. In the following weeks some goats had difficulties in eating and stayed with the mandible temporarily at one side during chewing. Only one goat had severe weight loss. All animals were killed after 270 days of ingestion (Tabosa *et al.*, 2000).

## Pathology

Gross and histological lesions reported in cattle were emaciation, muscular atrophy of the masseter, and spongiosis and gliosis of the central nervous system (Figueiredo *et al.*, 1996).

In a recent study of the experimental intoxication in goats, the main histological lesion involved the trigeminal motor nuclei. In these nuclei most neurons had loss of Nissl substance and fine vacuolation of the perikaryon with numerous vacuoles observed diffusely scattered in the perikaryon or at one pole of the cell. Some nuclei of these neurons were distended and displaced to the cell margin. Occasionally, ghost neurons, characterized by pale perikaryon with dissolution of the Nissl substance and undefined borders, were observed. A few reactive astrocytes with pale cytoplasm were also observed in the trigeminal motor nuclei. Occasionally, vacuolated neurons were also observed involving the oculomotor nuclei. Loss of neurons followed by proliferation of satellite cells and neuronophagia were observed in the trigeminal ganglia. Wallerian degeneration characterized by short chains of two to five vacuoles side by side, occasionally containing eosinophilic residues or some macrophages, was observed in the trigeminal and mandibular nerves of some goats. Denervation atrophy was observed on the masseter, temporal, hyoglossus, genioglossus, styloglossus, medial pterygoid, lateral pterygoid and mylohyoid muscles. These lesions were characterized by marked variation in the diameter of the fibres with many small fibres having angular shape and increased nuclei concentration. Some fibres had a hyaline sarcoplasm with infiltration by macrophages and proliferation of satellite cells. Other fibres were enlarged with central nuclei and some showed longitudinal myofibre splitting. In some areas connective tissue and/or fat had replaced disintegrated and atrophic fibres.

The neuronal vacuolation observed in the intoxication by *P. juliflora* is similar in some aspects to those observed in some of the acquired storage diseases suggesting that some material is stored in the affected neurons (Tabosa *et al.*, 2000).

Lesions in the trigeminal nuclei had not been described in cattle but clinical signs including masseter atrophy, tongue protrusion, mandible slackening,

profuse salivation and deglutition difficulties are characteristic of a cranial nerve impairment, suggesting that in this species the trigeminal nuclei and probably other cranial nerve nuclei are also involved.

Recently we did a necropsy of a cow with typical signs of mesquite beans intoxication. This animal was already affected when it was bought by the owner and probably did not ingest mesquite beans since, at least, some months ago. Gross lesions were characterized by yellow colour and severe reduction in the size of the masseter and temporal muscles. Histologically these muscles had a severe chronic denervation atrophy. Much of the fibres were replaced by adipose tissue or fibrous tissue and many of the remaining myofibres were very slender and hypercellular with only a minority of the fibres resembling normal muscle. The histology of the central nervous system revealed a loss of neurons in the trigeminal motor nuclei. The remaining neurons were not vacuolated. The glial fibrillary acidic protein (GFAP) stain revealed a widespread mild astrocytosis. The absence of neuronal vacuolation in the remaining neurons in the trigeminal nucleus suggests that these surviving neurons recovered from the vacuolation after the withdrawal of the plant.

## Active Principle

Piperidine alkaloids (julifloricine, julifloridine, juliflorinine, juliprosopine, juliprosine, juliprosinene and others) have been identified in the pods of *P. juliflora* (Ahmad *et al.*, 1978; Batatinha, 1997; Tabosa *et al.*, 2001). Some of these alkaloids are toxic for laboratory animals (Tabosa *et al.*, 2001) and cell cultures (Batatinha, 1997) but it is unknown if they induce neuronal vacuolation.

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## Chapter 53

# Screening Poisonous Plant Toxins for Cytotoxicity Using Bovine Embryos Produced by *In Vitro* Fertilization Techniques

K.E. Panter<sup>1</sup>, L.F. James<sup>1</sup>, S. Wang<sup>2</sup>, D.R. Gardner<sup>1</sup>, W. Gaffield<sup>3</sup>, R.J. Molyneux<sup>3</sup>, B.L. Stegelmeier<sup>1</sup> and T.D. Bunch<sup>2</sup>

<sup>1</sup>Poisonous Plant Research Laboratory, USDA-Agricultural Research Service, Logan, UT 84341, USA; <sup>2</sup>Animal, Dairy and Veterinary Science Department, Utah State University, Logan, UT 84322-4815, USA; <sup>3</sup>Western Regional Research Center, USDA-Agricultural Research Service, Albany, CA 94710, USA

### Introduction

Locoweeds (certain species of *Astragalus* and *Oxytropis*) cause reproductive dysfunction in livestock including early embryo loss (Panter *et al.*, 1999a). Negative effects on oestrous behaviour, length of oestrous cycle, breeding and conception were reported in cows after ingesting locoweed (*Oxytropis sericea*) as 20% of their diet (Panter *et al.*, 1999b). Locoweed poisoning occurs worldwide and causes tremendous losses annually to the livestock industry (James and Nielsen, 1988; Nielsen *et al.*, 1988).

Swainsonine, the toxin in locoweed, is an indolizidine alkaloid which inhibits lysosomal  $\alpha$ -mannosidases and Golgi mannosidase II, resulting in impaired lysosomal function and altered glycoprotein metabolism (Das *et al.*, 1995; Jacob, 1995). Swainsonine causes locoweed intoxication (locoism) when animals continuously graze locoweeds for extended periods of time (Stegelmeier *et al.*, 1995a). Concentrations of swainsonine in serum of over 200 ng ml<sup>-1</sup> have been measured in cattle and sheep after feeding locoweed (Stegelmeier *et al.*, 1995b).

While many of the effects of locoweed on reproduction have been described, the effects of swainsonine on preplacentation embryo development including oocyte maturation, fertilization and development through morula and blastocyst stages have only recently been reported (Wang *et al.*, 1999).

Ponderosa pine needles (PN) induce abortion in pregnant cows when ingested primarily during the last trimester (James *et al.*, 1989, 1994). Parturition often results in stillborn or small weak calves depending on the stage of gestation when cows ingest needles. Direct losses from PN in the western USA are estimated to exceed \$20 million annually (Miner *et al.*, 1987).



Isocupressic acid, a diterpene acid found in the PN of Ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), common juniper (*Juniperus communis*) and Monterey cypress (*Cupressus macrocarpa*), was identified as the abortifacient compound when ingested by late term pregnant cattle (Gardner *et al.*, 1994, 1998). Isocupressic acid induces abortions during the last trimester of pregnancy when administered orally or by intravenous infusion to cattle (Gardner *et al.*, 1996, 1999).

*Veratrum californicum* was responsible for the induction of congenital cyclopia, monkey faced lambs, in sheep grazing high mountain ranges in central Idaho. The cyclopic-type craniofacial birth defects occur when *Veratrum* is ingested by pregnant sheep on day 14 of gestation (Binns *et al.*, 1963). Jervine and cyclopamine were two important compounds isolated from *V. californicum* responsible for the induction of congenital cyclopia. Skeletal malformations and tracheal stenosis occur in lambs when ingestion occurs during 28-33 days gestation (Keeler and Stuart, 1987). Jervine, veratramine and cyclopamine are naturally occurring steroidal alkaloids from *V. californicum* and cyclopamine-4-ene-3-one is a synthetic analogue of cyclopamine (Keeler, 1978). Three of the four alkaloids, jervine, cyclopamine and cyclopamine-4-ene-3-one, inhibit Sonic hedgehog (Shh) signalling and produce cyclopia and holoprosencephaly in gastrulation-stage embryos (Gaffield *et al.*, 2000). Cyclopamine completely inhibits Shh at 0.12  $\mu\text{M}$ .

The objective of this research was to determine the effects of selected natural reproductive toxins on oocyte maturation and pre-implantation embryo development using *in vitro* fertilization (IVF) techniques. We believe these IVF techniques can be utilized to screen natural toxins for potential cytotoxicity.

## Materials and Methods

### Production of bovine embryos *in vitro*

Ovaries were collected from a local abattoir. Oocytes were aspirated from small antral follicles (3-8 mm in diameter) as described by Hawk and Wall (1994). Cumulus oocyte complexes (COCs) with evenly granulated ooplasm and surrounded by several layers (at least three layers) of compact cumulus cells were selected for use according to the oocyte grading system of Hawk and Wall (1994). Oocytes were washed three times with Hepes-TALP solution (Parrish *et al.*, 1988) and once with maturation medium. *In vitro* maturation (IVM) of oocytes followed the procedure of Sirard *et al.* (1988) and Bavister *et al.* (1992) with minor modifications. The maturation medium consisted of M-199 plus 10% (vol/vol) fetal bovine serum (FBS, A-1111, HyClone Laboratories, Inc., Logan, UT, USA), 25 mM HEPES, 2 mM glutamine, 0.25 mM sodium pyruvate, 0.5  $\mu\text{g ml}^{-1}$  ovine FSH (F-4520, Sigma Chemical Company, St Louis, MO, USA), 5.0  $\mu\text{g ml}^{-1}$  ovine LH (L-5269, Sigma) and 1.0  $\mu\text{g ml}^{-1}$  oestradiol (E-2258, Sigma). Polystyrene plastic 4-well culture petri dishes (Nunclon, Nunc Inc., Naperville,

IL, USA) were used for IVM culture. Each well contained 500  $\mu\text{l}$  IVM medium covered with paraffin oil (6358, Mallinckrodt Inc., Port, KY, USA). Approximately 40 to 65 oocytes were transferred to the IVM medium well<sup>1</sup> and cultured in a humidified 5% CO<sub>2</sub> atmosphere at 39°C for 24 h.

Cryopreserved bovine semen was used for this IVF. Live sperm were separated by Percoll (P-4937, Sigma) gradients (45% and 90% on the upper and lower layers, respectively) and centrifuged at 500 x g for 30 min. Motile spermatozoa were added to the fertilization medium (Fert-TALP, Parrish *et al.*, 1988) to provide a final concentration of  $1.5 \times 10^6 \text{ ml}^{-1}$ . Capacitation of spermatozoa occurred in Fert-TALP containing 10  $\mu\text{g}$  heparin  $\text{ml}^{-1}$  and 0.6% fatty acid free bovine serum albumin. IVM matured oocytes were added to Fert-TALP containing spermatozoa and cultured in plastic four-well petri dishes under paraffin oil in a humidified 5% CO<sub>2</sub> atmosphere at 39°C for 17 h. Each well contained 500  $\mu\text{l}$  Fert-TALP and approximately 40 to 65 oocytes.

Cumulus and corona cells were removed from ova by vortexing in Hepes-TALP supplemented with 0.3% (w/v) bovine serum albumin for 3 min. The presumptive zygotes were then cultured in plastic four-well petri dishes under paraffin oil at 39°C in a humidified 5% CO<sub>2</sub> atmosphere. A modified CR2 medium (Wang *et al.*, 1997) comprising 108.3 mM NaCl, 2.9 mM KCl, 24.9 mM NaHCO<sub>3</sub>, 2.5 mM hemicalcium lactate, 0.5 mM sodium pyruvate, BME amino acids (B-6766, Sigma), MEM non-essential amino acids (M-7145, Sigma), 0.5 mM glycine, 0.5 mM alanine, 1.0 mM glutamine, 1.0 mM glucose and antibiotics was used to culture embryos. Each well contained 500  $\mu\text{l}$  CR2 medium with approximately 40 to 60 oocytes. During culture, medium was changed every other day.

### Swainsonine

The experiment consisted of five replications (blocks) with oocytes ( $n = 3275$ ) from the same collection of abattoir ovaries. The concentrations of swainsonine supplemented to the media were based on data from feeding experiments where cows were fed 20% of their diet as locoweed. Swainsonine was supplemented to the IVM, IVF and *in vitro* culture (IVC) media at 0  $\mu\text{g ml}^{-1}$  (TRT 1, control), 0.8  $\mu\text{g ml}^{-1}$  (TRT 2), 1.6  $\mu\text{g ml}^{-1}$  (TRT 3), 2.4  $\mu\text{g ml}^{-1}$  (TRT 4), 3.2  $\mu\text{g ml}^{-1}$  (TRT 5), 4.8  $\mu\text{g ml}^{-1}$  (TRT 6) and 6.4  $\mu\text{g ml}^{-1}$  (TRT 7). The oocyte cleavage rate was determined 48 h after exposure of the *in vitro* matured oocytes to spermatozoa during IVF and embryos were examined for development on day 6, 8 and 10 of culture (IVF = day 0).

### Isocupressic acid (ICA)

The experiment consisted of 16 replicates (blocks) with oocytes ( $n = 2719$ ) from the same collection of abattoir ovaries. In each experiment, there were four IVC treatments (TRT). ICA was added into IVC medium at 2.6  $\mu\text{g ml}^{-1}$  (TRT 1) and 1.3  $\mu\text{g ml}^{-1}$  (TRT 2), 10% sera from ICA-dosed cattle (TRT 3), and only IVC

medium, which contained no ICA, served as control. The ICA serum (TRT 3) was collected from pregnant cattle used in feeding trials (Gardner *et al.*, 1998). The cattle received a daily dose of ICA ranging from 62 to 245 mg ICA kg<sup>-1</sup> bodyweight and had classical clinical signs of PN-induced abortion (Gardner *et al.*, 1996, 1998). Cleavage rates were determined at 48 h after the exposure of oocytes to spermatozoa. The pre-implantation embryo development was evaluated at days 6, 8 and 10 of *in vitro* culture (IVF = day 0).

### **Veratrum alkaloids**

The experiment consisted of five replicates using oocytes ( $n = 1198$ ) from the same collection of abattoir ovaries. There were five experimental treatments where IVC medium was supplemented with 12  $\mu\text{M}$  of jervine (TRT 1), cyclopamine (TRT 2), veratramine (TRT 3), cyclopamine-4-ene-3-one (TRT 4) and IVC medium only (TRT 5, control). Cleavage rates were determined 48 h after the exposure of oocytes to spermatozoa. The pre-implantation embryo development was evaluated at days 6, 9, and 10 of IVC (IVF = day 0).

### **Statistical analysis**

A complete randomized block experimental design was used to establish the effects of these toxins on *in vitro* culture of IVM/IVF derived bovine embryos. Percentage data were angularly transformed and analysed by the use of a general linear model (GLM) ANOVA. The Fisher's Least Significant Difference (LSD) at the 5% significant level ( $P < 0.05$ ) was used to test the differences between treatment means. The NCSS 97 (Number Cruncher Statistical System) computer software package (Hintze, 1997) was used for all statistical calculations.

## **Results**

The cleavage rates for swainsonine were 74.6%, 84.0%, 80.1%, 83.7%, 75.7%, 78.2% and 78.8%; the percentages of morulae at day 6 of IVC were 44.0, 48.8, 42.2, 47.1, 49.3, 50.6 and 41.0; and the percentage of blastocysts at day 8 of IVC were 27.6, 29.0, 25.4, 30.1, 30.3, 28.7, and 23.2 for TRTs 1 to 7, respectively. There was no significant difference ( $P > 0.05$ ) between treatments. Blastocysts from TRT1 (control), TRT 6 (3.2  $\mu\text{g ml}^{-1}$ ) and TRT 7 (6.4  $\mu\text{g ml}^{-1}$ ) were non-surgically transferred to synchronized recipients (2 embryos/recipient). One out of 12 recipients (8.3%) in control, one out of six recipients (16.7%) in TRT 6 and one out of eight recipients (12.5%) in TRT 7 were confirmed pregnant. No significant differences were determined between TRTs and control.

The cleavage rates for isocupressic acid were 85.4%, 84.5% 85.6% and 87.0%, and the percentages of morulae at day 6 of IVC were 58.3, 59.6, 55.8 and 50.3 for TRT 1, TRT 2, TRT 3 and control, respectively. There was no significant ( $P > 0.05$ ) difference with respect to oocyte cleavage and morula production

between treatments. The percentages of blastocysts at day 8 of IVC were 29.8, 22.5, 22.6 and 18.8 for TRT 1, TRT 2, TRT 3 and control, respectively. The number of embryos developing to the blastocyst stage was significantly higher ( $P < 0.01$ ) in ICA-containing medium compared to control. The percentages of expanded and hatched blastocysts at day 10 were 26.0, 18.9, 17.6 and 12.5 for TRT 1, TRT 2, TRT 3 and control, respectively. ICA TRTs 1 and 2 resulted in a higher ( $P < 0.01$ ) percentage of expanded and hatched blastocysts compared to control. The number of embryos from TRT 1 ( $2.6 \mu\text{g ml}^{-1}$ ) developing to the expanded and hatched blastocyst stage was greater than TRT 2 ( $1.3 \mu\text{g ml}^{-1}$ ;  $P < 0.01$ ). Therefore, isocupressic acid appears to promote bovine pre-implantation embryo development *in vitro* in a dose dependent manner.

The cleavage rates for *Veratrum* alkaloids were 78.1%, 83.8%, 72.6%, 59.6% and 81% for jervine (TRT 1), cyclopamine (TRT 2), veratramine (TRT 3), cyclopamine-4-ene-3-one (TRT 4) and control (TRT 5), respectively. The percentages of morulae at day 6 of IVC were 57.8, 48.5, 43.7, 20.3 and 50.8, respectively; the percentages of blastocysts at day 8 of IVC were 18.9, 19.0, 16.2, 5.6 and 22.6, respectively; and the percentages of expanded and hatched blastocysts at day 10 were 12.6, 8.2, 9.7, 8.0 and 21.8 for TRT 1, TRT 2, TRT 3, TRT 4 and TRT 5, respectively.

Development of pre-implantation embryos derived from oocytes matured in media containing steroidal alkaloids was inhibited compared to those of control ( $P < 0.05$ ). Cleavage rates were lower and numbers of embryos developing to morula, blastocyst, expanded blastocyst and hatching blastocyst stages were significantly reduced ( $P < 0.05$ ). Also, the adverse effects of cyclopamine-4-ene-3-one on oocyte maturation were greater than cyclopamine ( $P < 0.05$ ). The production of morula and blastocyst was lower ( $P < 0.01$ ) in TRT 4 compared to TRT 2. Furthermore, embryos derived from oocytes matured in medium containing cyclopamine-4-ene-3-one had a lower ( $P < 0.01$ ) production of expanded blastocysts and hatched blastocysts compared to cyclopamine.

## Conclusion

Swainsonine did not significantly affect the development and viability of pre-implantation bovine embryos. Blastocysts from controls and TRT 6 ( $4.8 \mu\text{g ml}^{-1}$ ) and TRT 7 ( $6.4 \mu\text{g ml}^{-1}$ ) were transferred to synchronized recipients (2 embryos/recipient). Pregnancy progressed normally for all embryos and the calves were normal. Furthermore, swainsonine calves were raised to maturity and produced normal offspring of their own. Isocupressic acid did not inhibit embryo growth and development but enhanced oocyte or embryonic development *in vitro*. While this phenomenon has significant utility in IVF systems, further evaluation is required, especially in light of the cardiovascular anomaly in two of the five calves produced from transfer of ICA embryos to recipient heifers. Exposure of oocytes and embryos to *Veratrum* alkaloids and a synthetic analogue of cyclopamine significantly inhibited embryo development during the late pre-

implantation stage of development. Transfer of *Veratrum* alkaloid-exposed embryos has yet to be done.

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## Chapter 54

# Growth of Bracken in Denmark and the Content of Ptaquiloside in Fronds, Rhizomes and Roots

L.H. Rasmussen and H.C.B. Hansen

*The Royal Veterinary and Agricultural University, Chemistry Department, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*

### Introduction

In Denmark, the bracken variety common bracken (*Pteridium aquilinum* ssp. *aquilinum* var. *aquilinum* (L.) Kuhn) is encountered as a weed on acid soils in plantation forestry and on recreational areas. As a part of an investigation of soil- and groundwater-contamination with the carcinogenic compound ptaquiloside, three typical common bracken populations situated in plantations were investigated from April 2000 until April 2001 to quantify the above-ground biomass production as well as ptaquiloside production in different plant compartments (fronds, frondbearing rhizomes, storage rhizomes and roots).

Frond growth commences around 1 May in Denmark. The fronds reach maturity in late summer (July and August) and finally die back from late September. In the case of early autumn night frost, the growth season may stop at an earlier time (Watt, 1976; Hansen, 1991; Øllgaard and Tind, 1993). The fronds reach a height of 100-250 cm. The highest fronds are typically found within forests and plantations, where the ferns are protected against wind and frost. In the UK where common bracken is widespread in forests, on moors and on heathlands, frond heights from 60 to 110 cm are typical on open terrain, while the fronds generally are higher (150 cm or more) in protecting ecosystems like forests (Nicholson and Paterson, 1976; Watt, 1976; Whitehead and Digby, 1997a). No investigations have been published regarding the frond density of common bracken in Denmark. In the UK, densities between 16 and 51 fronds m<sup>-2</sup> were measured (Nicholson and Paterson, 1976; Whitehead and Digby, 1997a).

Several frond biomass production functions have been established over the years (Paterson *et al.*, 1997; Alonso-Amelot *et al.*, 2000). Recently Alonso-Amelot *et al.* (2000) presented a simple exponential model for the neotropical bracken variety *caudatum* based on rachis length. Models like this and direct measurements have been used to estimate the annual frond dry matter production. The dry matter content in common bracken fronds reaches a maximum at frond maturity in late summer/early autumn before the fern begins retrieval of nutrients

to rhizomes (Watt, 1976; Williams and Foley, 1976). Common bracken has been reported to have an annual frond dry matter production of up to  $1400 \text{ g m}^{-2}$ , while neotropical brackens (var. *caudatum* and *arachnoidum*) with a whole year growth cycle have a standing biomass in the fronds between 25 and  $1439 \text{ g m}^{-2}$  (Alonso-Amelot *et al.*, 1995; Watt, 1976; Alonso-Amelot *et al.*, 2000). A few investigations have been performed regarding the belowground biomass found in the rhizomes. The dry matter content of common bracken rhizomes is lowest during late spring and early summer while the fronds are growing. From July to October the common brackens in England replenish the carbohydrate content of the rhizomes before going into dormancy by the end of October (Williams and Foley, 1976). The belowground biomass for common bracken can be very large ( $660 \text{ g-dry-matter m}^{-2}$ ,  $8630 \text{ g-fresh-matter m}^{-2}$ ), and it is estimated that the rhizomes make up around 80% of the total biomass of bracken populations. Of the rhizomes, the storage rhizomes make up around 60% of the rhizome biomass (Baker *et al.*, 1997; Whitehead and Digby, 1997b).

The ptaquiloside content in fronds is usually highest just after the croziers emerge from the ground. The content typically decreases during the growing season. In neotropical brackens less than 5% of the maximum content is found in mature fronds (Saito *et al.*, 1989; Alonso-Amelot *et al.*, 1992; Alonso-Amelot *et al.*, 1995). In common bracken contents between 60 and  $9800 \mu\text{g g}^{-1}$  were encountered in a worldwide collection held in Sydney (Smith *et al.*, 1994).

The ptaquiloside content in rhizomes lies between 5 and  $1200 \mu\text{g g}^{-1}$ . Only a few studies have been performed on rhizomes, and they were performed in the growth season on common bracken and neotropical bracken. Highest contents were found in common bracken rhizomes (Saito *et al.*, 1989; Alonso-Amelot *et al.*, 1992; Rasmussen *et al.*, 2003).

## Materials and Methods

Three bracken-dominated ecosystems situated all over Denmark on different kinds of soil materials were chosen for the investigation (Fig. 54.1). Descriptions of the sites can be found in Table 54.1. Frond heights (30 fronds per site) and dry matter content of fronds (three fronds per site) were measured once (Præstø Fed and Salten Langsø) or twice (Mørup Skov) a month from the time croziers emerged in April 2000 until the fronds reached maturity in September 2000. The dry matter content was measured after drying for 5 days at  $110^\circ\text{C}$ . In addition frond densities were measured at the end of the growing season (three times ( $1\text{m}^2$  each) per site). Several fronds, rhizomes and roots were sampled from each site for ptaquiloside analysis. A distinction was made between frondbearing (short shots) and storage rhizomes (long shots). Roots from both kinds of rhizomes were pooled to obtain a suitable sample size. Plant material was brought to the laboratory, cut into small pieces, and dried at  $50^\circ\text{C}$  for 5 days, milled (resulting in diameter less than 2 mm), and finally stored at  $4^\circ\text{C}$  until further analysis. Before drying, rhizomes and roots were gently washed free from soil. The ptaquiloside-



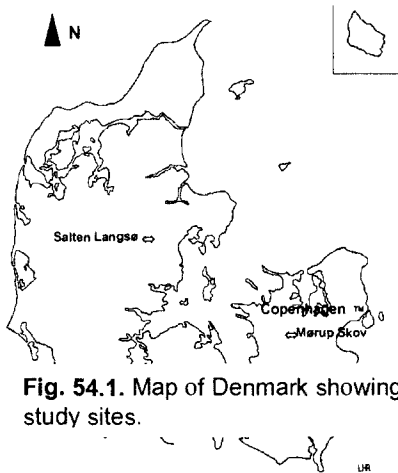


Fig. 54.1. Map of Denmark showing study sites.

content was measured in duplicate once or twice a month during the period of investigation following the water-extraction method of Agnew and Lauren (1991). Ptaquiloside levels were measured as ptaquiloside equivalents after conversion to pterosin B at 214 nm on a Perkin Elmer Series 10 liquid chromatographer, equipped with a Shimadzu SPD-10A UV Detector, a Perkin Elmer LCI-100 Laboratory Computing Integrator, and a Merck LiChroCART 250-4, Lichrospher 100 RP-8 as analytical column. Water-methanol (45:55 vol:%) was used as eluent with a flow rate of 1.50 ml min<sup>-1</sup>.

A 50 µl sample loop was used for all samples. Standards in the range of 0.0112-22.5000 µg ml<sup>-1</sup> ptaquiloside equivalents were used for quantification, resulting in measurement of both ptaquiloside and *iso*-ptaquiloside (Agnew and Lauren, 1991; Castillo *et al.*, 1997). A standard of ptaquiloside was obtained from Professor Ojika, Nagoya University.

Table 54.1. Site description according to FAO (1990) and Soil Survey Staff (1999).

Site	Parent material	Soil order	Drainage class	Land use	Herbal dominants
Mørup Skov	Glaciofluvial deposits	Spodosol	Well drained	Pedunculate Oak ( <i>Quercus robur</i> L.)	B,C,D,E,F G,H
Salten Langsø	Fine sandy till	Entisol	Well drained	Douglas Fir ( <i>Pseudotsuga menziesii</i> Franko.)	B,D,I,J,C K
Præstø Fed	Beach ridges	Spodosol	Somewhat excessively drained	Silver Birch ( <i>Betula pendula</i> Roth)	B,I

B = Bracken (*Pteridium aquilinum* (L.) Kuhn), C = Shield fern (*Dryopteris dilatata* (Hoffm.) A.Gray), D = Honeysuckle (*Lonicera periclymenum* L.), E = Wood anemone (*Anemona nemorosa* L.), F = Dutch rush (*Equisetum hyemale* L.), G = Raspberry (*Rubus idaeus* L.), H = Nettle (*Urtica dioica* L.), I = Wavy hair-grass (*Deschampsia flexuosa* (L.) Trin.), J = Blackberry (*Rubus fruticosus* L.), K = Wood sorrel (*Oxalis acetosella* L.).

## Results and Discussion

The fronds reached maturity 97-134 days after emergence from the ground (Fig. 54.2). The maximum frond lengths were 166-212 cm, which is in accordance with previous investigations of common bracken in Denmark. The frond densities were 9-14 fronds  $m^{-2}$ , which is rather low compared to investigations of common bracken in the UK (Nicholson and Paterson, 1976; Whitehead and Digby, 1997a). Exponential biomass equations based on frond length and dry matter content were established and yielded correlation coefficients between 0.9209 and 0.9277 (Fig. 54.2). Using these functions and the frond density, a total aboveground biomass production between 163 and 496 g dry matter  $m^{-2}$  was estimated at the end of the growth season, which is somewhat low compared to other investigations (Watt, 1976).

Ptaquiloside was found in all plant compartments all year round (Figs 54.3 and 54.4). The fronds had ptaquiloside contents between 360 and 3612  $\mu g g^{-1}$ . Salten Langsø and Præstø Fed showed maximum ptaquiloside content in June and July, while Mørup Skov showed maximum in April.

Both Mørup Skov and Salten Langsø exhibited a tendency for the ptaquiloside content to decrease during the growth season in accordance with other findings, while on Præstø Fed no marked difference in the ptaquiloside content was observed. The different kinds of rhizomes had ptaquiloside contents between 2 and 7046  $\mu g g^{-1}$ , with the lowest contents just after the fronds emerged from ground and during the frond growth season (Fig. 54.4). The highest contents were encountered after fronds had reached maturity as fully enrolled fronds, in late summer and autumn (after day number 210). Raised ptaquiloside content was also measured in late winter/early spring before the croziers emerged from the ground. The storage rhizomes had in general a higher content of ptaquiloside than

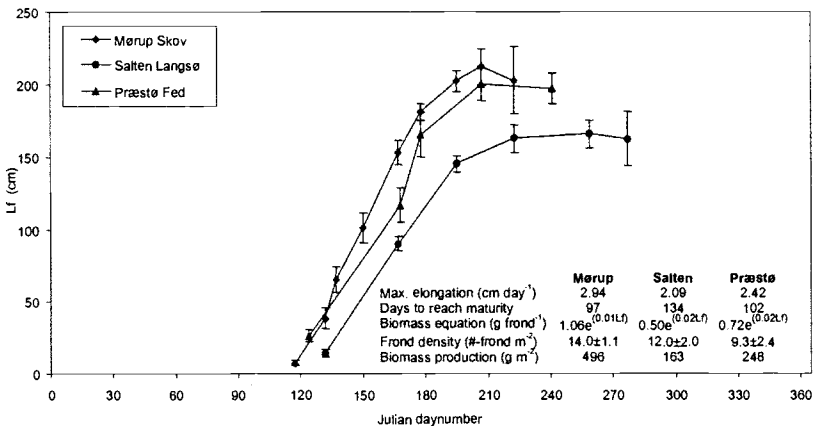


Fig. 54.2. Bracken growth. Lf = Length of frond (cm). The population at Mørup Skov tilted after heavy rain at day number 210.

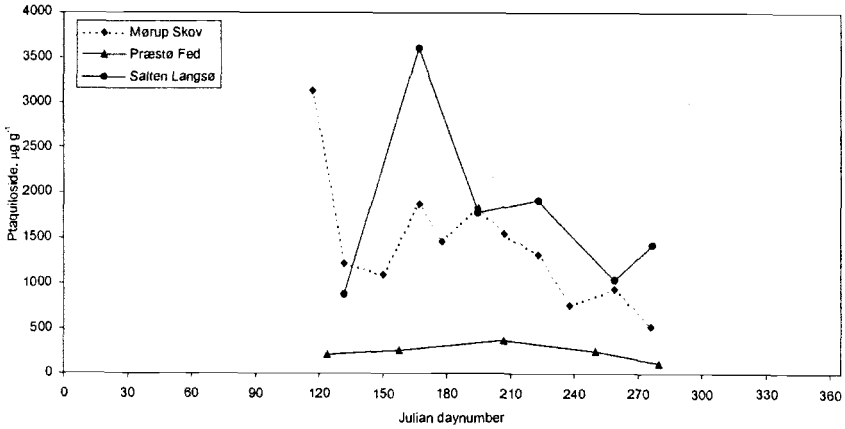


Fig. 54.3. Ptaquiloside content in bracken fronds. SDev < 10%.

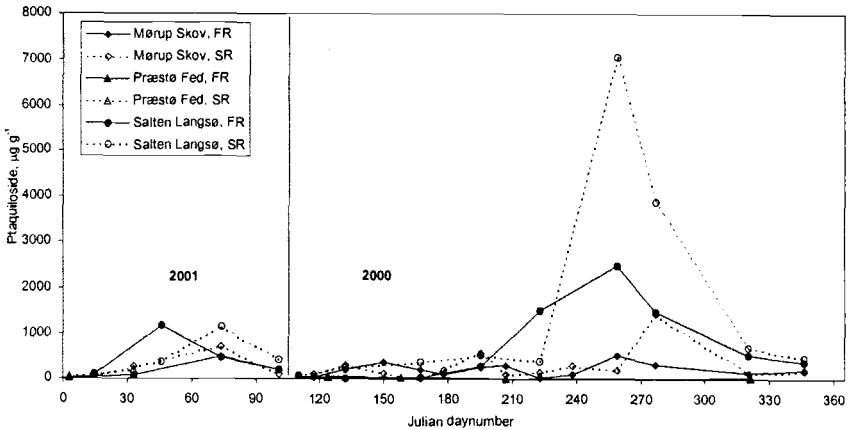


Fig. 54.4. Ptaquiloside in rhizomes. FR = Frond-bearing rhizome. SR = Storage rhizome. SDev < 10%.

the frond-bearing rhizomes, especially in the autumn. The content seem to be rather high compared to other investigations of rhizomes (Saito *et al.*, 1989; Alonso-Amelot *et al.*, 1992). The rhizome contents tend to be positively correlated with carbohydrate regeneration of rhizomes in the autumn, rhizome- and below-ground shoot-elongation, and negatively correlated with frond growth during summer (Williams and Foley, 1976).

The ptaquiloside content in roots was generally low compared to the other fern compartments ( $5\text{--}230 \mu\text{g g}^{-1}$ ). This is the first time ptaquiloside has been measured in bracken roots. The content does not seem to be correlated with the ptaquiloside content in fronds and rhizomes.

## Conclusion

Compared to other investigations, Danish populations of common bracken seem to have relatively low growth rates and frond densities resulting in a low biomass production. The content of ptaquiloside in the fronds is in accordance with other observations but does not follow the marked decline after frond emergence as reported elsewhere. The ptaquiloside contents found in roots and rhizomes are higher than previously reported. The high ptaquiloside contents found in the rhizomes in the autumn are probably caused by replenishment of the rhizomes with carbohydrates.

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## Chapter 55

# Poisonous Plant Research: Biomedical Applications

L.F. James<sup>1</sup>, K.E. Panter<sup>1</sup>, W. Gaffield<sup>2</sup>, R.J. Molyneux<sup>2</sup> and J. Weinzweig<sup>3</sup>

<sup>1</sup>Poisonous Plant Research Lab, USDA, Agricultural Research Service, Logan, UT 84341, USA; <sup>2</sup>Western Regional Research Center, Agricultural Research Service, Albany, CA 94710, USA; <sup>3</sup>Department of Plastic Surgery, Rhode Island Hospital, Providence, RI 02905, USA

Since the discovery that ingestion of false hellebore (*Veratrum californicum*) by pregnant sheep on day 14 of gestation induced cyclopic-type craniofacial birth defects in offspring, numerous investigations have attempted to characterize the mechanisms underlying the induction of teratogenic effects. Recent advances in molecular biology have demonstrated that the jerveratrum alkaloids from *Veratrum* exert effects on developing embryos by selectively blocking Sonic hedgehog signal transduction. Thus, cyclopamine has been used as a probe to understand the biological development of a variety of mammalian organs. Furthermore, cyclopamine or its derivatives have been proposed as potential therapeutic agents for the treatment of tumours arising from the disruption of the Hedgehog pathway. Models derived from research using cyclopamine as a probe or tool could provide insight into understanding the aetiological role of environmental agents in various human birth defects.

The primary toxin of the locoweeds *Swainsonia* and some *Ipomoea* spp. is the indolizidine alkaloid, swainsonine. Swainsonine is a potent inhibitor of the enzymes  $\alpha$ -mannosidase and Golgi  $\alpha$ -mannosidase II, which are essential in cell function. Ingestion of swainsonine by livestock produces a phenocopy of the genetic lysosomal storage disease, mannosidosis. Mannosidosis results from improper trimming of certain sugars from glycoproteins in cellular organelles affecting primarily *N*-linked glycoproteins involved in physiological functions. Abnormal glycoprotein processing inhibits cell to cell recognition critical to pathogenesis, inflammation, parasitism, embryo development, cell adhesion and symbiosis. Significant applications of the inhibitory activity of swainsonine include animal models for lysosomal storage diseases, such as mannosidosis, Pompe's disease, Gaucher's disease and Fabry's disease; anti-metastatic activity; anti-viral activity, suppression of the infectivity of many retroviruses, including HIV; immuno-suppressive activity, promoting heart and renal allograft survival in rats; and anti-parasitic activity, inhibiting infection by the causative agents of Chagas' disease and cerebral malaria, respectively.

*Lupinus* spp., poison-hemlock (*Conium*) and *Nicotiana* spp. induce contracture birth defects and cleft palate in livestock. A goat model was developed to study the mode of action of lupin-induced 'crooked calf disease' in cattle. This model, using *Nicotiana glauca* to induce fetal cleft palate, has now become an important tool to study the mechanism of cleft palate induction in humans. Using this model, biomedical research is focusing on the privileged period of fetal scarless healing and development of *in utero* surgical procedures to repair human cleft palates early in gestation. This model is highly reproducible, closely simulates the etiopathogenesis of the human anomaly and represents an ideal model for management of the cleft palate anomaly in humans. This *in utero* methodology results in scarless healing of the palatal mucoperiosteum and velum and unimpaired development after repair.

### ***Veratrum californicum***

*Veratrum californicum* grows throughout the Western USA in mountainous areas that are grazed by livestock and wildlife (Binns *et al.*, 1965). During the 1950s up to 25% of pregnant ewes grazing in the mountains of Central Idaho gave birth to lambs suffering from serious craniofacial defects (James, 1999). These malformations varied from the extreme, cyclopia, to mildly deformed upper jaws. Research demonstrated that ingestion of *Veratrum* by sheep on day 14 of gestation induced grotesque craniofacial birth defects in offspring, dramatically highlighted by cyclopia (Binns *et al.*, 1965). The primary *Veratrum* alkaloid responsible for the terata induction was 11-deoxojervine, which R.F. Keeler named cyclopamine (Keeler, 1984, 1986). Keeler further discovered that cyclopamine induced not only craniofacial malformations on day 14, but also induced limb defects on days 28-31 (Keeler and Stuart, 1987) and tracheal stenosis on days 31-33 (Keeler *et al.*, 1985).

Recent advances in molecular biology and genetics have provided insight into the mechanisms underlying the induction of teratogenic expressions by cyclopamine. Investigation of a variety of Sonic hedgehog-dependent cell types, derived from the neural tube and somites of chick embryo explants with cyclopamine-induced malformations has shown clearly that virtually all aspects of Sonic hedgehog signalling are interrupted in these tissues upon exposure to cyclopamine (Cooper *et al.*, 1998; Incardona *et al.*, 1998).

### **Potential biomedical applications of cyclopamine**

Hedgehog proteins are intimately involved in diverse processes such as the development of limbs, skin, eye, lung, teeth, nervous system and the differentiation of sperm and cartilage (Hammerschmidt *et al.*, 1997).

A variety of diseases and clinical disorders result from mutations in the human Sonic hedgehog gene and in additional downstream genes such as Patched or Smoothed that comprise its intracellular signalling pathway (Ming *et al.*,

1998; Hahn *et al.*, 1999). Included among these diseases are not only holoprosencephaly and various tumours, but also several forms of polydactyly that are derived from genetic defects in network genes. Holoprosencephaly can occur in families as part of rare but inherited disorders that encompass a spectrum of malformations from mild cognitive effects to severe physical impairment (Roach *et al.*, 1975). The syndrome is relatively common in early embryogenesis, occurring in one of 250 spontaneous abortions (Matsunaga and Shiota, 1977). Cyclopamine's ability, both to induce holoprosencephaly in experimental animals and to strongly inhibit Sonic hedgehog signal transduction, offers the potential to enhance understanding the development of the human brain and spinal cord at the cellular and molecular level (Gaffield *et al.*, 2000).

Patched has been implicated as the gene involved in basal cell nevus syndrome, with mutations observed in approximately 30-40% of afflicted patients (Gailani *et al.*, 1996). Basal cell nevus syndrome, an autosomal dominant condition known also as Gorlin's syndrome, displays a wide spectrum of phenotypes including general overgrowth, polydactyly and fused or bifid ribs (Gorlin, 1995; Kimonis *et al.*, 1997). Basal cell nevus syndrome is characterized by a large number of basal cell carcinomas and patients are at further risk for the muscle tumour rhabdomyosarcoma and a greatly increased incidence of the brain tumour medulloblastoma (Gorlin, 1987; Kimonis *et al.*, 1997). Several mutations in the human analogue of Patched have been detected both in sporadic medulloblastomas and primitive neuroectodermal tumours (Xie *et al.*, 1997; Reifenberger *et al.*, 1998). In addition, Patched mutations have been identified in breast carcinoma, a meningioma (Xie *et al.*, 1997), esophageal squamous carcinoma (Maesawa *et al.*, 1998) and trichoepithelioma (Vorechovshy *et al.*, 1997), another type of skin cancer. In addition, activating mutations in Smoothed are found in appreciable instances of sporadic basal cell carcinomas and primitive neuroectodermal tumours (Xie *et al.*, 1998; Reifenberger *et al.*, 1998). Upon inactivation of Patched, Smoothed may become active and independent of Sonic hedgehog control leading in turn to activation of Sonic hedgehog target genes that might play a role in tumour development.

Cyclopamine or its derivatives are proposed as potential mechanism-based therapeutic agents for the treatment of tumours arising from disruption of components of the Hedgehog pathway. Hedgehog inhibitors such as cyclopamine might be effective in controlling the onset of progression of certain lesions or disease states in non-pregnant adults because of the low toxicity of drug levels that are effective in blocking Sonic hedgehog (Taipale *et al.*, 2000).

Because of its propensity to interfere with cholesterol metabolism, cyclopamine was evaluated as an inhibitor of multi-drug resistance in tumour cells. Intrinsic or acquired resistance of tumour cells to cytotoxic drugs is a major cause of failure of cancer chemotherapy. Both cyclopamine and the spirosolane alkaloid tomatidine were observed to act as potent and effective chemosensitizers in multi-drug resistance cells (Lavie *et al.*, 2001). Both of these steroidal alkaloids are comparable in potency and efficacy to verapamil, a common reversal agent used in multi-drug research. Thus, plant steroidal alkaloids such as



cyclopamine and tomatidine, or their analogues, might serve as chemosensitizers in combination with chemotherapy and conventional cytotoxic drugs for treating multi-drug resistant cancer (Lavie *et al.*, 2001).

### **Altered expression of organ tissue**

Expression of organ tissue either can be enhanced or inhibited upon administration of cyclopamine. For example, exposure of embryonic chick to cyclopamine promotes pancreatic development (Kim and Melton, 1998). Cyclopamine inhibition of Sonic hedgehog signalling apparently permits expansion of portions of the endodermal region of the foregut where Sonic hedgehog signalling does not occur, resulting in pancreatic differentiation in a larger area of the foregut endoderm (Kim and Melton, 1998). Understanding the mode of action of drugs such as cyclopamine may further the development of cell-replacement therapies for pancreatic diseases such as diabetes mellitus.

An example of inhibition of organ tissue formation is provided by cyclopamine-treated mice that showed striking inhibition of hair follicle morphogenesis (Chiang *et al.*, 1999). The hair follicle is a source of stem cells and the site of origin for several epithelial skin cancers (Hansen and Tennant, 1994). Since basal cell carcinoma appears to be caused by mutations in genes involved in the Sonic hedgehog signalling pathway, future research may reveal how constitutive activation of the pathway in keratinocytes contributes to the formation of basal cell carcinomas (Oro *et al.*, 1997).

### **Future research**

Cyclopamine is considered the primary prototype of a small molecule that reveals the logic and timing of vertebrate development (Peterson *et al.*, 2000). Models derived from research using cyclopamine as a probe or tool could provide insight into understanding the aetiological role of environmental agents in various human birth defects. Dissection of the Sonic hedgehog network might further reveal how this network interacts with other signal transduction pathways, for example those of the transforming growth factor- $\beta$  (TGF- $\beta$ ) and others.

## **Locoweeds Research (Swainsonine and Other Polyhydroxy Alkaloids)**

The major toxic constituent of locoweeds has been established as the indolizidine alkaloid, swainsonine (Molyneux and James, 1982). The compound was first identified in *Swainsonia* species in Australia (Colegate *et al.*, 1979) and was subsequently found in the locoweeds (*Astragalus* and *Oxytropis* species) of North America and other regions of the world (Molyneux *et al.*, 1994).

The chemical structure of swainsonine is not complex and has many similarities to the simple sugar mannose, which it mimics (Tulsiani *et al.*, 1989). Swainsonine suppresses the action of the enzyme  $\alpha$ -mannosidase, essential for proper cell function. This enzyme trims sugar molecules from complex glycoproteins, and failure of proper trimming results in an accumulation of abnormal molecules within the cell, vacuolation and cell death.

The recognition that swainsonine is an inhibitor of  $\alpha$ -mannosidase suggested that structurally similar alkaloids might have similar properties. One such alkaloid, castanospermine (Hohenschutz *et al.*, 1981), had been isolated from seeds of the Moreton Bay chestnut (*Castanospermum australe*), an Australian rainforest tree. The leguminous seeds, which litter the ground beneath the trees, are toxic to livestock. When tested, castanospermine was found to be a potent inhibitor of  $\alpha$ - and  $\beta$ -glucosidase, enzymes which are essential for glycoprotein processing (Saul *et al.*, 1984). Signs of poisoning are different from swainsonine with pronounced gastrointestinal disturbances, as might be expected from inhibition of digestive glucosidases.

A chemical examination of *C. australe* seeds resulted in the identification of several structurally related indolizidine analogues of castanospermine and some pyrrolizidine alkaloids called australines. These new alkaloids inhibited  $\alpha$ - and  $\beta$ -glucosidases to a greater or lesser extent but were present at significantly lower levels than castanospermine (Elbein and Molyneux, 1998).

Studies of compounds with structural similarities have led to the identification of hydroxylated alkaloids belonging to the tropane class, known as calystegines, which inhibit  $\alpha$ - and  $\beta$ -galactosidase and  $\beta$ -glucosidase (Molyneux *et al.*, 1993). This group consists of 14 alkaloids differing by the number, disposition, and stereochemistry of the hydroxyl groups, with calystegines B2 and C1 being the most commonly found (Elbein and Molyneux, 1998). Various combinations of these alkaloids have been discovered in several plant families - *Convolvulaceae*, *Solanaceae* and *Moraceae*.

An interesting livestock toxicity occurs with respect to *Ipomoea* species which have been found to poison sheep in Australia and goats in Africa. Analysis of *Ipomoea calobra* and *Ipomoea polpha* from Queensland showed that the plants contained not only calystegines B2 and C1 but also swainsonine. An analogous pattern of alkaloids was detected in *Ipomoea carnea* from Mozambique (de Balogh *et al.*, 1999). The toxicity of these plants results from the effect of the toxins on at least three different enzymes and many of the symptoms correlated with those observed in locoism induced by  $\alpha$ -mannosidase inhibition, exacerbated by inhibition of  $\alpha$ -galactosidase and  $\beta$ -glucosidase.

Glycoproteins are involved in numerous essential physiological functions, especially cell-cell recognition reactions critical to pathogenesis, inflammation, parasitism, development, cell adhesion and symbiosis. Consequently, the polyhydroxy alkaloids exhibit a diversity of biological effects, including insecticidal, herbicidal, antimicrobial and therapeutic activities. Discovery and isolation of many of the alkaloids has been a result of observations of the ultimate

clinical effects which result from the consumption by animals of plants containing these bioactive compounds.

### **Animal models for lysosomal storage diseases**

Collectively, genetic diseases in animals occurring as a consequence of an insufficiency of glycoprotein processing enzymes are known as lysosomal storage diseases and have counterparts in humans. These are mannosidosis, Pompe's disease, Gaucher's disease and Fabry's disease, which arise from a deficiency of the enzymes  $\alpha$ -mannosidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase and  $\alpha$ -galactosidase, respectively. The availability of specific inhibitors of these enzymes provides a mechanism for induction of phenocopies of these genetic diseases in animal models. For example, feeding experiments with castanospermine in rats resulted in vacuolation of hepatocytes and skeletal myocytes, and glycogen accumulation, consistent with Pompe's disease (Saul *et al.*, 1985). Young rats treated with swainsonine developed axonal dystrophy in the CNS as a consequence of lysosomal storage of incompletely processed mannosides, which has a parallel in genetic mannosidosis (Huxtable and Dorling, 1985). Experiments using these and other alkaloids should provide useful information for early diagnosis and possible means of intervention to interrupt the progression of such diseases.

### **Anti-metastatic activity**

Although the mammalian toxicity of the polyhydroxy alkaloids is an obvious concern with regard to their medicinal application, the capability to disrupt the general cellular function of glycoprotein processing leads to the expectation that these compounds should have therapeutic potential for the treatment of various disease states. However, many drug candidates have significant toxicity and it is well-recognized that an appropriate dose-response relationship can often be achieved which minimizes harmful side effects. Moreover, adverse effects, such as the neurological damage caused by swainsonine, often develop quite slowly and appear to be reversible if ingestion of the alkaloids is terminated, as would be the situation with most drug regimens. Investigation of these alkaloids for therapeutic potential has so far concentrated on four major disease states, namely the treatment of cancer, inhibition of metastasis, as anti-viral agents, and as anti-parasitics. Structurally related compounds have also been used as anti-diabetic drugs (Taylor *et al.*, 1986).

Swainsonine has received particular attention as an anti-metastatic agent and this effect has been shown to be due to enhancement of natural killer T-cells and increased susceptibility of cancerous cells to their effect (Humphries *et al.*, 1988). *In vivo* experiments with mice have shown that in animals provided with drinking water containing  $3 \mu\text{g ml}^{-1}$  of swainsonine for 24 hours prior to injection with B16-F10 murine melanoma cells had an 80% reduction in pulmonary colonization (Humphries *et al.*, 1990). Pharmacokinetic studies indicate that the levels of alkaloid and period of administration would be insufficient to produce

neurological damage (Bowen *et al.*, 1993). It has been suggested that post-operative metastasis of tumour cells in humans could be suppressed by intravenous administration of the alkaloid prior to and following the surgery. Clinical trials in humans with advanced malignancies showed that lysosomal  $\alpha$ -mannosidases and Golgi mannosidase II were inhibited and improvement in clinical status occurred (Goss *et al.*, 1997).

### **Anti-viral activity**

Castanospermine suppresses the infectivity of a number of retro viruses, including the human immunodeficiency virus (HIV) responsible for AIDS (Gruters *et al.*, 1987; Tyms *et al.*, 1987; Walker *et al.*, 1987). This effect is a direct consequence of glycoprotein processing inhibition, resulting in changes in the structure of the glycoprotein coat of the virus. Cellular recognition of the host is prevented and syncytium formation is suppressed. In spite of this effect, the alkaloid suffers from the disadvantage that it is highly water soluble and therefore rapidly excreted. This problem has been overcome by derivatization to give a lipophilic derivative which has undergone clinical trials against AIDS in humans, either alone or in combination with AZT, with the only significant side effect being gastrointestinal disturbances, as might be predicted (Taylor *et al.*, 1991; Bridges *et al.*, 1995).

### **Immuno-suppressive and anti-parasitic activity**

The ability of polyhydroxy alkaloid glycosidase inhibitors to prevent cellular recognition has resulted in their use in studies of clinical situations where suppression of an immune response would be desirable, or for use against parasitic disease. *In vivo* experiments have shown that castanospermine can be used as an immuno-suppressive drug, promoting heart and renal allograft survival in rats (Grochowicz *et al.*, 1996). Similarly, parasitic diseases may be controlled by altering cellular recognition processes. Swainsonine has been demonstrated to inhibit the association of *Trypanosoma cruzi*, the cause of Chagas' disease, with host cells by formation of defective mannose-rich oligosaccharides on the cell surface (Villalta and Kierszenbaum, 1985), while castanospermine provides protection against cerebral malaria by preventing adhesion of *Plasmodium falciparum* to infected erythrocytes (Wright *et al.*, 1991).

There is no doubt that polyhydroxy alkaloids have considerable potential for treatment of a variety of disease states in humans and animals. The primary challenge in introducing them as commercial drugs is to minimize their toxicity and enhance the specificity of their beneficial effects.

## Lupin, *Conium* and *Nicotiana* Research

The establishment of appropriate animal models for biomedical application is essential if new techniques and procedures are to be applied to human conditions. Briefly, the syndrome of plant-induced cleft palate and contracture skeletal malformations in livestock is the same whether it is induced by *Lupinus*, *Conium* or *Nicotiana* spp. However, the piperidine and or quinolizidine toxins from each plant inducing these malformations possess different toxic potency because of their respective chemical structural components (Panter *et al.*, 1998b). In general the teratogenic piperidines meet specific structural criteria for teratogenesis (Keeler *et al.*, 1981). These differences in structural characteristics are important in the manifestation of the mechanisms of action, such as reduction of fetal movement and fetal malpositioning (Panter *et al.*, 1990). The chemical structural differences and mechanism of action will be discussed as we focus upon the evolution and establishment of the goat model for human biomedical applications.

The cleft palates induced by toxic plants in the goat model closely mimic the human cleft condition (Weinzweig *et al.*, 1999a, b). This model is also useful for histological comparison of the prenatal and postnatal repaired cleft palate and comparison of craniofacial growth and development. Therefore this model provides an ideal congenital model to study the aetiology of cleft palate in humans, for development of fetal surgical techniques *in utero*, and to compare palate histology after prenatal or postnatal repair. The impetus for the biomedical application using these plants and the specific animal model selected has evolved over time and occurred because of the discovery of certain specific biological effects in the goat and their relationship with similar conditions in humans (Panter *et al.*, 1990; Panter and Keeler, 1992; Weinzweig *et al.*, 1999a, b; Panter *et al.*, 2000).

### Description of the syndrome

Research at the Poisonous Plant Research Lab on plant-induced skeletal malformations and cleft palate began in the late 1950s when musculoskeletal defects in newborn calves known as 'crooked calf disease' were attributed to maternal ingestion of *Lupinus* spp. (Palotay, 1959; Wagon, 1960; Binns and James, 1961; Shupe *et al.*, 1967a, b). A high incidence of cleft palate was also associated with lupin-induced crooked calf disease (Shupe *et al.*, 1968a, b). Other significant craniofacial deviations such as asymmetry of the skull, maxillary hypoplasia, brachygnathia or malocclusion often accompany cleft palate. Lupin-induced cleft palate and skeletal malformations continue to cause heavy losses to the cattle industry in the west (Panter *et al.*, 1997; Dr Clive Gay, WSU, 1997, and Gary Walker, Lewisville, ID, 2001, personal communications).

In the late 1960s and early 1970s in the midwestern and southern states, epidemic proportions of skeletal malformations from *Conium maculatum* and *Nicotiana tabacum* in pigs were recorded (Crowe, 1969; Edmonds *et al.*, 1972). It

was determined that poison-hemlock (*C. maculatum*) and burley tobacco (*N. tabacum*) were responsible. The induced malformations in newborn pigs appeared similar to and were eventually described as the same as those in lupin-induced crooked calf disease, i.e. contracture-type skeletal malformations (arthrogryposis, scoliosis, kyphosis and torticollis) and cleft palate.

Of the three genera, lupins have been the most economically important to the livestock industry and continue to cause large losses (Panter *et al.*, 1997; Gay, C.C., Panter, K.E., Motteram, E., Gay, J.M., Wierenga, T., Hantz, H. and Platt, T., unpublished observations). While the economic losses to the livestock industry are significant and often devastating, the potential benefits to human medicine through spin-off research and discovery of biomedical tools that may provide medical breakthroughs in treatment of certain diseases may somewhat negate the adverse effects.

Thus, as the research in this area evolved, two key factors have surfaced in the study of the mechanism of action and advancement of research on the human condition: the goat as a preferred animal model and *N. glauca* and anabasine as a preferred test plant/alkaloid. With these two factors in hand, the advancements in studying the human cleft palate condition are rapidly moving forward (Weinzweig *et al.*, 1999a, b; Panter *et al.*, 2000).

### **Mechanism of action and insult periods**

The proposed mechanism of action for *Lupinus*-induced malformations and cleft palate involves a chemically induced reduction in fetal movement much as one would expect with a sedative, neuromuscular blocking agent, or anaesthetic (Panter *et al.*, 1990). The mechanism of action of the teratogenic effects of *Lupinus*, *Conium* and *Nicotiana* spp. is believed to be similar if not identical (Panter *et al.*, 1994). This mechanism of action was supported by experiments using radio ultrasound where a direct relationship between reduced fetal activity and severity of contracture-type skeletal defects and cleft palate in sheep and goats was recorded (Panter *et al.*, 1990).

Cleft palate induction by these plants is believed to result from mechanical interference by the tongue between the palatal shelves at the programmed time of closure (day 38 in goats; between days 40 and 50 in cows (Panter and Keeler, 1992; Panter *et al.*, 1998a)).

While the mechanical mechanism can be a major factor of cleft palate formation in goats, the molecular mechanism is yet unknown. We hypothesize, however, that it may be due to a unique fetal pharmacological neuromuscular blockade. This hypothesis is supported by preliminary experiments in which curare extract with known pharmacological activity was infused via osmotic minipumps into the amniotic sac of fetal goats during susceptible periods of gestation (Panter, unpublished data, 1996). Cleft palate and severe multiple congenital contracture (MCC)-type skeletal defects were induced. This evidence is preliminary and further research is needed to verify the biochemical mechanisms of action.

### Use of anabasine to induce cleft palate in goats

While the mechanism of action of the piperidine alkaloid-induced contractures and cleft palate is the same, the potency of the alkaloids differs based on structural characteristics (Panter *et al.*, 1998b). These chemical differences among these piperidine alkaloids have led us to focus on anabasine, the piperidine teratogen from *N. glauca*, because of its potency and consistent activity among different livestock species, i.e. cattle, pigs, sheep and goats. Therefore, we have selected *N. glauca*, extracts therefrom and the pure alkaloid anabasine as we advanced investigations about the mode of action of this group of plants to induce contracture malformations and cleft palate. Similarly, goats were selected as the animal model of choice to study the mechanism of action because of their susceptibility to the teratogenic effects, especially cleft palate (Panter *et al.*, 2000), and their small size, ease of handling, consistent response and availability (Panter and Keeler, 1992; Panter *et al.*, 2000). Most recently, goats have been demonstrated to be the ideal model for the study of the aetiology of cleft palate induction and development of fetal biomedical procedures for human application (Weinzwieg *et al.*, 1999a, b; Panter *et al.*, 2000).

### Biomedical Application

While the development of a small ruminant model (goat) was primarily to study the mechanism of action of crooked calf disease in cattle, this goat model, using *N. glauca* and anabasine-rich extracts therefrom to induce fetal cleft palate, has become an important tool in the study of the mechanism of cleft palate induction in humans and fetal biomedical research (Weinzwieg *et al.*, 1999a, b). This research is currently focused on the privileged period of fetal scarless healing and development of *in utero* surgical procedures to repair human cleft palates early in gestation. Children born with cleft palate often undergo a series of operations to correct the ensuing deformities, only the first of which is the actual palate repair at the age of 6-12 months. For many children, speech remains a major problem as well as craniofacial development. Our goal is to eliminate the need for any of these reconstructive procedures by performing the cleft palate repair *in utero* and this is a real possibility. Despite this, what is truly exciting is that we now have a congenital goat model of cleft palate as well as the model of *in utero* cleft repair. The biomedical value of this goat model using *N. glauca* plant or anabasine-rich extracts therefrom to induce cleft palates has been demonstrated.

While fetal surgical intervention in life-threatening circumstances has been demonstrated (Harrison *et al.*, 1990), the role of fetal intervention (surgery) in the treatment of non-life-threatening congenital anomalies remains a source of much debate (Longaker *et al.*, 1991). In 1999 a surgical team at Vanderbilt University operated on a human fetus *in utero* at 21 weeks of gestation to repair a spinal defect, spina bifida (Bruner *et al.*, 2000). Even though the fetal surgery was successful the debate continues. Regardless of the debates over fetal surgical

intervention, procedures and techniques must be established in animal models that closely resemble the human anomaly to demonstrate the utility and safety of the *in utero* procedures.

Recently, we described and characterized a congenital model for cleft palate in the goat (Weinzweig *et al.*, 1999a, b). We also presented the methodology and techniques used to successfully repair congenital cleft plates *in utero* and demonstrated successful scarless palatal healing and development after repair (Weinzweig *et al.*, 1999b). This model closely simulates the etiopathogenesis of the human anomaly. Thus, *in utero* cleft palate repair early in gestation (on or before day 85 in the goat) is feasible and results in scarless healing of the mucoperiosteum and velum. Furthermore, this congenital cleft palate goat model is highly reproducible with little variation representing an ideal animal model (Panter *et al.*, 2000).

In conclusion, this research has significant implications in the management of fetal cleft palates in humans. This research also has important applications in agricultural research. Understanding periods of fetal susceptibility, elucidating teratogenic plants and toxins therefrom and understanding mechanisms of action will provide information for livestock managers whereby losses might be reduced.

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## Chapter 56

# **Essential Amino Acids and Antimetabolites in the Seed Kernel of an Unconventional Legume, Gila Bean (*Entada phaseoloides* Merrill)**

P. Siddhuraju<sup>1</sup>, H.P.S. Makkar<sup>2</sup> and K. Becker<sup>1</sup>

<sup>1</sup>*Department of Animal Nutrition and Aquaculture, Institute for Animal Production in the Tropics and Subtropics (480), University of Hohenheim, D-70593 Stuttgart, Germany;* <sup>2</sup>*Animal Production and Health Section, International Atomic Energy Agency, PO Box 100, Wagramerstrasse 5, A 1400, Vienna, Austria*

### **Introduction**

The incidence of malnutrition or under nutrition among people living in the developing countries and the limited protein supply from animal sources mostly due to their exorbitant cost, and the inadequate availability of protein from conventional food grains, has led to the search for new protein sources. Research efforts are being directed to identify and evaluate under-utilized sources including tribal pulses (Siddhuraju *et al.*, 1995; Makkar *et al.*, 1998) because they are well adapted to adverse environmental conditions, are highly resistant to disease and pests, and exhibit good nutritional qualities. *Entada phaseoloides* Merrill (Gila bean) is widely distributed throughout the tropics. The soaked seed kernels are roasted/boiled and eaten as such or mixed with salt by Northeast Indian tribals such as Garo, Khasi, Naga and Karbis (Arora, 1981). In India, ground seeds are taken internally for an incredible variety of remedies, including contraception, snake bites and as aphrodisiacs. Occasionally, villagers use the seeds as natural shampoo and as a fish poison. Although the legumes are considered to be potential protein and carbohydrate sources for humans and animals, the biologically available nutrients such as amino acids, minerals and carbohydrates are relatively poor due to the presence of various antiphysiological/antimetabolic and toxic substances (Liener, 1994) and the deficiency of sulphur amino acids. Since only limited information is available on the nutritive value and food potential of Gila beans, an attempt has been made in the present study to investigate the physical characteristics of pods and seeds and the detailed nutritional, antinutritional and toxic properties of the seed kernels.

## Materials and Methods

### Samples

The mature and dry raw seeds and pods of *E. phaseoloides* were collected during the month of January, 1999 from Salem district, Tamil Nadu, India. The dried seeds were taken from matured pods and stored at room temperature (25°C) until further analyses.

### Proximate composition

The moisture content of the samples was determined by oven drying to a constant weight at 105°C. Crude protein, lipid, crude fibre and ash were determined in accordance with the standard methods of AOAC (1990). Carbohydrates (nitrogen free extractives) were obtained by difference. Gross energy was estimated by an adiabatic bomb calorimeter (IKA C7000) using benzoic acid as a standard.

### Extraction and estimation of seed kernel protein fractions

The albumin and globulin protein fractions of defatted seed kernel flour were extracted and separated according to the procedure described by Sze-Tao and Sathe (2000). The respective separated protein fractions were freeze-dried. The prolamin protein fraction was extracted from the residual pellet by treating the pellet with 80% ethanol (80 ml EtOH + 20 ml water) (1:10 w/v) overnight at 25°C under constant magnetic stirring at low speed. After centrifugation (12000 × g for 20 min at 4°C) the supernatant containing prolamins was dialysed against deionized-water at 10°C for 24 h (with four water changes), freeze-dried and dissolved in 0.1 M NaOH. The remaining pellet was extracted with 0.01 M NaOH (1:10 w/v) overnight and centrifuged at 12000 × g for 20 min at 4°C. The supernatant thus obtained was designated as glutelins and dialysed against deionized-water at 10°C for 24 h (with four water changes) and freeze-dried. All four fractions so obtained were redissolved in 0.2 M NaOH and the protein content was determined by the method of Lowry *et al.* (1951). A portion of the freeze-dried albumin and globulin protein fractions was stored at -18°C for further analysis of amino acids. These results are not reported here.

### Total phenolics, tannins and hydrogen cyanide

Total phenolics and tannins were extracted and determined by spectrophotometric methods described by Makkar *et al.* (1998). Hydrogen cyanide concentration of the seed kernel was determined according to the method of Jackson (1967).

### Trypsin, chymotrypsin and $\alpha$ -amylase inhibitors analyses

Trypsin inhibitor activity was essentially determined according to Smith *et al.* (1980), except that the enzyme was added last, as suggested by Liu and Markakis (1989). Chymotrypsin inhibitor activity of defatted kernel meal was assayed in a 0.1 M Borate buffer, pH 7.6 by the procedure of Kakade *et al.* (1970).  $\alpha$ -Amylase assay and  $\alpha$ -amylase inhibitor activity was determined according to the procedure outlined by Deshpande *et al.* (1982).

### Estimation of phytic acid and assay of lectin activity

The phytate content of the sample was determined by a colorimetric procedure described by Vaintraub and Lapteva (1988). Results are expressed as percentage phytic acid by using standard phytic acid. Lectin activity was conducted according to the procedure described by Makkar *et al.* (1997) against 2% trypsinized cattle erythrocytes. The lectin activity against 3% trypsinized human erythrocytes (type O) also was performed.

### Total saponin estimation

Total saponin content was determined using a spectrophotometric method described by Hiai *et al.* (1976). The results are expressed as diosgenin equivalent from a standard curve of different concentrations of diosgenin in 80% aqueous methanol.

### Extraction, partial purification and haemolytic analysis of saponins

The seed kernel flour (25 g) was defatted with petroleum ether in a Soxhlet apparatus (10 h, 300 ml). After air-drying, the saponin extract was prepared from the defatted sample (10 g) obtained after Soxhlet extraction in 200 ml of 50% MeOH (18 h). The extract was centrifuged at  $3000 \times g$  for 20 min in a room temperature and methanol was removed by a rotary vacuum evaporator at 50°C. The aqueous phase was extracted three times with methanol:n-butanol (0.5:9.5, v/v). The combined extract was concentrated using a rotary vacuum evaporator at a temperature not higher than 50°C. The dried residue was dissolved in water and freeze-dried. The qualitative analyses were conducted by thin layer chromatography (TLC) (Burbano *et al.*, 1999) using Merck (1.05721) TLC plates (20 cm  $\times$  20 cm, silica gel 60). Each lane was loaded with 5  $\mu$ l of saponin extract (5 mg dissolved in 1 ml of methanol:water (4:6, v/v)). TLC separation was performed by using a solvent mixture of chloroform:methanol:water (65:35:10, v/v/v). After air-drying, the saponin spots on the developed plates were identified by spraying with *p*-anisaldehyde:glacial acetic acid:H<sub>2</sub>SO<sub>4</sub> (1:100:2, v/v/v) reagent and heated at 85°C for 15 min. To evaluate the haemolytic nature of saponins, another set of TLC plates was developed in the same manner and the



plates were sprayed with 6% trypsinized cattle and human (O type) blood erythrocytes suspension in phosphate buffered saline (PBS), pH 7.0.

For the haemolytic unit (HeU) activity determination, saponins enriched fraction (10 mg) was dissolved in 1 ml of PBS, pH 7.0. The fresh cattle red blood cells were washed three times with saline phosphate solution and a 3% suspension of red blood cells was prepared by using the same saline solution. A 50  $\mu$ l suspension of red blood cells was placed in separate wells of a microtitre plate. Fifty  $\mu$ l of twofold-diluted solutions of saponins (1 ml) were added into each well containing blood erythrocytes (50  $\mu$ l) and the mixture was incubated at room temperature ( $24 \pm 1^\circ\text{C}$ ) for 2 h. The haemolytic activity was expressed as the minimum amount of saponins extract per ml of the assay medium in the highest dilution, which was positive for haemolysis. One haemolytic unit activity (HeU) was defined as the least amount of saponins extract per ml in the last dilution giving positive haemolysis. For comparing the haemolytic activity of Gila bean saponins extract, a commercial standard triterpenoid saponin (S-2149, Sigma Chemical Co., St Louis, MO, USA) from Quillaja bark was used as a reference.

### **Fish assay**

Tilapia (*Oreochromis niloticus* L.) used were reared in the aquaculture laboratory of the Institute. The fish were maintained in aerated and good quality water for 2 days prior to the test. The crude saponins were extracted from the defatted Gila bean seed kernels by using the procedure described in the above section. A group of nine fish (average weight of 200 mg; length of 19–21 mm each) were placed in a 200 ml beaker containing 90 ml of water. A 0, 1, 2, 3, 4 and 5 mg of freeze-dried saponin extracts dissolved separately in 10 ml of water were added to the respective beaker. A similar experiment was also performed for standard saponins from Quillaja bark (Sigma). The experimental tests were conducted at room temperature ( $25^\circ\text{C}$ ) for 24 h with maintenance of above 75% oxygen saturation in the water, using an aerator.  $\text{LC}_{50}$  was calculated from the three independent experiments performed for each sample.

### ***In vitro* protein digestibility**

The *in vitro* protein digestibility (IVPD) of raw Gila bean seed kernel was measured according to a multienzyme technique (Satterlee *et al.*, 1979).

## **Results and Discussion**

### **Proximate composition**

The crude protein ( $256.7 \text{ g kg}^{-1}$ ), ash ( $27.3 \text{ g kg}^{-1}$ ), crude fibre ( $22.3 \text{ g kg}^{-1}$ ) and carbohydrate ( $585.7 \text{ g kg}^{-1}$ ) contents are comparable with those of commonly

consumed legumes. However, the level of oil ( $108.1 \text{ g kg}^{-1}$ ) in the seed kernel appears to be much higher than that in conventional pulses and moreover, due to the presence of such a high proportion of lipid, the kernel has a high energy value ( $20.21 \text{ MJ kg}^{-1}$ ).

### Protein fractionation

From the results of kernel fraction it is evident that albumins (predominant one;  $160.2 \text{ g kg}^{-1}$ ) and globulins ( $58.0 \text{ g kg}^{-1}$ ) together form the major seed proteins. This is in agreement with reports on *Pisum sativum* (Murray, 1979), whereas in other legumes globulin proteins have been reported to be a major fraction of the total seed proteins (Derbyshire *et al.*, 1976). However, the concentration of prolamin ( $2.5 \text{ g kg}^{-1}$ ) and glutelin ( $9.1 \text{ g kg}^{-1}$ ) proteins appears to be very low. The relative proportion of each fraction in a seed strongly affects the nutritional quality of the total seed proteins. In the present investigation, the presence of a major proportion of albumin fractions might also have been responsible for the higher content of essential amino acids in Gila bean kernel flour.

### Amino acid profiles

As has been reported in other legumes, Gila bean kernel protein has a relatively high concentration of aspartic and glutamic acid residues. It is noteworthy that all the essential amino acids of the seed kernel from *E. phaseoloides*, except tryptophan, register a higher value than the FAO/WHO (1990) recommended pattern and these compare well with that of soybean protein (Vasconcelos *et al.*, 1997). Moreover, cystine + methionine contents are comparable to hen egg and higher than the levels found in soybean. Thus, *E. phaseoloides* seeds could be a very good complement to other legumes and cereals, which usually are deficient in sulphur containing amino acids and lysine respectively. However, each of the fractions is distinct with respect to its amino acid composition. Among the water soluble and salt soluble fractions, albumin protein contains more threonine, cystine, methionine, isoleucine, tyrosine, histidine, lysine and tryptophan (essential amino acids) than the globulin fraction. Overall, the essential amino acid profiles of the kernel flour and albumin proteins of *E. phaseoloides* are better balanced than the profiles of the globulin proteins.

### Antinutritional factors and toxicity

Though food legumes are important sources of dietary protein in the developing countries, the acceptability and utilization of unconventional and little-known legumes has particularly been limited due to the presence of a relatively high concentration of certain antimetabolic factors. The total phenolics content ( $25.5 \text{ g kg}^{-1}$ ) is comparable to that of different cultivars of faba beans, however when compared to the level of tannins in different coloured-flowering cultivars of faba beans, the content in Gila bean kernels is very low ( $4 \text{ g kg}^{-1}$ ; Makkar *et al.*,

1997). Phytic acid decreases the bioavailability of certain minerals and may interfere with the utilization of proteins due to the formation of phytate-protein and phytate-mineral-protein complexes and also inhibits the digestive enzymes. In the present study the seed kernels of Gila bean contain  $13.7 \text{ g kg}^{-1}$  phytic acid and this value is relatively lower than those of commonly consumed legumes like faba bean and soybean (Makkar *et al.*, 1997). The cyanogen content of Gila bean seed kernels ( $0.021 \text{ g kg}^{-1}$ ) seems to be much lower than the value found in varieties of lima bean that are safe for human consumption (Conn, 1973) and comparable to those of *Vigna sinensis* and *Pisum sativum* (Montgomery, 1980).

The trypsin inhibitor activity of Gila bean seed kernels ( $96.7 \text{ mg g}^{-1}$ ) is three times higher than the values reported in soybeans ( $29.1\text{--}30.2 \text{ mg g}^{-1}$ ; Smith *et al.*, 1980) and about 30–40 times higher than of different cultivars of *Vicia faba* ( $1.72\text{--}3.35 \text{ mg g}^{-1}$ ; Makkar *et al.*, 1997). The activity of another inhibitor of protease enzyme, chymotrypsin, appears to be much higher ( $30 \text{ g kg}^{-1}$ ) when compared to the pigeon pea ( $0.2 \text{ CIU mg}^{-1}$ ) (Mulimani and Paramjyothi, 1995). Protease inhibitors in raw legumes impair their nutritional quality. It has been reported that upon feeding they depress animal growth, decrease dietary protein digestibility, reduce the sulphur and nitrogen absorption, stimulate pancreatic enzyme secretion, induce pancreatic enlargement and increase the synthesis and release of hormonal factors (Liener, 1994). Even though the underexploited legumes contain relatively high levels of protease inhibitors, these could be inactivated, either partially or completely, by adopting indigenous hydrothermal processing techniques. Haemagglutination activity ( $\text{HU mg}^{-1}$  sample) of Gila bean kernel against cattle erythrocytes and O-type human erythrocytes shows very weak agglutination of 0.02 and 0.01, respectively. Similarly, Grant *et al.* (1995) reported only weak lectin activity in the *E. phaseoloides* seeds against pre-treated rat erythrocytes. Hence the presence of such a low level of lectin activity in the present seed kernel could be nutritionally desirable and moreover, being a heat labile factor, it can be easily removed when subjected to dry or wet thermal treatments. The presence of the trypsin inhibitors and haemagglutinins in the Gila bean may account in part for the relatively poor *in vitro* coefficient of digestibility of the protein (0.67).

Saponins in general (either steroid or triterpenoid) have a lower surface tension and possess emulsifying properties. They are a diverse group of compounds possessing an aglycone moiety linked to one or more sugar or oligosaccharide residues. These, at high levels, can damage intestinal mucosal cells by altering cell membrane permeability and interfering with active transport and they also significantly reduce the food conversion efficiency (Gee *et al.*, 1993). Their haemolytic and antilipidaemic activities and their capacity to lower the serum cholesterol levels can be considered to be their most important characteristics. These effects are dependent upon the structure of the individual saponin molecules. Saponins are present as triterpene glycosides in significant amounts in legume grains and impart bitter taste to these plant foods (Oakenfull, 1981). The saponin content in Gila bean kernels ( $32.2 \text{ g kg}^{-1}$ ) appears to be much higher than the values reported in different conventional legume seeds such as

mung bean and chick pea (Fenwick *et al.*, 1991) and different cultivars of *V. faba* beans (Makkar *et al.*, 1997).

By the application of spraying reagents, five individual saponin compounds separated on the TLC plates were recognized visually at Rf value of 0.05, 0.15, 0.38, 0.45 and 0.48. Moreover, separate TLC plates showed three distinct heavy haemolytic spots at Rf values of 0.38, 0.45 and 0.48 by spraying with diluted cattle blood (6% trypsinized erythrocytes suspension in PBS). This is in good agreement with the previous report of three haemolytic saponins including saponin A and saponin B isolated from *E. phaseoloides* seed kernel (Watt and Breyer-Brandwijk, 1962). Furthermore, the efficiency of the haemolytic activity (HeU) of the above concentrated saponins extract determined, using the twofold dilution technique ( $128.0 \text{ HeU mg}^{-1}$ ), appears to be four times higher than the value found in Quillaja saponins ( $32.0 \text{ HeU mg}^{-1}$ ). These results have further confirmed the very strong haemolytic nature of saponins from Gila bean seed kernels (Watt and Breyer-Brandwijk, 1962). Fish are used as a biological method to evaluate toxicity of certain plant extracts. In the present study fish were exposed to different concentrations of Gila bean saponin extract and Quillaja saponin containing water ( $1\text{--}5 \text{ mg } 100 \text{ ml}^{-1}$ ; period of 24 h at  $25^\circ\text{C}$ ). A 50% mortality of fish was observed at a concentration of  $2.5 \text{ mg } 100 \text{ ml}^{-1}$ , whereas no mortality occurred in the Quillaja saponin-treated groups. Similarly, Khalil and El-Adawy (1994) also reported toxicities of saponins isolated from different legumes in fish and erythrocyte assays. Chopra *et al.* (1940) reported that *Entada pursaetha* seed kernel saponins, at doses of  $0.0005\text{--}0.002 \text{ g kg}^{-1}$  bodyweight in mammals, result in a sharp decrease of blood pressure, associated with an increase in the intestinal volume and to a lesser extent of the kidney. They also found that the respiratory system is depressed by the saponins and death seems to result from respiratory failure. Moreover, the smooth muscle of the intestine and of the uterus is also inhibited. However, the traditional method of processing by aqueous washing has been shown to reduce the saponin content and consequently not only the bitterness of the sample and its adverse activity against the intestinal mucosa (Gee and Johnson, 1988). Boiling and cooking could reduce the saponin concentration further to below toxic levels.

The *in vitro* digestibility coefficient (0.67) of Gila bean proteins observed in the present study is comparable to the levels reported for *Glycine max* (Gross, 1982). The digestible protein content ( $172 \text{ g kg}^{-1}$ ) is considerably lower than that of soybeans. The resistance of native legume proteins to proteolysis by mammalian digestive enzymes is an important factor contributing to the poor nutritive value of the unheated protein. The main contributing factors from native legume proteins for such poor digestibility are: (1) their structural characteristics, (2) the relative proportion of the globulin fraction and (3) the presence of protease inhibitors and other antinutrients (Liener, 1994). Hence, in the present study the high level of trypsin inhibitor activity might be one of the major barriers to proteolysis, rather than the other antinutrients.

## Conclusions

*Entada phaseoloides* seed kernel is rich in protein, has a well-balanced essential amino acid profile, a major proportion of albumin proteins and has a high lipid content. Among various antimetabolic factors investigated, trypsin and chymotrypsin inhibitor activities and saponin levels were found to be very high. After inactivation of heat labile and stable antinutrients through adopting cost effective and viable processing techniques, used by aborigines and village people, the Gila bean could serve as a cheap and additional protein source to alleviate protein malnutrition widely prevailing among the poorer section of the population in India.

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## Chapter 57

# The Detection and Estimation of Pyrrolizidine Alkaloids in Plants and Feeds using an ELISA

S.T. Lee<sup>1</sup>, T.K. Schoch<sup>2</sup>, B.L. Stegelmeier<sup>1</sup>, D.R. Gardner<sup>1</sup>, K.A. Than<sup>3</sup> and R.J. Molyneux<sup>2</sup>

<sup>1</sup>Poisonous Plant Research Laboratory, ARS, USDA, Logan, UT, USA; <sup>2</sup>Western Regional Research Center, ARS, USDA, Albany, CA, USA; <sup>3</sup>Australian Animal Health Laboratory, CSIRO Livestock Industries, Geelong, Victoria, Australia

### Introduction

Pyrrolizidine alkaloid-containing plants are found throughout the world and are common in the genus *Senecio*. Many pyrrolizidine alkaloids are toxic and cause poisoning in livestock and in humans (Bull *et al.*, 1968; Mattocks, 1986; Johnson *et al.*, 1989; Prakash *et al.*, 1999; Stegelmeier *et al.*, 1999). The alkaloid types and concentrations vary between plant species. In addition, within a species of plant concentrations vary with environment and location. Most pyrrolizidine alkaloid-containing plants produce mixtures of the free bases and their corresponding *N*-oxides in varying concentrations. In general, the free base and *N*-oxide forms are similar in toxicity if absorbed via the gut (Mattocks, 1986; Molyneux *et al.*, 1991). Rapid, sensitive and specific diagnostic techniques are needed to determine the plants and conditions under which livestock are likely to be poisoned and to monitor feeds and food supplies that are intended for livestock or human consumption. In this study a competitive inhibition (CI)-ELISA for riddelliine, riddelliine *N*-oxide and other closely related pyrrolizidine alkaloids was developed. This assay allowed us to estimate the total pyrrolizidine alkaloid content in *Senecio riddellii* and *Senecio madagascarensis* plant material and in *S. riddellii* admixtures with lucerne.

### Materials and Methods

#### Chemicals

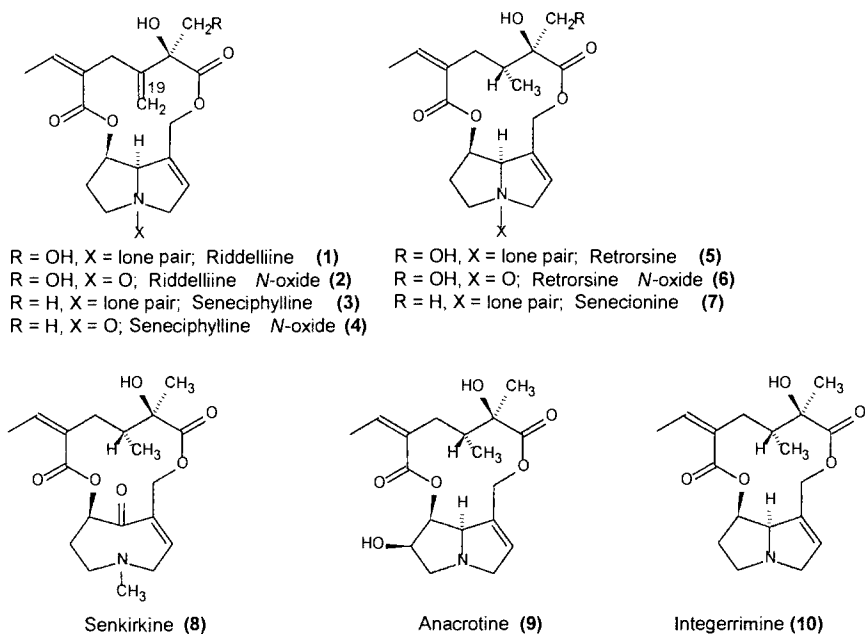
The pyrrolizidine alkaloids anacrotine (9), echinatine, heliotrine, integerrimine (10), lasiocarpine, retronecine and seneciphylline (3) were gifts from John A. Edgar (CSIRO, Livestock Industries, Geelong, Victoria, Australia). Junceine,



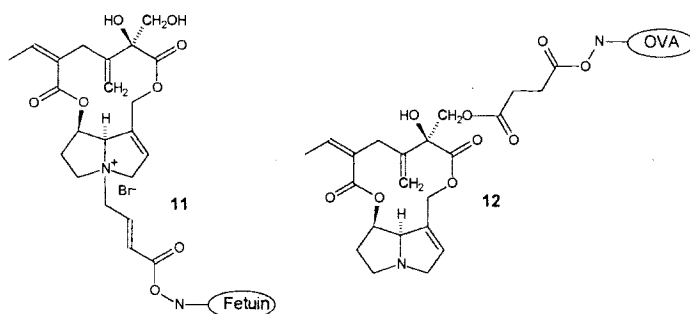
retrorsine (5), and senecionine (7) were obtained from Russell J. Molyneux (Western Regional Research Center, Albany, CA). Monocrotaline was purchased from Sigma Chemical Co. (St Louis, MO). Riddelliine (1) was extracted from *S. riddellii* collected near Hobbs, NM (PPRL collection 96-3,4) and senkirkine (8) was extracted from *S. madagascarensis* collected near Makau, HI. These alkaloids were isolated from plant material using methods previously described (Molyneux *et al.*, 1979). Monocrotaline *N*-oxide, retrorsine *N*-oxide (6), riddelliine *N*-oxide (2), and seneciophylline *N*-oxide (4) were synthesized by treatment of a solution of the alkaloid in chloroform-ethanol with 30% hydrogen peroxide (Mattocks and White, 1971). Figure 57.1 shows the chemical structures of these pyrrolizidine alkaloids.

### Immuno-conjugate and plate coating conjugate

Riddelliine (1) and riddelliine *N*-oxide (2) are not large enough molecules to be immunogenic *per se*. Riddelliine (1) was conjugated to a high molecular weight protein to elicit an immune response. Conjugation of riddelliine (RID) to fetuin (FET) was accomplished using the alkyl halide, *N*-[(4-bromocrotonyl)oxy]succinimide, to *N*-alkylate riddelliine with subsequent



**Fig. 57.1.** Chemical structures of 12-membered macrocyclic pyrrolizidine alkaloids.



**Fig. 57.2.** Riddelliine immuno-conjugate (11) and plate coating conjugate (12).

reaction with fetuin to form a 4-atom linker (11) (Fig. 57.2). This immuno-conjugate is referred to as RID-CROT-FET.

Riddelliine was conjugated to ovalbumin (OVA) to create a plate coating. The plate coating conjugate was achieved by reaction of the C-18 primary hydroxyl group on the molecule with succinic anhydride (SA) to form a hemisuccinate followed by activation of the carboxylic acid moiety with *N*-hydroxysuccinimide and reaction with the protein to form a 5-atom length succinate linker (12) (Fig. 57.2). This plate coating conjugate is referred to as RID-SA-OVA.

### Immuno-conjugate injections

Two ewes were initially injected subcutaneously with a primary injection solution (2 ml) of 0.4 mg of riddelliine-fetuin conjugate solution that contained DEAE-dextran, Quil A and Montanide 888. Booster injections with one-half the concentration of hapten-fetuin conjugate in the above injection solution were given after two 6-week intervals. Blood samples were drawn immediately before the initial injection and 14 days after the second booster injection. Sera was collected for use in the ELISA procedure.

### CI-ELISA

Antibody titres were determined. The sera from the sheep that resulted in the highest titres were selected for further ELISA development. A CI-ELISA was developed with the antisera raised against RID-CROT-FET immuno-conjugates, and the RID-SA-OVA conjugate was used as a coating conjugate. The optimum dilutions for both the coating conjugate and the antiserum were determined by checkerboard assays. The absorbances were measured at 450 nm ( $OD_{450}$ ).

## Plant material preparation

*Senecio riddellii* and lucerne plant material were air-dried, ground and 500 mg weighed into 50 ml extraction tubes in ratios of 0:100; 0.5:99.5; 5:95; 50:50; and 100:0 *S. riddellii*:lucerne. Methanol (25 ml) was added and the plant material extracted by continuously inverting the samples for 16 h. The samples were then centrifuged (25,000 rpm, 10 min, RT). The methanol extract (10  $\mu$ l) was diluted into 1990  $\mu$ l of assay buffer and 50  $\mu$ l applied to the microtitre plates. *Senecio madagascarensis* plant material was extracted in the same manner except it was not admixed with lucerne.

## Results and Discussion

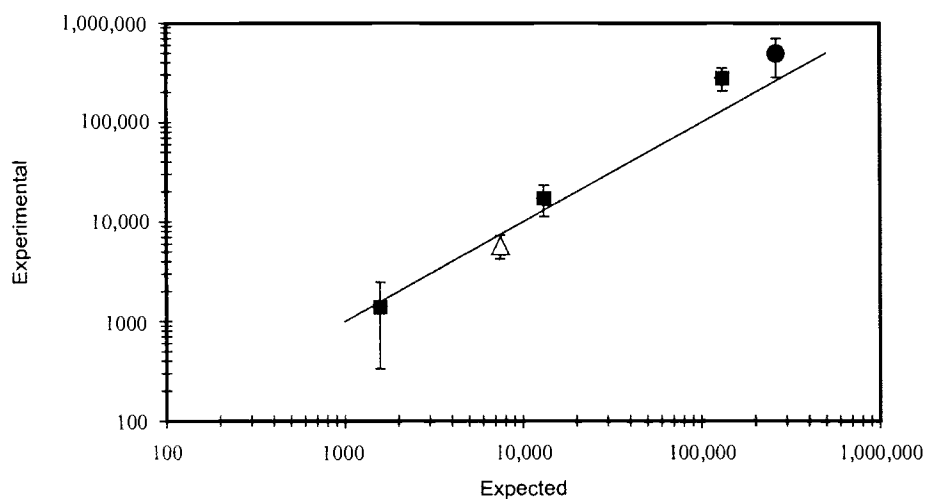
### Cross-reactivity

Sixteen pyrrolizidine alkaloids were selected for cross-reactivity studies: riddelliine *N*-oxide (2), seneciphylline (3), seneciphylline *N*-oxide (4), retrorsine (5), retrorsine *N*-oxide (6), senecionine (7), senkirkine (8), anacrotine (9), integerrimine (10), monocrotaline, monocrotaline *N*-oxide, junceine, echinatine, heliotrine, retronecine and lasiocarpine. Figure 57.1 shows the chemical structures of 12-membered macrocyclic pyrrolizidine alkaloids. The alkaloids were tested for cross-reactivity over the range of 1.95-1.024  $\times 10^6$  pg well<sup>-1</sup>. Table 57.1 reports the  $I_{50}$  values and the limit of detection (LOD) ( $I_{80}$ ). The  $I_{50}$  and  $I_{80}$  values are the mass of the alkaloid at which the absorbance is 50% and 80% of the maximum absorbance (or blank), respectively.

Riddelliine (1), riddelliine *N*-oxide (2), seneciphylline (3) and seneciphylline *N*-oxide (4) cross-react with the antibodies raised against the RID-CROT-FET immunogen (Table 57.1). The antibodies in this assay have some affinity to anacrotine (9), integerrimine (10), retrorsine (5), retrorsine *N*-oxide (6), senecionine (7) and senkirkine (8). All the pyrrolizidine alkaloids that cross-react with the antibodies are 12-membered macrocyclic diesters. Echinatine, heliotrine, junceine, lasiocarpine, monocrotaline, monocrotaline *N*-oxide and retronecine are not 12-membered macrocyclic diesters and showed no cross-reactivity with the antibodies over the concentration range studied in this assay. The *N*-oxide forms of riddelliine (1), seneciphylline (3) and retrorsine (5) have similar cross-reactivities to their corresponding free base pyrrolizidine alkaloids. Riddelliine *N*-oxide (2), seneciphylline (3) and seneciphylline *N*-oxide (4) have the highest cross-reactivity with riddelliine (1) and have the most similar macrocyclic diester structure of the compounds examined with this assay. Thus, we conclude the 12-membered macrocyclic diester and the methylene group at the C-19 carbon on these compounds are primary antigenic sites for the antibodies raised against the RID-CROT-FET antisera.

**Table 57.1.** Pyrrolizidine alkaloid cross-reactivity ( $I_{50}$ ) and limit of detection (LOD,  $I_{80}$ ).

Pyrrolizidine alkaloid	$I_{50}$ (pg)	LOD ( $I_{80}$ ) (pg)
Riddelliine (1)	$1.96 \times 10^3$	325
Riddelliine <i>N</i> -oxide (2)	$3.11 \times 10^3$	284
Seneciphylline (3)	$3.49 \times 10^3$	534
Seneciphylline <i>N</i> -oxide (4)	$2.11 \times 10^3$	31.3
Retrorsine (5)	$3.92 \times 10^4$	$6.11 \times 10^3$
Retrorsine <i>N</i> -oxide (6)	$3.32 \times 10^4$	$5.34 \times 10^3$
Senecionine (7)	$8.91 \times 10^5$	$1.38 \times 10^4$
Senkirkine (8)	$1.76 \times 10^4$	$1.74 \times 10^3$
Anacrotine (9)	$1.10 \times 10^5$	$7.93 \times 10^3$
Intergerrimine (10)	$7.29 \times 10^4$	$1.28 \times 10^4$



**Fig. 57.3.** Correlation between expected levels based on gravimetric determination of total pyrrolizidine alkaloids in plant material using extraction/isolation methods described in Molyneux *et al.* (1979) and the level detected by CI-ELISA in *Senecio riddellii* (●), *S. riddellii*-lucerne admixtures (■) and *Senecio madagascarensis* plant material (□).

## **Pyrrrolizidine alkaloids in plant material and lucerne admixtures**

This assay was evaluated for the detection and estimation of riddelliine (1), riddelliine *N*-oxide (2) and senkirkine (8) in plant material. Samples containing riddelliine (1) and riddelliine *N*-oxide (2) were quantified against a 10-point riddelliine *N*-oxide (2) standard curve over the range of 200 pg to 500,000 pg. *Senecio madagascarensis* samples were quantified against a 10 point senkirkine (8) standard curve over the range of 200 pg to 500,000 pg. Average recoveries for 0.263, 2.63, 26.3 and 52.66 parts per thousand riddelliine in *S. riddellii*-lucerne plant material ranged from 88 to 212% while relative standard deviations were 26-76%. Average recoveries for 1.50 parts per thousand senkirkine in *S. madagascarensis* plant material was 77% while the relative standard deviation was 26%. See Fig. 57.3.

A CI-ELISA for riddelliine (1), riddelliine *N*-oxide (2) and other closely related pyrrolizidine alkaloids was developed. The assay showed cross-reactivity to the *N*-oxide forms of riddelliine and closely related pyrrolizidine alkaloids in addition to the free base forms. The cross-reactivity between the *N*-oxide and free base forms of this assay allowed us to estimate the total pyrrolizidine alkaloid content in *S. madagascarensis* and in *S. riddellii*-lucerne admixture samples. The simple extraction-ELISA methods described in this chapter demonstrate the potential of using these techniques for the identification of toxic plants and rapid screening of feed and food supplies.

## **Acknowledgements**

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## Chapter 58

# Monocrotaline Pyrrole Protein Targets in Pulmonary Artery Endothelial Cells

M.W. Lamé<sup>1</sup>, A.D. Jones<sup>3</sup>, D.W. Wilson<sup>2</sup>, S.K. Dunston<sup>2</sup> and H.J. Segall<sup>1</sup>

<sup>1</sup>*Department of Molecular Biosciences and* <sup>2</sup>*Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA;* <sup>3</sup>*Department of Chemistry and Intercollege Mass Spectrometry Center, 152 Davey Laboratory, The Pennsylvania State University, University Park, PA 16802, USA*

### Introduction

Monocrotaline (MCT)-induced pulmonary hypertension remains a principal model for the biology and development of intervention strategies for the human disease. Monocrotaline, a pyrrolizidine alkaloid, is a phytotoxin used experimentally to cause a pulmonary vascular syndrome in rats characterized by proliferative pulmonary vasculitis, pulmonary hypertension (PH), and cor pulmonale (Kay and Heath, 1969; Chesney and Allen, 1973; Wilson *et al.*, 1992). Current concepts of pulmonary hypertension assign a primary pathogenetic role to the pulmonary endothelial cell in both human PH and that induced by MCT. Although MCT intoxication is used as a model for studying human PH, the initiating mechanism(s) by which this agent produces PH have remained elusive. To produce pulmonary insult MCT must first be activated by the liver to the putative electrophile monocrotaline pyrrole (MCTP) (Mattocks, 1968; Segall *et al.*, 1991), which has characteristics of a bifunctional crosslinking agent and is quite unstable (Mattocks and Jukes, 1990b). Stabilization of MCTP by red blood cells facilitates subsequent transport to the lung (Pan *et al.*, 1991). Previous work has supported the involvement of endothelial cells as the target for MCT induced PH, however the mechanism(s) by which these cells lose their ability to function correctly is unknown. The evidence for the involvement of the pulmonary endothelium as the target for MCT intoxication is supported by the circulatory proximity of the liver to the lung endothelium, evidence of increased thymidine uptake and decreased 5-hydroxytryptamine clearance by endothelial cells, and extravasature leakage of large macromolecules (Meyrick and Reid, 1982; Hoorn and Roth, 1992; Wilson *et al.*, 1992). With respect to pyrrole adduct formation this has been restricted to the measurement of covalent binding to endothelial cell DNA (Hoorn *et al.*, 1993; Thomas *et al.*, 1996). Monocrotaline pyrrole has been shown to react in a facile manner with the thiol groups of

cysteine and glutathione (Robertson *et al.*, 1977; Mattocks and Bird, 1983; Mattocks and Jukes, 1990a; Reed *et al.*, 1992; Lamé *et al.*, 1995). A carbonium ion can be generated at both the C7 and the C9 positions on the pyrrole ring with the pyrrole structure being stabilized by resonance structures that share charge with the bridge head nitrogen (Huxtable, 1979). This delocalization of charge for MCTP confers soft electrophile characteristics in line more with reactivity toward soft nucleophile protein side chains than with nucleic acids, which are harder nucleophiles. It has previously been shown that MCTP reacts with thiol groups on proteins such as haemoglobin (Mattocks and Jukes, 1990a; Seawright *et al.*, 1991; Lamé *et al.*, 1997). Of the limited number of proteins identified as specific targets for MCTP, cytochrome P450 3A, which is responsible for the dehydrogenation of MCT, has also been shown to form adducts with pyrroles (Reid *et al.*, 1998).

We have coupled the use of two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization (MALDI) to identify five major MCTP target proteins in human lung endothelial cells and discuss their potential relevance to the enigmatic process of pulmonary hypertension.

## Materials and Methods

### Production of $^{14}\text{C}$ -monocrotaline pyrrole

*Crotalaria spectabilis* was grown under a confined atmosphere of  $^{14}\text{CO}_2$  and the  $^{14}\text{C}$ -monocrotaline ( $^{14}\text{C}$ -MCT, purity > 98%) was extracted and purified as described before (Lamé *et al.*, 1996).  $^{14}\text{C}$ -MCT was converted to  $^{14}\text{C}$ -monocrotaline pyrrole ( $^{14}\text{C}$ -MCTP, 1.95 mCi mmol $^{-1}$ ) by the method of Mattocks *et al.* (1989).

### Tissue culture and sample preparation

Human pulmonary artery endothelial cells (HPAEC, passage 7 to 8) (Clonetics, San Diego, CA) from a 34-year-old female were grown to 80-90% confluence prior to treatment. Cells were incubated at 37°C, 5% CO $_2$  with humidity in EGM-2 medium (Clonetics). Following the application of  $^{14}\text{C}$ -MCTP, cells were removed from flasks, pelleted, washed with isotonic PBS and lysed. The bicinchoninic acid method was used to determine protein concentrations.

### Two-dimensional gel electrophoresis and autoradiography

Protein samples (60  $\mu\text{l}$ , 400  $\mu\text{g}$  protein) were diluted to 250  $\mu\text{l}$  with 8 M urea, 2% CHAPS, 18 mM DTT, and IPG buffer pH 4-7 was added directly to give a final concentration of 2%. A trace of bromophenol blue was added and samples (250  $\mu\text{l}$ ) were placed in a Immobiline DryStrip reswelling tray, and Immobiline DryStrips (IPG) (13 cm, pH 4-7) were placed gel side down in sample and a layer of mineral oil was added. The samples were allowed to equilibrate with the strips



overnight. Isoelectric focusing was performed on a MultiPhor II platform at 15°C. Further details regarding the isoelectric focusing conditions, transfer to membranes, extraction from the membranes and tryptic digests are available in Lamé *et al.* (2000).

### **Mass spectrometry**

Molecular masses of tryptic peptides were determined using a Voyager-DE STR MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA), using a nitrogen laser (337 nm) for ionization. To determine the identity of selected spots MS-Fit and in some cases MS-Tag were used to search databases for peptide mass fingerprints and to match fragment ions observed in post-source decay (PSD) spectra, respectively (Qiu *et al.*, 1998).

### **Western analysis of PDI and galectin-1**

An additional blot and the corresponding autoradiographic analysis were performed for antibody detection of protein disulphide isomerase (PDI) and galectin-1. PVDF membranes were stained with sulphorhodamine B and the proteins matched with autoradiographic spots. The primary antibodies were polyclonal rabbit raised against rat galectin-1 (generously provided by D.N.W. Cooper from UCSF) and a mouse monoclonal raised against rat protein disulphide isomerase synthetic peptide (StressGen Biotechnologies Corp., Victoria BC Canada).

## **Results**

### **Two-dimensional gel electrophoresis, autoradiography and peptide mass fingerprinting**

Of the 13 labelled spots indicating covalent adducts with MCTP, seven were chosen for analysis based on the amount of radioactivity associated with them and the intensity of the corresponding sulphorhodamine B stain. Of these seven spots, five were identified as probable protein disulphide isomerase precursor ER-60 (EC 5.3.4.1, Swiss-Prot P30101), protein disulphide isomerase precursor (PDI, EC 5.3.4.1, Swiss-Prot P07237), beta or gamma-cytoplasmic actin (Swiss-Prot P02570, P02571, respectively), cytoskeletal tropomyosin (TM30-NM, Swiss-Prot P12324) and galectin-1 (Swiss-Prot P09382).

### **Antibody recognition of galectin-1 and PDI**

Since these are two of the more interesting proteins found to be adducted by pyrroles, we determined if for future experiments they could be more simply identified through the use of antibody techniques. The commercially available

antibody to PDI was found to react with the protein previously identified using MALDI. The polyclonal rabbit antibody raised against rat lung galectin-1 was found to also cross react with human endothelial cell galectin-1 previously identified by MALDI.

## Discussion

Autoradiographic analysis showed that MCTP forms covalent adducts with specific proteins. The degree of selectivity observed is not surprising considering earlier work. Dehydroretronecine, a pyrrole formed from the reaction of water with MCTP, could alkylate, in decreasing order of reactivity, cysteine, tryptophan and histidine. Adduct formation with glutathione was more pronounced than with cysteine alone, indicating that the environment of a thiol group can increase its reactivity to pyrrole (Mattocks and Bird, 1983). Adduct formation would thus be more favourable for proteins containing a higher percentage of accessible cysteine residues and a micro environment which aids in thiol activation.

Galectin-1 is a lectin or carbohydrate binding protein, which in its dimeric state possesses two galactoside binding sites (Perillo *et al.*, 1998). The dimeric state can thus participate in both intramolecular and intermolecular crosslinkings through the interaction of more than one sugar residue (Perillo *et al.*, 1998). In rat lung, the  $\beta$ -galactoside-binding proteins are expressed in smooth muscle cells, type I alveolar epithelial cells and concentrated extracellularly in elastic fibres of the pulmonary parenchyma and blood vessels (Cerra *et al.*, 1984; Leffler and Barondes, 1986). Human aortic (HAECs) and umbilical vein (HUVECs) endothelial cells have also been shown to express galectin-1 *in vitro* (Baum *et al.*, 1995). In HAECs, the bulk of galectin is located within the interior of the cell with approximately 5% on the surface.

Galectins have been implicated as participants in cell adhesion, cell growth, immunomodulation, inflammation, embryogenesis, apoptosis, pre-mRNA splicing and metastasis. Although there are numerous biochemical and molecular studies relative to galectins, their *in vivo* role is basically unknown (Rabinovich, 1999), however the properties of galectin-1 make it an attractive and never before considered participant in the pathology induced by MCT treatment.

Protein disulfide isomerase (PDI) is located in the lumen of the endoplasmic reticulum at concentrations approaching mM levels (Lyles and Gilbert, 1991) and is responsible for the insertion of disulfides into folding proteins as well as correcting errors in disulfide formation (Gilbert, 1997). Protein disulfide isomerase also has been shown to act as a chaperone that binds to unfolded proteins and prevents their aggregation with other proteins (Cai *et al.*, 1994; Quan *et al.*, 1995; Song and Wang, 1995; Yao *et al.*, 1997). It also acts as a subunit for prolyl 4-hydroxylase as well as microsomal triglyceride transfer protein (Wilson *et al.*, 1998b). Because of the numerous functions of PDI, alteration or loss of activity due to alkylation or indirect perturbation in the redox environment by pyrroles could rapidly endanger the homeostasis of the endothelial cell.

Peptide mapping of spot four indicated the presence of either the beta or gamma form of cytoskeletal actin, but could not distinguish between the two. Previously we have shown, using SDS-PAGE, Western blotting and antibodies, that beta-actin was a potential target for pyrrole adduct formation (Wilson *et al.*, 1998a). Actin is important in the maintenance of the endothelial permeability barrier. Actin's contractile interactions with myosin regulates the endothelial permeability barrier and serves as an effector for inflammatory and procoagulant induced vascular leak. At cell-cell adherens junctions, actin is linked to the plasma membrane through interactions with  $\alpha$ -actinin, which associates with vinculin; vinculin is linked to catenins and plakoglobin which both interact with membrane associated cadherins. Cadherins in turn associate with cadherins from other cells through their extracellular domains. In the subendothelial matrix, fibronectin or vitronectin are bound to integrins which bridge the plasma membrane and in turn bind through talin to vinculin which is linked to  $\alpha$ -actinin thus to actin filaments (Lum and Malik, 1994). Alterations in actin or other proteins involved in the normal functioning of the endothelial barrier could result in extravascular leakage of plasma proteins and fibrin, which could stimulate vascular wall remodelling.

Studies with non-muscle isoforms of cytoskeletal tropomyosin have shown it to be important in the process of actin filament formation and structure and in the defining of domains along the actin filament (Fowler, 1990). Tropomyosin can block the bundling of actin by villin (Burgess *et al.*, 1987), the interaction of actin with filamin and  $\alpha$ -actinin (Zeece *et al.*, 1979; Nomura *et al.*, 1987) and inhibits fragmentation by gelsolin (Fattoum *et al.*, 1983). This protein, like the actin it influences, could be essential for preservation of the osmotic barrier provided by functional endothelial cells and, along with DNA adducts, could potentially explain some of observed perturbations observed in the cell cycle following MCTP treatment.

In the search for important molecular target(s) for MCTP, it should be considered that only small quantities of electrophile (MCTP) are essential to elicit pulmonary insult. Proteins that are found in low levels or function in enzymatic or signalling processes are potentially better candidates. Alterations in a small percentage of the PDI pool may be amplified if adducted PDI can still participate in enzymatic processes resulting in the incorrect formation of disulfides or fails to act as a proper chaperone causing the aggregation of unfolded proteins. ER60 has previously been found to be associated with the internal nuclear matrix and participates in the anchorage of the DNA loops at the matrix (Ferraro *et al.*, 1999). Such matrix DNA interactions have been shown to be important in the control of gene expression (van Driel *et al.*, 1991).

The proteins observed to form covalent adducts with MCTP and their resulting partial or total loss of function could be important elements in the puzzling MCT model induced PH. Further exploration of these proteins and their roles in both chemically precipitated or natural occurring PH warrant future investigation.

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## Chapter 59

# Analysis of Corynetoxins: a Comparative Study of an Indirect Competitive ELISA and HPLC

K.A. Than, Y. Cao, A. Michalewicz, V. Olsen, N. Anderton, P. Cockrum, S. Colegate and J.A. Edgar

*Plant Toxins Unit, CSIRO Livestock Industries, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria, Australia 3220*

### Introduction

Corynetoxins (CTs) are extremely poisonous and cumulative. These toxins are produced by a bacterium *Rathayibacter (Clavibacter) toxicus* which colonizes galls formed by a nematode, *Anguina* sp., in the seedheads of annual ryegrass/wimmera ryegrass (*Lolium rigidum*), annual beard grass (*Polypogon monspeliensis*) and blown grass (*Agrostis avenacea*). They are responsible for annual ryegrass toxicity (ARGT) in Western Australia and South Australia (Vogel *et al.*, 1981; Edgar *et al.*, 1982), Stewart's Range Syndrome in South Australia and floodplain staggers (FPS) in New South Wales (Bryden *et al.*, 1994; Edgar *et al.*, 1994). CTs have also caused poisoning of livestock feeding on contaminated hay (Roberts and Bucat, 1992; Nogawa *et al.*, 1997).

CTs have molecular masses of 800-900 daltons and are closely related, in structure, to the tunicamycin (TM) antibiotics, produced by *Streptomyces* (Edgar *et al.*, 1982) and to other tunicaminy-uracil toxins (Cockrum *et al.*, 1987). They differ from each other only in regard to the fatty acid side chain linked to the

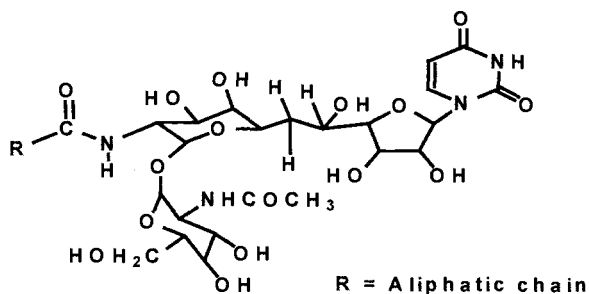


Fig. 59.1. Generic structure of CTs.

amino group of the central C<sub>11</sub> amino-sugar, tunicamine (Fig. 59.1). A total of 16 CTs with the fatty acid chain length of 15 to 19 carbon atoms have been identified (Cockrum and Edgar, 1983; Frahn *et al.*, 1984).

The objectives of this work were twofold: firstly to test the detection limit of a newly developed ELISA for the known CTs; and secondly to compare the levels of CTs in annual ryegrass galls using the high-performance liquid chromatography (HPLC) and the CT ELISA. Animal experiments were conducted under protocols approved by the CSIRO Livestock Industries, Australian Animal Health Laboratory animal ethics committee.

## Generation of Anti-CT Antisera

Two conjugation methods were applied. Method 1: Diamino derivatives of TM were conjugated to keyhole limpet haemocyanin and Australian foetal calf serum proteins using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Method 2: Australian fetal calf serum proteins were modified to generate free sulphhydryls by using N-succinimidyl S-acetylthioacetate (SATA) and coupled to diamino derivative of TM through a sulpho-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate heterobifunctional cross linker (sulpho-SMCC) (Than and Edgar, 1998a).

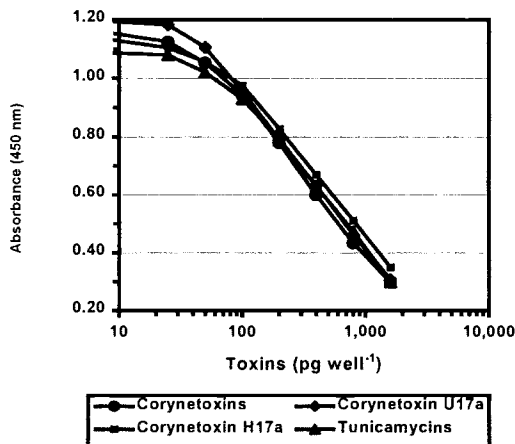
Each conjugate was administered to four sheep as an emulsion with 5 mg DEAE-dextran + 0.5 mg Quil A saponin per dose in 60% Montanide ISA 50V oil (Seppic) (Than and Edgar, 1998b). The total volume of 2 ml was injected under the skin at the back of the neck. The initial injections were followed by two boosters at 4 and 6 week intervals. From each sheep, 10 ml of blood was collected before and 2 weeks after each injection to monitor the antibody response. The suitability of the anti-CT antibodies produced by each sheep for CT ELISA development was determined by screening antisera for CT binding and competition with the free CT in the well on 4 ng chemically modified TM coated ELISA plates (Than *et al.*, 1998).

Sheep were again re-boosted twice at 2-monthly intervals and then after 5 months. The antiserum most suitable for the CT ELISA, described in the present paper, was obtained 1 year after the start of initial injection from a sheep injected with diamino derivative of TM conjugated to Australian fetal calf serum proteins using the EDC method. The serum can be diluted more than 1 in 10,000 and still showed an ELISA absorbance of 1.0 at 0 ng CT 100  $\mu\text{l}^{-1}$  well<sup>-1</sup> and the difference absorbance of 70-80% between 0 and 1.6 ng.

## Indirect Competitive ELISA

The CT ELISA utilizes an indirect competitive inhibition enzyme immunoassay system (Than and Edgar, 1998a; Than *et al.*, 1998). Free CTs are detected by the competitive inhibition of the binding reaction between solid-phase synthetic TM





**Fig. 59.2.** Comparison of absorbance of the wells with different amounts of mixed CTs, CT U17a, CT H17a, and mixed TM standards, showing competition for binding with 1/20,000 diluted sheep serum in glutaraldehyde-treated microtitre plates coated with 4 ng chemically modified TM well<sup>-1</sup>.

derivative (4 ng coated) and anti-TM/CT antibody generated in sheep. The unbound reagents and soluble toxin-antibody complex were washed away. The anti-CT antibodies bound to the surface of the wells are detected and quantitated by the addition of the appropriate dilution of anti-sheep IgG conjugated to horseradish peroxidase enzyme. After incubation the plates were washed and tetramethylbenzidine (TMB) substrate added and incubated for a further 15 min, before 0.05 M sulphuric acid is added to stop the colour reaction.

The concentration of free CTs present in the sample is directly proportional to the amount of inhibition. The degree of inhibition, compared to uninhibited controls is visualized by the intensity of colour development resulting from addition of TMB substrate. A comparison of the absorbances of the wells at 450 nm for different amounts of CTs, CT U17a, CT H17a, and TMs, shown in Fig. 59.2 indicates that the ELISA detects each to a similar extent.

## Detection

One gram of toxic annual ryegrass galls was extracted with 5 ml of 80% methanol in water (4 ml methanol + 1 ml water). A 25  $\mu$ l sample of the extract was filtered through 0.45  $\mu$ m Gelman hydrophilic polypropylene membrane and chromatographed on C-18 reversed phase HPLC column (Supelco discovery C18, 5  $\mu$ m particle size, 2.1 mm internal diameter and 150 mm in length). Fractions were collected at 1 min intervals and the collection run was performed at 0.2 ml min<sup>-1</sup> at the gradient programme, routinely used for crude extracts. Solvent A is

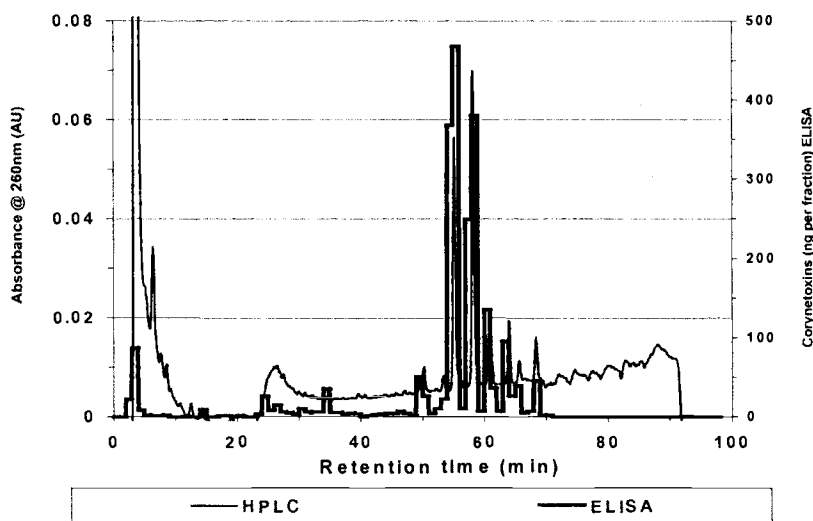
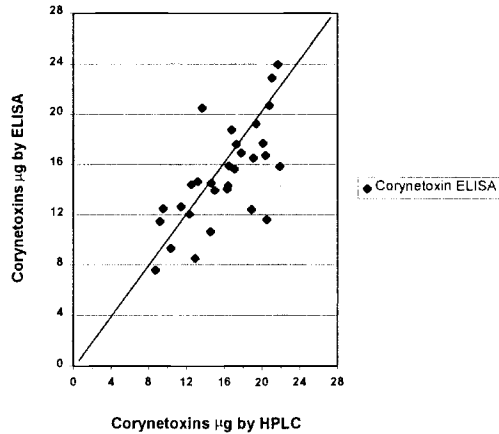


Fig. 59.3. The indirect competitive CT ELISA, using 1/20,000 dilution of sheep serum and HPLC profile of an extract of toxic annual ryegrass gall at 260 nm.

water, solvent B is methanol, both containing 0.1% v/v trifluoroacetic acid. The HPLC elution profile used was 40% B from 0 to 18 min, 40 to 65% B from 18 to 20.5 min, 65% B from 20.5 to 23 min, 65 to 95% B from 23 to 83 min, 100% B from 83 to 87 min, 100 to 40% B from 87 to 88 min and 40% B from 88 to 99 min. The fractions were assayed using the indirect competitive CT ELISA. The ELISA detects all the known CTs as well as an extra peak, eluting very early in the HPLC run, which is possibly a water-soluble precursor or metabolite of the CTs (Fig. 59.3)

## Validation

Deionized water (200  $\mu$ l) was added to each of 30 tubes containing six annual ryegrass bacterial galls still within the seed husk. After 1 hour, the swollen and softened bacterial galls were crushed using a polyethylene rod. Methanol (800  $\mu$ l) was added, washing down the rod in the process. The samples were shaken for 20 hours, centrifuged and the supernatants removed. The supernatants were then assayed by HPLC (Cockrum and Edgar, 1985) and by indirect competitive CT ELISA. The mean corynetoxin content ( $\mu$ g sample<sup>-1</sup>) of six crushed galls extracted with 80% methanol was 16.00 ( $\pm$  3.98 SD) when assayed using HPLC and 15.10 ( $\pm$  4.01 SD) by indirect, competitive CT ELISA. Paired data were



**Fig. 59.4.** Comparison of the analysis of CT content ( $\mu\text{g sample}^{-1}$ ) of six crushed galls extracted by 80% methanol and assayed by HPLC and indirect competitive ELISA.

analysed by using a *t*-test: paired two sample for means. The data showed that means of CT content ( $\mu\text{g sample}^{-1}$ ) assayed by HPLC and by ELISA were not significantly different:  $P(T \leq t)$  one-tail = 0.06,  $P(T \leq t)$  two-tail = 0.13. When a linear relationship with zero intercept was fitted to the data, the regression slope of the line estimated by least squares method was 0.929 and the corresponding correlation coefficient between HPLC and ELISA was estimated as 0.907,  $P < 10^{-6}$  (Fig. 59.4). Therefore, the CT ELISA detected and quantitated the CTs in the samples as accurately as the HPLC technique.

## Conclusion

The CT ELISA is sensitive to CT above  $400 \text{ pg ml}^{-1}$  (400 parts per trillion) in the sample after being diluted at 1:10 in assay buffer. The CT ELISA is robust and showed no adverse effect due to the presence of extractives from the matrix when wheat, barley and fodder samples were extracted with 70-80% methanol or 1% to 2% methyl- $\beta$ -cyclodextrin. The CT ELISA has been successfully used for the analysis of CTs in crude extracts of 77 wheat, 77 barley and 113 fodder samples.

## Acknowledgements

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## Chapter 60

# Pavetamine: an Inhibitor of Protein Synthesis in the Heart

R.A. Schultz<sup>1</sup>, N. Fourie<sup>2</sup>, K.M. Basson<sup>1</sup>, L. Labuschagne<sup>1</sup>,  
L.D. Snyman<sup>1</sup> and L. Prozesky<sup>3</sup>

<sup>1</sup>Onderstepoort Veterinary Institute, Onderstepoort, 0110, Republic of South Africa; <sup>2</sup>Intervet, Private Bag x2026, Isando, 1600, Republic of South Africa; <sup>3</sup>University of Pretoria, Pretoria, 0001, Republic of South Africa

### Introduction

One of the six most important plant poisonings in southern Africa is gousiekte, a disease of ruminants characterized by acute heart failure without premonitory signs 6-8 weeks after the initial ingestion of certain rubiaceaceous plants (Kellerman *et al.*, 1988, 1996). The active principle of gousiekte plants has been isolated (Fourie, 1994; Fourie *et al.*, 1995) and identified (R. Vleggaar, Pretoria, 1997, personal communication) as pavetamine. The yield from one of the causative plants (*Pavetta harborii*) is extremely low and in order to address this problem a synthesis of pavetamine is currently underway.

The group of compounds (polyamines) to which pavetamine belongs is much studied and contains highly biologically active substances affecting many functions in the body, including cell growth. An investigation of the relative toxicity of pavetamine towards cancer cells (HELA cells) compared to lymphocytes gave promising results (C. Medlen, Pretoria, 2000, personal communication). A literature survey has shown that synthetic polyamines are being investigated as possible chemotherapeutics against HIV (Bergeron *et al.*, 1999). Pavetamine is the only naturally-occurring member of this group to be incriminated in the poisoning of stock.

### Materials and Methods

Young male rats (Sprague-Dawley strain, live mass 180-307 g) were individually housed in cages and supplied *ad libitum* with standard rat pellets and water. Sixteen hours prior to use, food was removed from the animals' cages.

Three trials were conducted to investigate the rate of protein synthesis in rat organs over a period of time, specifically at 4 h, 24 h and 48 h after administration of pavetamine. Each group of Sprague-Dawley rats comprised seven controls and seven test animals and each rat was injected intraperitoneally with 10 mg kg<sup>-1</sup> pavetamine

as an aqueous solution. A flooding dose of 10  $\mu\text{Ci}$  ( $^3\text{H}$ )phenylalanine was administered intraperitoneally in every case 2 h before termination of the experiment. Samples of the different rat organs were analysed for rate of protein synthesis by measuring protein content and the level of radioactivity using liquid scintillation, according to the methods of Garlick *et al.* (1980) and Thompson and Wannemacher (1990). The results for treated rats, measured as  $\text{dpm mg}^{-1}$  protein, were plotted as a percentage of the level found for control rats for each tissue at each period.

## Results

These experiments show that one of the effects of pavetamine is inhibition of protein synthesis, more pronounced in certain organs than in others. Figure 60.1 indicates the pavetamine treatment group computed as a percentage of the control group, while the number of asterisks (\*, \*\* and \*\*\*) shows the level of significant difference from the control at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$  respectively.

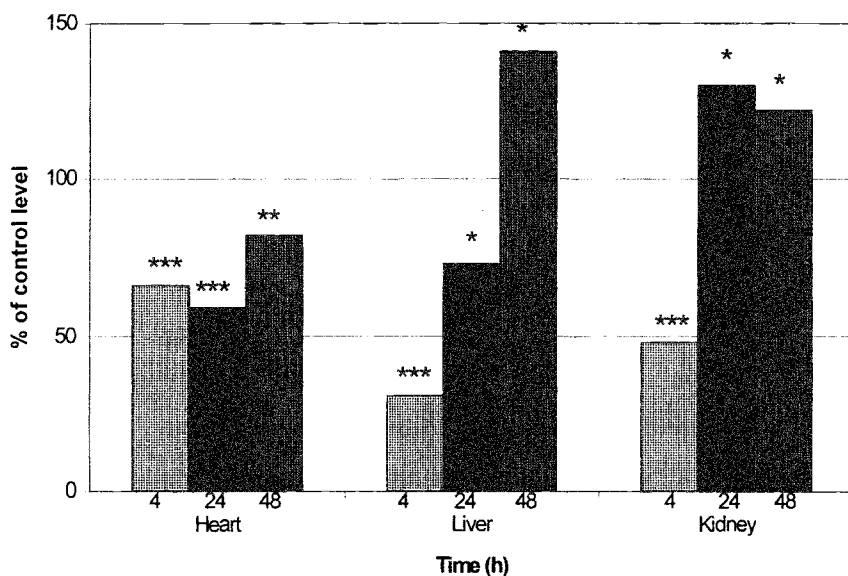


Fig. 60.1. Effect of pavetamine on the synthesis of protein in rat tissue.

Protein synthesis levels were less than 62% of those of controls in the heart, liver and kidney tissue of rats 4 h after exposure to a lethal dose of pavetamine. In contrast to this, the levels in muscle, spleen and intestine tissues were hardly affected. In the liver and kidneys of rats the protein synthesis returned to normal (or overcompensated) between 24 and 48 h after pavetamine was administered. Protein synthesis in the rat heart, however, remained suppressed (less than *c.* 80% of controls) for as long as 48 h after administration. Control rats died 5 days after injection of pavetamine.

## Discussion

Sheep hearts affected by gousiekte sometimes show macroscopic lesions such as endocardial fibrosis. Electron microscopy studies show a reduction in the number of myofilaments, especially myosin (Kellerman *et al.*, 1988). The contractile proteins in the myocardium of normal hearts are constantly broken down and re-synthesized (Swick and Song, 1974). Results from the current study in rats enable us to postulate that while the body is breaking down myosin in its ongoing turnover of myocardial proteins, pavetamine is inhibiting the formation of new myosin. Inhibition of protein synthesis can thus explain the typical latent period of gousiekte. The seemingly specific action of pavetamine on the heart can most probably be attributed to the fact that, as indicated by this study, the other organs recover much faster (Schultz *et al.*, 2001).

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## Chapter 61

# Effects of the Mycotoxins Lolitrem B and Paxilline on Gastrointestinal Smooth Muscle, Cardiovascular and Respiratory Systems and Temperature in Sheep

B.L. Smith<sup>1</sup>, L.M. McLeay<sup>2</sup> and G.W. Reynolds<sup>3</sup>

<sup>1</sup>Toxicology and Food Safety Research Group, AgResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand; <sup>2</sup>Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand;

<sup>3</sup>Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand

### Introduction

Certain fungi produce toxins that elicit tremoring in animals and among these are the lolitrems and paxilline which are classified in a group based on their common indole nucleus linked to a diterpenoid unit (Steyn and Vleggaar, 1985). Lolitrem B is responsible for ryegrass staggers in livestock (Gallagher *et al.*, 1981, 1984), but its activities have not been studied extensively in animals due to difficulties in its extraction and purification. Paxilline produced both by *Penicillium* species of fungi (Steyn and Vleggaar, 1985) and *Acremonium lolii* (Weedon and Mantle, 1987) is thought to be a precursor in the biosynthesis of lolitrem B by *A. lolii* (Penn and Mantle, 1994; Munday-Finch *et al.*, 1995). It is more readily available, causes similar but shorter-lasting clinical signs in animals, and provides a valuable comparison in studies on the lesser known activities of lolitrem B. While most studies in ruminants have been related to tremoring, we have investigated the effects of paxilline and lolitrem B on smooth muscle of the stomach and intestine (Smith *et al.*, 1997; McLeay *et al.*, 1999) and now on other physiological activities including blood pressure, heart rate, respiration and body temperature.

### Materials and Methods

Lolitrem B and paxilline were prepared by the methods described in McLeay *et al.* (1999). Both tremorgens were dissolved in acetone (2 ml) and dose rates chosen (0.66-1.5 mg kg<sup>-1</sup> for paxilline and 25-110 µg kg<sup>-1</sup> for lolitrem B) that produced moderate tremors in sheep (Smith *et al.*, 1997).

Eight animals were used in the experiments on gastrointestinal smooth muscle and full details of the methods have been previously described (Smith *et al.*, 1997; McLeay *et al.*, 1999). Electromyogram (EMG) activity was recorded from electrodes surgically implanted in the reticulum, cranial dorsal rumen, abomasal antrum and duodenum. EMG activity was integrated and used as an index of motility. For the experiments on other physiological functions, three 9-month-old castrated male Romney x Dorset sheep (30-35 kg) were maintained indoors in pens on a daily diet of 1200 g dried chaffed meadow hay and 100 g concentrate pellets. Each sheep was prepared under general anaesthesia with a catheter inserted via a branch of the femoral artery, so that the tip lay in the abdominal aorta. The catheter was tunnelled subcutaneously and exteriorized in the mid-lumbar region. It was filled with heparinized saline (5 IU ml<sup>-1</sup>) and usually flushed at 2 day intervals. Such preparations were maintained for at least 6 months without blockage. At the start of each experiment the catheter was flushed with sterile saline and connected to a physiological pressure transducer and preamplifier, for the recording of blood pressure. The transducer was mounted at the estimated level of the abdominal aorta in the sheep's standing position.

Before each tremorgen challenge, three platinum cap electrodes were sutured to the skin over the left shoulder muscles, which had produced good electromyographic recordings (Smith *et al.*, 1997). The electrodes from skeletal muscle and gut smooth muscle were connected to AC preamplifiers and the EMGs integrated using an integrator (McLeay *et al.*, 1990). Movement of each animal's head was monitored over the experimental period with an Actiwatch activity monitoring system. The Actiwatch was secured to the top of the head with Velcro and the recording downloaded for analysis.

Temperature was continuously recorded with a 20 gauge needle thermistor inserted into a Longissimus dorsi muscle and connected to a preamplifier at a range of 35-45°C. Respiration was recorded by strapping corrugated bellows around the abdomen and connecting them to a pressure transducer. Outputs from the preamplifiers for blood pressure, respiration and temperature and from the integrator for EMG activity were recorded using a chart recorder. Respiration rate and heart rate were measured by running the chart faster for a 10 s interval. All outputs were captured on disc and analysed using a program, which integrated volts under the trace over time.

At least 24 h prior to each experiment, an in-dwelling intravenous catheter was inserted into the jugular vein for administration of tremorgens. Prior to each administration, a normal recording was collected for 30 min followed by a further 30 min immediately after the administration of acetone and immediately prior to the administration of tremorgen in the same volume of acetone. In addition, as separate controlled experiments, two consecutive administrations of 2 ml of acetone, 30 min apart were given. The 2 ml of infusate was given slowly over 2 min into the jugular catheter and washed in with 2 ml heparinized saline.

Blood pressure, heart rate, respiration rate, temperature and EMG activity of skeletal and gut smooth muscles were observed for each sheep from the chart recordings and on head movement from the Actiwatch records. Quantitative data were normalized for each animal in volts over 15 min periods, as a percentage of

the average of the first two 15 min periods. The maximal increases in heart rate, blood pressure, respiration rate and temperature above control values before the first administration of acetone were averaged for the three sheep. The statistical significance of mean maximum changes for each tremorgen, compared with the corresponding period of the acetone control was tested using Student's *t*-test. Clinical observations of all animals were recorded.

This work was approved by the ethics committees of both Waikato University and the Ruakura Research Centre.

## Results

Both tremorgens produced tremors and increased movement of the head as measured by the Actiwatch and increased EMG activity of skeletal muscle. The effects of paxilline and lolitrem B on activity of skeletal muscle differed in that there is a very short latency (2 min) and relatively short longevity (2 h) with paxilline, whereas with lolitrem B the onset is delayed for about 30 min, but effects persisted for at least 12 h.

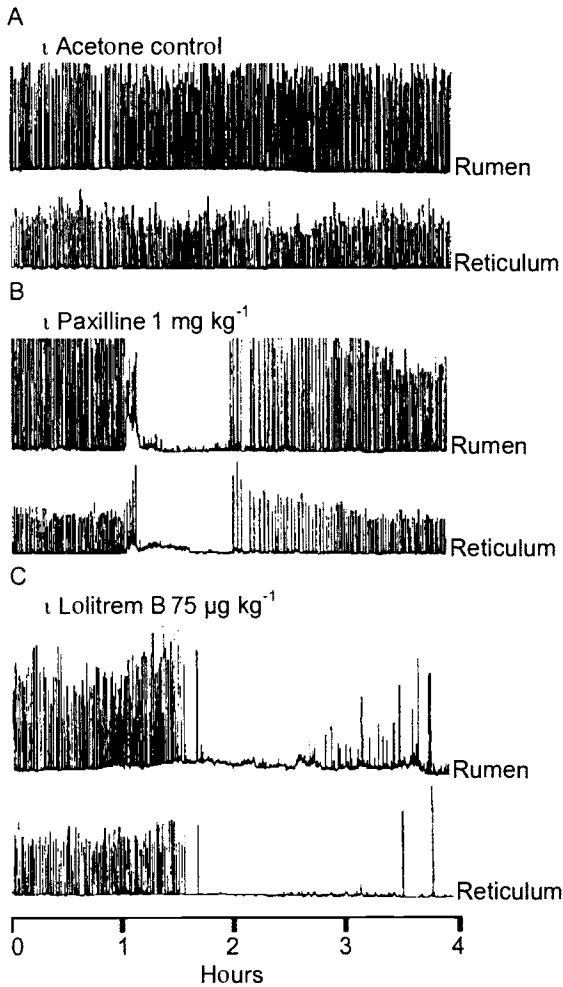
Paxilline was inhibitory on the antrum in two of the five animals, an effect that was immediate and lasted for up to 1 h. A reduction in duodenal activity was obtained in four animals and in the other marked stimulation of phase III MMC-like activity occurred. Paxilline was both excitatory and inhibitory on the reticulum and rumen. The excitatory effects occurred within the 2 min infusion time and took the form of elevations in baseline of both the reticulum and the rumen EMG and increases in the amplitude of the reticulum EMG associated with A sequences, both before and after, inhibitory responses occurred (Fig. 61.1).

An inhibitory effect on A and B sequences of contraction became apparent soon after the elevation in baseline activity and these were completely abolished for up to about 1 h (Fig. 61.1). A and B sequences of contraction were maximally reduced in frequency (A sequences  $P < 0.01$ , B sequences  $P < 0.001$ ) from 30-60 min and were reduced in frequency for a total period of about 2.5 h (Fig. 61.1).

Lolitrem B inhibited activity of the antrum in three sheep, the effect commenced within 30 min and continued for up to 2 h. Duodenal activity was reduced in two animals and stimulated in a third animal. Lolitrem B inhibited the frequency of A and B sequences of contraction of the reticulum and rumen ( $P < 0.001$ ). Compared with paxilline the inhibition was delayed, becoming apparent after about 20-30 min (Fig. 61.1), and was associated with the appearance of tremors. A and B sequences of contraction were virtually completely inhibited or markedly reduced in frequency (Fig. 61.1) for up to 11-15 h following administration of lolitrem B. In two sheep an elevation in baseline EMG of the reticulum changed to activity which took the form of lower amplitude, faster frequency activity than that associated with A sequences (defined as 'chaotic' activity), and this marked excitatory activity was sustained for many hours during which time A and B sequences were abolished. The re-appearance of A and B sequences after giving lolitrem B coincided with decreased 'chaotic' activity and

gradually normal motility and rumination returned after about 12 h. In contrast to the reticulum, the rumen did not exhibit 'chaotic' activity.

Both tremorgens induced tremor and caused marked increases in respiration rate, heart rate and blood pressure, but had relatively little effect on body temperature (Table 61.1).



**Fig. 61.1.** Integrated electromyograms of the rumen and reticulum recorded over 4 h in the same sheep during administration at 1 h of (A) acetone control, (B) 1 mg kg<sup>-1</sup> paxilline and (C) 75 µg kg<sup>-1</sup> lolitrem B. The recordings are computer printouts of the stored data.

**Table 61.1.** Maximal increases (means and standard deviations for three animals) in systolic (BPS) and diastolic (BPD) blood pressure, heart rate (HR), respiration rate (RR) and body temperature (T) after administration of acetone or tremorgens.

	HR (min <sup>-1</sup> )	RR (min <sup>-1</sup> )	BPS (mm Hg)	BPD (mm Hg)	T (°C)
Acetone 0	0	34 ± 19	4 ± 17	4 ± 7	0.17 ± 0.3
Paxilline	88 ± 27**	122 ± 19**	83 ± 7**	65 ± 25*	0.73 ± 1.1
Lolitre B	92 ± 28**	102 ± 24*	52 ± 26*	38 ± 18*	1.13 ± 0.8

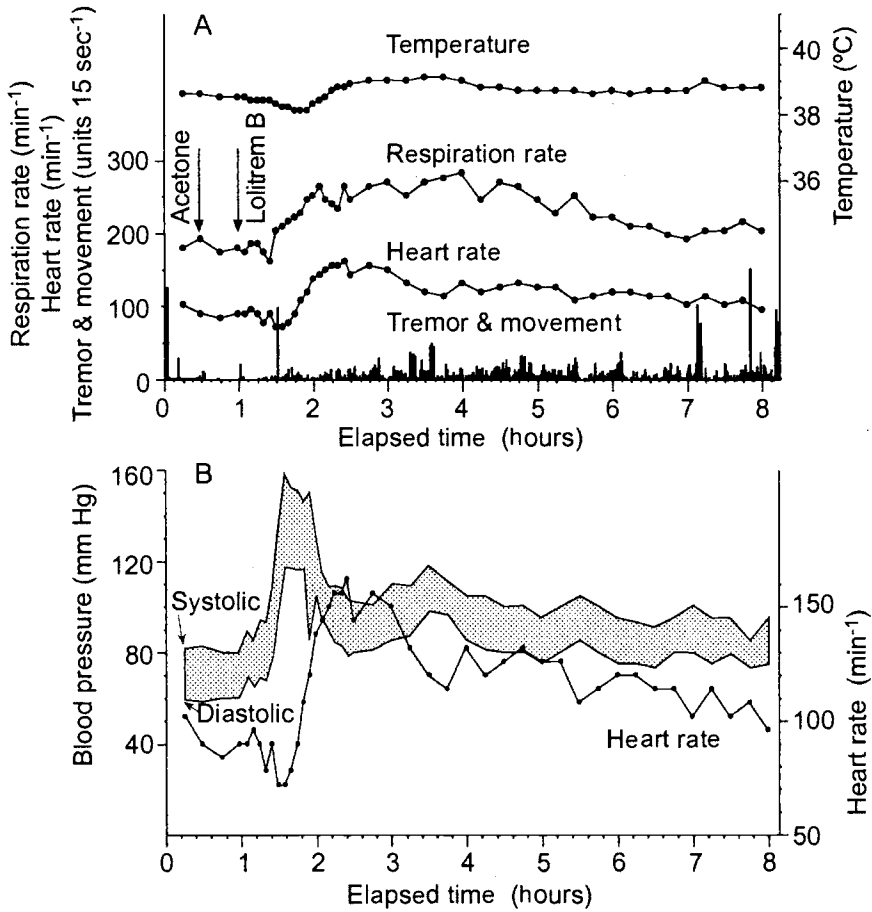
\* Indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ , between acetone and tremorgens.

Increases in blood pressure in animals given paxilline doubled to reach a maximum within 5 min in all three animals and gradually declined over the next 2 h. In one animal, heart rate had doubled by 10 min and gradually declined over the next 2 h, showing a similar trend to blood pressure. In a second animal, heart rate remained stable and in the third animal, heart rate increased 1 h after paxilline, at a time when blood pressure was dropping. Blood pressure increased between 15 and 25 min and reached a maximum 1-1.5 h after lolitre B (Fig. 61.2B). Thereafter blood pressure gradually declined, but remained above control levels for at least 7 h (Fig. 61.2B). Heart rate showed similar responses, but the rises and maxima reached were delayed, so that responses in blood pressure always preceded those in heart rate (Fig. 61.2B).

Respiration rate and body temperature increased in association with tremoring, which was short term (1-2 h) with paxilline and long term (up to 7-8 h) with lolitre B (Fig. 61.2A). Respiration rates in these animals were relatively high under control conditions, but increased further with administration of the tremorgens and took the form of high frequency, shallow depth panting.

## Discussion

Paxilline and lolitre B had consistent and profound excitatory and inhibitory effects on smooth muscle of the reticulum and rumen, whereas their effects on the abomasal antrum and duodenum were less marked and variable. In contrast to motility of the abomasum and duodenum, the cyclical A and B sequences of contractions of the reticulorumen are dependent upon the release of acetylcholine from the terminals of the vagus nerve (Titchen, 1968). Previous work has indicated that excitatory effects of paxilline on smooth muscle of guinea pig intestine involved acetylcholine release (Selala *et al.*, 1991). If the tremorgens influence neurotransmitter release or action, effects on motility of the reticulorumen should have been clearly evident and this was the case. Paxilline has been shown to inhibit high-conductance calcium-activated K<sup>+</sup> channels



**Fig. 61.2.** Changes in physiological activities over 10 h following the administration of control acetone at 30 minutes and  $75 \mu\text{g kg}^{-1}$  lolitrem B at 1 h in Sheep 1. In A, tremor and movement (T and M activity), heart rate, respiration rate and body temperature. In B, systolic and diastolic blood pressures and heart rate.

(Knaus *et al.*, 1994) and these have been shown to regulate neurotransmitter release (Robitaille and Charlton, 1992; Stretton *et al.*, 1992). Consequently it has been argued that this is one possible mechanism for the excitatory effects of paxilline and lolitrem B on the reticulorumen via an atropine-sensitive pathway (McLeay *et al.*, 1999).

The inhibitory effects of paxilline and lolitrem B on the cyclical motility of the reticulorumen may arise through a number of pathways (McLeay *et al.*, 1999). These contractions are stimulated by excitation of vagal efferent fibres

originating from gastric centres in the medulla oblongata of the brain, which in turn are excited or inhibited by afferent stimulation from many areas of the gut, including the reticulorumen itself (Titchen, 1968; Harding and Leek, 1971). Reflex inhibition of motility results from pharmacologically-induced increased local activity of the reticulorumen (Ruckebusch, 1989). Increased intrinsic activity was a feature of the excitatory effects of paxilline and lolitrem B on the reticulum and rumen in our experiments and may have been responsible for the inhibitory effects on cyclical contractions.

The responses in blood pressure and heart rate to paxilline and lolitrem B indicate the initial effect was on peripheral vasoconstriction, followed by increased heart rate. Vasoconstriction may have arisen through blockade of high-conductance calcium-activated  $K^+$  channels, as these have been shown to regulate smooth muscle tone of arteries, their activation causing relaxation (Braydon and Nelson, 1992; Nelson *et al.*, 1995). Increases in heart rate occurred at a time when blood pressure was dropping from its maximal level (see Fig. 61.2B), but was still above basal levels and thus was unlikely to be a reflex response to lowered blood pressure. Generally, increases in heart rate, respiration rate and body temperature were closely associated with tremoring and included discrete changes in association with the 'clonal tremors' (groups of tremors interspersed with quiescent periods) of lolitrem B, for example 7 h after its dosing (see Fig. 61.2A). These responses were probably indirect, reflecting the greater oxygen demands and heat production resulting from the increased skeletal muscle activity of tremoring. Panting is an important mechanism whereby sheep control body temperature. An initial drop in temperature after lolitrem B administration had a similar time course to the rise in blood pressure (see Fig. 61.2A and B), and may have been due to peripheral vasoconstriction reducing blood flow to the region in which the thermistor was located.

These experiments have shown that as well as affecting tremoring and producing the well known staggers syndrome, paxilline and lolitrem B have widespread effects on other physiological activities. Their effects on smooth muscle of the gut are likely to have serious consequences for digestion in animals ingesting these tremorgenic mycotoxins. Evidence of impaired digestion of animals grazing endophyte-infected pastures has been reported as diarrhoea, dag formation and illthrift (Fletcher, 1993). While our present experiments have investigated effects on smooth muscle of the gut, one likely mechanism of action of the mycotoxins involving ion channels in cell membranes and their effects on neurotransmitter release and action suggests that they may also affect the secretory activities of the gut, which, together with alterations in motility, would contribute to clinical features such as diarrhoea. It is also likely that the vascular effects of the tremorgens will exacerbate the effects of such ergopeptides present in ryegrass as ergovaline or those present in other species such as fescue.

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## Chapter 62

# Conditioned Feed Aversion as a Means of Preventing Sheep from Grazing Vermeerbos (*Geigeria ornativa*)

L.D. Snyman and J.P.J. Joubert

*Onderstepoort Veterinary Institute, P/bag X5, Onderstepoort, 0110, South Africa*

### Introduction

Vermeerbos (*Geigeria ornativa*, family Asteraceae) is a sesquiterpenoid-lactone containing plant that occurs in the Northern Cape Province of South Africa. Ingestion of this plant causes vermeersiekte, which amongst other symptoms is characterized by regurgitation of rumen contents through the mouth and nose (Kellerman *et al.*, 1988). Although it is one of the most important plant poisonings of sheep on the sub-continent, no means for the prevention or treatment of vermeersiekte in sheep has yet proved possible.

Conditioned feed aversion is potentially a means of teaching stock to avoid poisonous plants (Provenza *et al.*, 1992). The induced aversion, however, is easily broken down by the social influence of non-averted animals (Ralphs and Olsen, 1990). In a previous study (Snyman *et al.*, 2001), the hypothesis was made that aversion to vermeerbos could be maintained if the initial aversion conditioning is followed by sustained conditioning, accomplished by means of continuous exposure to an aversive mixture. The aversive mixture, which contained an aversive substance and the identification factors of vermeerbos, was much more palatable than vermeerbos and therefore tempted sheep to frequently eat small amounts of the aversive mixture whereby sheep were persistently kept averted to the vermeerbos. Results of the investigation proved the hypothesis to be workable. Three sheep that were averted to an established vermeerbos pasture persistently refused grazing the vermeerbos over a 42-day period, despite the social influence of three non-averted control sheep that grazed vermeerbos on an adjacent site.

The objective of the present study was to investigate the workability of the hypothesis under a mixed grazing situation with averted and non-averted sheep together on natural field grazing.

## Methods and Results

Three trials were performed. The sheep were averted by subjecting them to initial aversion conditioning followed by sustained conditioning to vermeerbos. All animal work done was approved by the animal ethics committee of the institute.

### Initial aversion conditioning

Prior to aversion conditioning, the sheep were firstly made accustomed to the non-vermeerbos plants on the field to provide a 'learned-safety status' to these plants. This was accomplished by introducing the sheep to a small field grazing, cleansed of vermeerbos, for 7 days. The grazing was supplemented with hay (*ad libitum*). The sheep were also familiarized with maize meal by feeding them 100 g maize meal per sheep day<sup>-1</sup> for at least the last 2 weeks before conditioning. The above-mentioned treatments were directly followed up by an initial aversion conditioning to vermeerbos with LiCl as follows. Sheep were withheld from feed for 24 h and then exposed to a small field grazing (4 m<sup>2</sup> per sheep) heavily infested with vermeerbos until all plants, including vermeerbos, had been grazed down. This took approximately 6 h, whereafter half of the sheep (randomly allotted to the aversion group) were each dosed by stomach tube with LiCl (160 mg kg<sup>-1</sup> bodyweight (BW)) dissolved in 100 ml water. This was immediately followed by dosing with 200 ml of a vermeerbos water extract to strengthen the taste-association with vermeerbos. The extract was prepared by stirring 100 g freshly chopped vermeerbos for 15 min with 1 litre of water, brought to boiling point just before mixing. The sheep were exposed to the surrounding vermeerbos-infested field grazing the next morning.

### Sustained aversion conditioning

The averted sheep were subjected to sustained conditioning to vermeerbos from the second day after the initial aversion conditioning. This was accomplished by continuous exposure to an aversive mixture, consisting of an aversive concentrate mixed with maize meal. Averted sheep had free access to the aversive mixture that was presented *ad libitum* in a feeding trough. The aversive concentrate consisted of 5 parts LiCl, 1 part sodium chloride, and a dried hexane extract equivalent to 2.5 parts fresh vermeerbos. The vermeerbos-hexane extract represented the identification factors of vermeerbos. Exposure to the aversive mixture was started with a concentrate content equivalent to 1% LiCl. This was increased by 1 percentage units, each time the mean intake of the aversive mixture exceeded 100 g, to a maximum concentration of 5%. In trials where averted sheep and non-averted control sheep grazed together, the two groups were separately penned overnight, during which time the averted sheep were exposed to the aversive mixture.

## **Trials on field**

### *Observation trial in movable pens on vermeerbos-infested field*

In this trial, the behaviour of five averted sheep in a 3 x 3 m movable pen in the field was compared with that of five non-averted control sheep in an adjacent pen. The averted sheep were exposed full-time to the aversive mixture. Both pens were simultaneously moved on to new grazing every time more than approximately 75% of the vermeerbos in the control's pen had been grazed down.

The averted sheep persistently refused grazing vermeerbos. Withdrawal of the aversive mixture on Day 16 led to vermeerbos intake on Day 28. This was most likely prompted by the behaviour of the control sheep in the adjacent pen. One of the control sheep showed clinical signs consistent with vermeerbos poisoning on Day 22 while none of the averted sheep exhibited signs of poisoning.

### *Ingestion of vermeerbos after joint grazing on vermeerbos-infested field*

Nine averted sheep, which had free access to the aversive mixture overnight, grazed together with nine non-averted control sheep on vermeerbos-infested field during the day. The aversive mixture was freshly prepared and provided every evening and the remaining mixture collected and weighed the next morning. After 18 days the two groups of sheep were fasted overnight and separately tested over 2 days for vermeerbos intake in two small pens (5 x 10 m each) adjacent to each other. The grazing in both pens was heavily infested with vermeerbos.

The averted sheep had a mean LiCl intake of  $1.4 \pm 0.6$  g per sheep day<sup>-1</sup> (44 mg kg<sup>-1</sup> BW day<sup>-1</sup>). None of the sheep showed clinical signs of vermeerbos poisoning during the 18-day grazing period on the field. When tested for vermeerbos intake on Days 19 and 20, the control group grazed down more than 50% of the vermeerbos while the averted sheep in the adjacent pen totally avoided vermeerbos. The observations show that the sheep remained averted to vermeerbos on field, despite grazing together for 18 days with the non-averted controls.

### *Vermeerbos poisoning during joint grazing on vermeerbos-infested field*

Twenty-five averted and 25 non-averted control sheep jointly grazed on vermeerbos-infested field. The averted sheep were separately penned overnight, during which time they had free access to the aversive mixture. The aversive mixture was freshly prepared and provided every evening and the remaining mixture collected and weighed the next morning. Exposure to the aversive mixture was ceased after 42 days and the sheep slaughtered 2 days later. Samples for LiCl analysis were taken from the kidney and skeletal muscle of the sheep.

Vermeerbos poisoning was diagnosed in eight of the control sheep while no poisoning occurred amongst the averted sheep. These results are in agreement with observations in the preceding trials and indicate that sheep could be kept averted to vermeerbos on field, despite the social influence of non-averted

animals. A mean LiCl intake of  $2.2 \pm 0.7$  g per sheep day<sup>-1</sup> ( $44 \text{ mg kg}^{-1} \text{ BW day}^{-1}$ ) was measured. Therapeutic doses for humans (treatment of mania and manic depressive psychoses) range between 0.6-2.4 g LiCl per patient day<sup>-1</sup> and are sometimes administered for years (Reynolds, 1982). It appears that daily ingestion of LiCl at this level might be safe to sheep, however, long-term studies need to be performed. Mean LiCl contents of  $3.6 \pm 3.3 \text{ mg kg}^{-1}$  and  $1.5 \pm 2.7 \text{ mg kg}^{-1}$  were determined in the kidney and skeletal muscle, respectively, of the 25 averted sheep, compared to  $0.5 \pm 0.0 \text{ mg kg}^{-1}$  and  $0.5 \pm 0.1 \text{ mg kg}^{-1}$  in the kidney and skeletal muscle, respectively, of five control sheep. In light of the therapeutic doses referred to above, these residue levels should not pose any risk following human consumption.

## Conclusion

The results of this investigation show that initial aversion conditioning followed by sustained conditioning, as performed by means of continuous exposure to an aversive mixture, persistently averted sheep to vermeerbos on field, despite the social influence of non-averted sheep. The results suggest that this technique might be useful for maintaining aversion in a mixed grazing situation on field.

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## Chapter 63

# **Prenatal Toxicity of Cyanide in Goats – a Model for Teratological Studies in Ruminants**

B. Soto-Blanco and S.L. Górnaiak

*Research Centre for Veterinary Toxicology (CEPTOX), Department of Pathology, School of Veterinary Medicine, University of São Paulo, Av. Dr. Prof. Orlando Marques de Paiva 87, São Paulo, 05508-900, Brazil*

### **Introduction**

Several plants for human and animal feeding contain cyanogenic glycosides that release cyanide. The most important one is cassava, a very important food staple for animals in the world, especially in the tropics (Tewe, 1992). Fetal malformations have been linked to maternal consumption of cyanogenic plants by animals, such as pigs, horses, sheep and cattle (Prichard and Voss, 1967; Selby *et al.*, 1971; Rodel, 1972; Seaman *et al.*, 1981; Bradley *et al.*, 1995), and by humans (Kumbhani *et al.*, 1990). However, there are few studies evaluating the effects of the prenatal exposure to cyanide, cyanogenic glycosides and cyanogenic plants. In this way, studies were performed in monogastric species such as rats (Tewe *et al.*, 1977; Olusi *et al.*, 1979; Miller *et al.*, 1981; Singh, 1981; Sousa, 2000), hamsters (Doherty *et al.*, 1982; Willhite, 1982; Frakes *et al.*, 1985), and pigs (Tewe and Maner, 1981). In all these experimental studies it was verified that exposure to cyanide itself and/or its products of biotransformation can produce fetotoxicity and/or teratogenesis. On the other hand, to our knowledge, studies of prenatal development with cyanide in ruminants were not performed until the present study.

Thus, the aim of the present work was to develop a model for prenatal toxicological studies in ruminants and evaluate the effects of the maternal exposure to potassium cyanide (KCN) during pregnancy in both the dams and the litter. Such study can contribute to the knowledge of the effects of the ingestion of cyanogenic plants by pregnant ruminants.

### **Materials and Methods**

Twenty-four mixed breed female goats, 1–3 years old, were bred to two Alpine bucks; the day of breeding was established as the first day of gestation. The

pregnant goats were divided into four groups, which were dosed with 0 (seven animals), 1.0 (five animals), 2.0 (five animals) or 3.0 (seven animals) mg KCN (Merck Co, Darmstadt, Germany)  $\text{kg}^{-1}$  bodyweight  $\text{day}^{-1}$  administered orally with tap water. The experimental animals received KCN twice a day, between 7:30 and 8:00 and between 16:30 and 17:00. The KCN administration started at the 24th gestational day and lasted at term.

Cyanide antidote was administered only when any animal was hardly poisoned. It was comprised by intravenous administration of sodium nitrite (10 g  $100 \text{ ml}^{-1}$  of water, at  $20 \text{ mg kg}^{-1}$  bodyweight) and sodium thiosulphate (20 g  $100 \text{ ml}^{-1}$  of water, at  $600 \text{ mg kg}^{-1}$  bodyweight). Sera from goats that had aborted were collected from the jugular vein and tested for levels of antibodies against the main abortive pathogens in Brazil (*Micoplasma* sp., *Brucella* sp., *Toxoplasma* sp. and *Leptospira* sp.).

At birth, the sex of each newborn goat was determined and each individual was weighed and examined carefully for gross abnormalities, as described by Szabo (1989). From birth to 3 months of lactation, each dam and its respective litter were weighed weekly to evaluate postnatal development. At the end of the experimental period, one doe from each group and all the male goats from every litter were euthanized for histopathological analyses. Data is reported as mean  $\pm$  SEM and was analysed statistically by two-way and one-way analysis of variance (ANOVA), followed by Dunnett's test. The level of significance was set at  $P < 0.05$ .

## Results

Vocalizations and ataxia were observed immediately after cyanide dosing in one dam from the group that received  $3.0 \text{ mg KCN kg}^{-1}$  over the period from the 34th to the 42nd gestational day. Another dam from the same group presented generalized tremors and ataxia on the 86th and 89th gestational days and convulsions on the 93rd day, which were treated by intravenous administration of nitrite and thiosulphate. Bodyweight of dams from all experimental groups did not differ significantly ( $P > 0.05$ ) from controls during both gestational and lactation periods.

Cyanide ingestion did not affect the length of gestation or the number of live kids (Table 63.1). Two kids with *prognathia inferior*, born from different dams, were born in the group that received the largest cyanide dose. One dam from the same group aborted two fetuses, one of them was *prognata*; this dam was the one that had presented vocalizations and ataxia after cyanide administration. The goat that presented convulsions delivered a normal male kid. One weak kid was born from the control group and another one was born from the group receiving  $3.0 \text{ mg KCN kg}^{-1} \text{ day}^{-1}$ . Furthermore, a stillbirth occurred in the control group.

The bodyweight of male kids showed no interaction between age and KCN doses, and the treatment did not affect the result. On the other hand, the bodyweight was affected by the age. The same was found in the bodyweight from

**Table 63.1.** Reproductive parameters from dams treated with KCN from 24th gestational day to term.

	Control	KCN (mg kg <sup>-1</sup> day <sup>-1</sup> )		
		1.0	2.0	3.0
Days of gestation	151 ± 1.17	149 ± 1.30	148 ± 1.21	148.9 ± 0.82
Live kids	9	8	7	7
males	5 (55.6%)	2 (25%)	3 (42.9%)	3 (42.9%)
females	4 (44.4%)	6 (75%)	4 (57.1%)	4 (57.1%)
Live kids/litter	1.29	1.60	1.40	1.20
Twins	3 (33.3%)	3 (60%)	2 (40%)	2 (33.3%)
Birth weight (kg)				
males	3.65 ± 0.34	3.77 ± 0.08	2.85 ± 0.45	3.28 ± 0.44
females	3.05 ± 0.09	3.05 ± 0.11	3.02 ± 0.28	3.42 ± 0.21
Weight at 90th day				
males	12.9 ± 0.59	16.1 ± 0.05	15.8 ± 0.93	18.2 ± 1.72 <sup>a</sup>
females	12.9 ± 2.32	13.9 ± 0.87	12.3 ± 1.16	16.4 ± 1.40

<sup>a</sup>*P* < 0.05 (one-way ANOVA followed by Dunnett's test).

female kids. Sex did not affect the weights of the kids, and no interaction between sex and treatment was found.

In the pathological study, no lesions were found in pancreas, thyroid glands, and central nervous system (CNS) from both dams and kids of all groups.

## Discussion

Maternal toxicity is a known factor causing alterations in fetal development (Khera, 1984). The use of three experimental groups objectively determined whether a teratogenic effect was a result from direct action of the chemical upon the embryo or fetus, or was a secondary effect from maternal toxicity. In our study, two pregnant goats from the 3.0 mg KCN kg<sup>-1</sup> day<sup>-1</sup> group presented clinical symptoms of cyanide toxicity: one of them presented vocalizations and ataxia whereas the other one had convulsions. Thus, it may be concluded the largest KCN dose promoted maternal toxicity.

Abortion is a drastic effect that is highly important in reproductive toxicology. There are a number of compounds that are found to be abortive to livestock, including poisonous plants (James *et al.*, 1994). In this way, mares and ewes fed cyanogenic *Sorghum* crops and pastures presented increased incidence of abortion (Prichard and Voss, 1967; Bradley *et al.*, 1995). Furthermore, stillborns



and cases of possible fetal resorptions or even abortions were found in pregnant ewes grazing on *Cynodon aethiopicus* (Rodel, 1972). In our study, a goat from the group receiving 3.0 mg KCN kg<sup>-1</sup> day<sup>-1</sup> aborted. The search for the most important aetiological agents of infectious abortion in Brazil was negative. Although many other causes, infectious or not, could be responsible for the abortion observed here, and even taking in to account that one control dam delivered a dead kid and a healthy one, it is plausible to consider that cyanide would be responsible for the abortion. Since mothers treated with this KCN dose showed clinical signs of toxicity, this effect could be a secondary consequence of the maternal toxicity.

Fetal/birth weight is an important parameter for teratological studies. It was found that female rodents treated with cyanide or the cyanogenic glycoside linamarin during pregnancy gave birth to litters with reduced fetal weight (Tewe *et al.*, 1977; Olusi *et al.*, 1979; Frakes *et al.*, 1985; Sousa, 2000). In the present study, no significant difference in birth weight of kids from KCN treated gestating goats was also found.

Some works have pointed out that cyanide could cause fetal malformations. In this manner, the ingestion of *Sorghum* by pregnant mares, ewes and cows was associated to the birth of kids with contracture type skeletal defects and stillbirths (Prichard and Voss, 1967; Seaman *et al.*, 1981; Bradley *et al.*, 1995). In the same way, pregnant sheep grazing on forage-containing cyanogenic glycosides (*Cynodon aethiopicus*, *Cynodon nlemfluensis nlemfluensis*, *Panicum coloratum* and *Paspalum dilatatum*) give birth to lambs with palpable goitre and skeletal deformities unspecified by the author (Rodel, 1972). Furthermore, pregnant sows with free access to wild black cherries (*Prunus serotina*), which contain the cyanogenic glycoside prunasin, farrowed pigs with aplasia of the tail, atresia ani, contracted hind legs and rudimentary external genitalia and some were stillborn (Selby *et al.*, 1971). In addition, some studies have associated the administration of cyanide itself (Doherty *et al.*, 1982), cyanogenic glycosides amigdalín (Willhite, 1982) and linamarin (Frakes *et al.*, 1985), and cyanogenic plants cassava (Singh, 1981) and apricot (Miller *et al.*, 1981) to pregnant laboratory rodents with malformations. Additionally, 16 cases of children in Nigeria with severe limb malformations were born to mothers that ingested cassava in early pregnancy (Kumbnani *et al.*, 1990). On the other hand, no malformations were found in rats (Tewe *et al.*, 1977; Sousa, 2000) and pigs (Tewe and Maner, 1981) receiving cyanide during pregnancy.

In the present work, two kids with *prognathia inferior* were born to mothers treated with the largest KCN dose. It should be considered spontaneous jaw defects could occur (Szabo, 1989). To our knowledge, there is no work associating cyanide exposure to development of this birth defect in any animal species. Thus, to better establish the association between cyanide exposure and the lower jaw defect found here, it is necessary to conduct additional experiments.

Tropical pancreatic diabetes has been associated with chronic cyanide exposure through consumption of cassava in man (McMillan and Geevarghese, 1979). As verified earlier in male goats (Soto-Blanco *et al.*, 2001b) no significant alterations were found in the morphology of the pancreas from any animal. These

data reinforce our previous supposition that cyanide itself does not induce a diabetogenic effect (Soto-Blanco *et al.*, 2001b).

Previous work on goats performed in our laboratory revealed an increase in the number of resorption vacuoles in the follicles of the thyroid following prolonged administration of KCN (Soto-Blanco *et al.*, 2001a) and degenerative lesions on the CNS (Soto-Blanco and Górnjak, unpublished data). However, in the present study both thyroid and CNS were evaluated after the lactation phase (3 months) and did not present morphological alterations, which supports the view that this period was sufficient for the regeneration of these tissues.

Summarizing, cyanide exposure of goats during pregnancy probably promotes toxic effects to both mothers and offspring. Furthermore, this work proposes a new animal model for prenatal toxicological assay, which could be important to evaluate poisonous plants. Data obtained from these studies could be extrapolated to other ruminant species, especially for substances with similar metabolism (absorption, biotransformation, distribution and excretion).

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## Chapter 64

# The Clinical and Morphological Changes of Intermittent Locoweed (*Oxytropis sericea*) Poisoning in Sheep

B.L. Stegelmeier<sup>1</sup>, L.F. James<sup>1</sup>, K.E. Panter<sup>1</sup>, R.J. Molyneux<sup>2</sup>,  
D.R. Gardner<sup>1</sup>, S.T. Lee<sup>1</sup>, M.H. Ralphs<sup>1</sup> and J.A. Pfister<sup>1</sup>

<sup>1</sup>Poisonous Plant Research Laboratory USDA Agricultural Research Service, Logan UT 84341, USA; <sup>2</sup>Western Regional Research Center, USDA Agricultural Research Service, Albany CA 94710, USA

### Introduction

Locoweed (*Astragalus* and *Oxytropis* spp.) poisoning costs the US livestock industry millions of dollars annually. Poisoning has an insidious onset, with signs of poisoning not becoming apparent until animals have grazed the plant for several weeks. Toxicity has been attributed to the locoweed toxin, swainsonine that inhibits several cellular mannosidases resulting in lysosomal dysfunction and altered glycoprotein metabolism. Clinical signs of poisoning include anorexia, lethargy, muscular weakness, intention tremors, proprioceptive deficits and emaciation. Although microscopic lesions such as subtle vacuolation of pancreas, thyroid and renal tubular epithelium develop after several days of poisoning, these lesions quickly resolve after discontinuing exposure (Van Kampen and James, 1972; Stegelmeier *et al.*, 1999). With prolonged exposure, poisoned animals develop permanent lesions including neuronal vacuolation, degeneration, pyknosis and death with axonal degeneration and dystrophy (James and Van Kampen, 1971). It has been hypothesized that exposing animals for brief intervals interspersed with recovery or withdrawal periods may minimize the effects of locoweed poisoning. The purpose of this study was to determine the effects of intermittent locoweed poisoning on the development of clinical and histological lesions.

### Materials and Methods

Forty mixed-breed wethers weighing about 50 kg each were randomly divided into ten groups of four sheep. Nine groups were dosed twice daily with finely ground *Oxytropis sericea* at a dose of 1.0 mg swainsonine kg<sup>-1</sup> bodyweight day<sup>-1</sup> for a total of 45 days interrupted by varying length recovery periods. A positive

control group was dosed with locoweed continuously for 45 days and the negative control group was dosed with an equal volume of ground lucerne for 45 days. Variable dosing periods and withdrawal times, as outlined in Table 64.1, were given to the remaining eight study groups. All animals were monitored daily, and bled and weighed weekly to characterize the clinical and biochemical responses associated with intermittent locoweed ingestion. The wethers in all groups were necropsied 7 days following the final dosing period. At necropsy tissues were weighed, collected, fixed and prepared for histological studies. Animal weights, serum biochemical data and histological grades were compared using an analysis of variance (ANOVA) with both mixed and generalized linear models for a repeated measures design. This work was done with the approval and under the supervision of the Utah State University Animal Care and Use Committee. The dose and duration of poisoning were chosen to produce histological lesions with minimal animal discomfort. Mean separations were done using Duncan's method (SAS Statistical Software 1986, SAS Institute Inc., Cary, NC). The level of significance was set at  $P < 0.05$ .

## Results and Discussion

The positive control sheep were reluctant to stand, were anorexic and had prominent intention tremors when they moved. They also gained significantly less ( $P = 0.001$ ) weight per day than any of the other groups after a cumulative 45 dosing days (Table 64.1). Similar reduced weight gains have previously been associated with locoweed poisoning (Ralphs *et al.*, 2000). As age-matched growth rates are better indicators of production, comparison on study day 41 showed positive control wethers (group 10) had similar weight gains as groups dosed for 15, 9 and 5 day durations (groups 5, 6, 7, 8 and 9). These groups gained significantly less ( $P = 0.004$ ) than control sheep. Sheep dosed at durations of 3 and 5 days (groups 2 and 4) with 14 day recovery periods had weight gains similar to those of control sheep on day 41 (Table 64.1). Wethers dosed at durations of 15 and 9 days (groups 6, 7, 8 and 9) were also depressed and reluctant to move at the end of their dose periods. Intention tremors were not observed in these animals. No clinical signs were observed in animals dosed with 0, 3 and 5 day durations (groups 1, 2, 3, 4 and 5). These findings indicate that intermittent locoweed ingestion reduces weight gains and the reduction is proportional to the dosing duration.

Serum swainsonine concentrations were directly correlated to dosing as serum concentrations quickly returned to normal within 5 days of the end of the dosing period. Other serum changes in poisoned animals included moderate increases in aminotransferase (AST) and alkaline phosphatase (ALP) activities and decreases in alpha-mannosidase activities. These activities tended to cycle according to the dosing schedule though they often did not return to normal activities. At necropsy 7 days after the last dose, AST activities were higher than controls; ALP activities tended to be higher and no serum swainsonine was detected (Table 64.1). It has

**Table 64.1.** Dose schedule of wethers dosed with locoweed (*Oxytropis sericea*) given by gavage at a rate of 1.0 mg swainsonine kg<sup>-1</sup> bodyweight day<sup>-1</sup> or equal volumes of lucerne for negative control animals. Weight gains and serum biochemical changes in wethers dosed with locoweed (*O. sericea*) or lucerne. Data are reported as means ± standard deviation. Significantly different (*P* < 0.05) means are indicated with different letters.

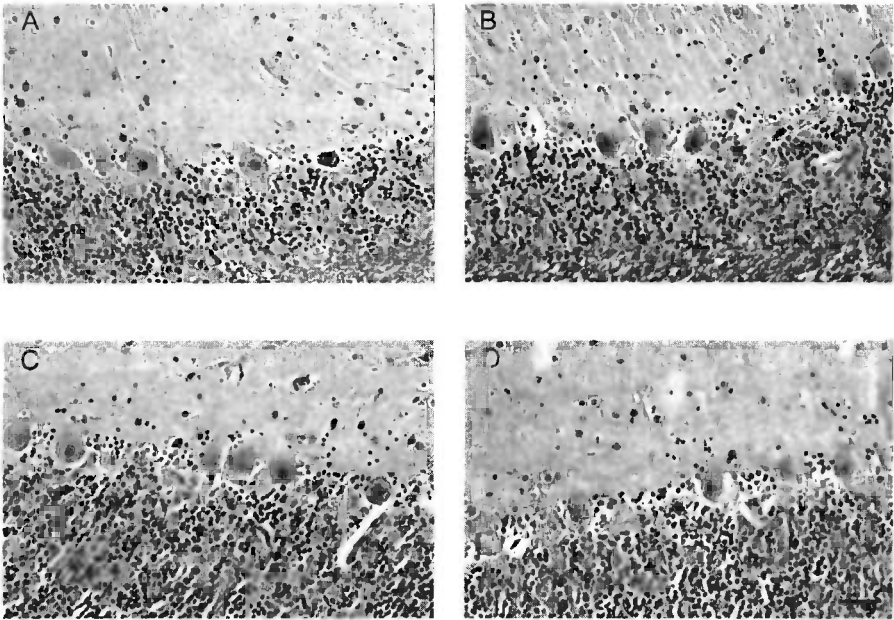
Group	Duration (days)	Recovery (days)	Necropsy day	Daily gain on study day 41 (kg day <sup>-1</sup> )	Daily gain at 45 total days dosing (kg day <sup>-1</sup> )	AST (IU) <sup>a</sup>	ALP (IU) <sup>a</sup>	Swainsonine (ng ml <sup>-1</sup> ) <sup>b</sup>
1	0	0	52	0.115±0.013 <sup>A</sup>	0.197±0.026 <sup>A</sup>	71±42 <sup>A</sup>	191±164 <sup>A</sup>	0±0 <sup>A</sup>
2	3	14	248	0.086±0.034 <sup>AB</sup>	0.115±0.029 <sup>B</sup>	218±54 <sup>B</sup>	91±29 <sup>A</sup>	327±26 <sup>B</sup>
3	3	7	150	0.019±0.006 <sup>D</sup>	0.055±0.020 <sup>CD</sup>	217±28 <sup>B</sup>	340±232 <sup>A</sup>	242±7 <sup>B</sup>
4	5	14	164	0.068±0.018 <sup>ABC</sup>	0.084±0.034 <sup>BCD</sup>	340±25 <sup>B</sup>	239±47 <sup>A</sup>	153±41 <sup>B</sup>
5	5	7	108	0.022±0.015 <sup>BCD</sup>	0.063±0.050 <sup>BCD</sup>	177±48 <sup>B</sup>	330±18 <sup>A</sup>	259±88 <sup>B</sup>
6	9	14	108	0.006±0.086 <sup>CD</sup>	0.105±0.037 <sup>BC</sup>	341±102 <sup>AB</sup>	336±25 <sup>A</sup>	239±67 <sup>B</sup>
7	9	7	80	0.016±0.027 <sup>BCD</sup>	0.052±0.007 <sup>CD</sup>	286±21 <sup>B</sup>	271±152 <sup>A</sup>	382±49 <sup>B</sup>
8	15	14	80	0.033±0.076 <sup>BCD</sup>	0.037±0.038 <sup>DE</sup>	259±44 <sup>B</sup>	350±47 <sup>A</sup>	420±109 <sup>B</sup>
9	15	7	66	0.005±0.037 <sup>D</sup>	0.048±0.048 <sup>D</sup>	292±40 <sup>B</sup>	287±91 <sup>A</sup>	296±120 <sup>B</sup>
10	45	0	52	0.007±0.031 <sup>D</sup>	0.007±0.024 <sup>E</sup>	255±40 <sup>B</sup>	252±107 <sup>A</sup>	273±48 <sup>B</sup>

<sup>a</sup>Serum enzyme activities at necropsy (7 days after 45 cumulative dosing days).

<sup>b</sup>Serum swainsonine concentration on last of 45 cumulative dosing days.

been suggested that serum AST and ALP activities reflect continued swainsonine-induced cellular damage. The AST and ALP activities detected at necropsy are probably a reflection of enzyme kinetics as they appeared to be correlated to the reported serum life of these enzymes (Duncan and Prasse, 1994).

Histological changes in the positive control wethers (group 10) included vacuolation, eosinophilic swelling, pyknosis and necrosis of neurones in the cerebellum (especially evident in Purkinje cells), basal ganglia, hypothalamus, medulla and ventral horns of the spinal cord. Dystrophic axons (spheroids) were found in the white tracts of the cerebellum and medulla. Vacuolation in visceral tissues resolved with minimal vacuolation of the exocrine pancreas. Sheep that were dosed with durations of 15 and 9 days had less Purkinje cell vacuolation with no neuronal pyknosis and much less axonal dystrophy (Fig. 64.1). Animals dosed at durations of 5 days or less (with both 7 and 14 day recovery periods) and the negative controls did not have detectable Purkinje cell vacuolation. Further, no neuronal pyknosis and axonal dystrophy were observed in these animals. Similar quick reversal of visceral vacuolation has been seen in other studies (James and Van Kampen, 1971; Stegelmeier *et al.*, 1999). Locoweed-induced permanent lesions are generally neuronal and include subtle swelling and vacuolation followed by more severe vacuolation, chromatolysis and eosinophilia, pyknosis and cellular degeneration, focal satellitosis and



**Fig. 64.1.** Photomicrograph of cerebellar Purkinje cells from locoweed-poisoned sheep with dosing durations of (A) 45 days, (B) 15 days, (C) 9 days and (D) 5 days. Sections are 5  $\mu$ m thick and stained with haematoxylin and eosin. Bar is 20 microns.

ultimately neuronal death (Stegelmeier *et al.*, 1999). Animals dosed at durations longer than 5 days in this study had neuronal changes that appeared to be progressing in this sequence. Shorter durations with both 7- and 14-day recovery periods did not have such neuronal changes. This suggests that sheep exposed to locoweed for durations of 5 days or less are not likely to develop permanent locoweed-induced sequelae.

Previous dose response studies using sheep and cattle suggest that cattle are likely to have similar responses to intermittent poisoning (Stegelmeier *et al.*, 1999). Studies also suggest that higher doses do not result in additional or more severe lesions (Stegelmeier *et al.*, 1995, 1999). In locoweed endemic areas, animals are not likely to be exposed to locoweed for durations of longer than 45 days. However, horses are highly susceptible to locoweed poisoning and it is likely that they will respond differently (James *et al.*, 1969; James and Van Kampen, 1971).

## Conclusion

Our findings suggest that sheep and possibly cattle may ingest locoweeds for short periods, 5 days or less, if allowed withdrawal periods of 7 to 14 days without developing behavioural or functional lesions. This supports the hypothesis that intermittent use of locoweed-infested ranges and pastures may allow use of valuable forages without permanently damaging animals.

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## Chapter 65

# Biological Control of the Toxic Shrub Juniper

E. Straka<sup>1</sup>, C.B. Scott<sup>2</sup>, C.A. Taylor, Jr<sup>3</sup> and E.M. Bailey, Jr<sup>4</sup>

<sup>1</sup>Angelo State University, Box 10890 ASU Station, San Angelo TX 76909, USA;

<sup>2</sup>Angelo State University, Box 10888 ASU Station, San Angelo TX 76909, USA;

<sup>3</sup>TAES, PO Box 918, Sonora, TX 76950, USA; <sup>4</sup>300 VMA Bldg, College of Veterinary Medicine, TAMU, College Station, TX 77843, USA

### Introduction

Ashe (*Juniperus asheii*) and redberry (*Juniperus pinchottii*) juniper are increasing in density throughout central and western Texas (Ansley *et al.*, 1995; Smeins *et al.*, 1997). Both reduce carrying capacity for livestock and wildlife, increase erosion, and reduce aquifer recharge because of reduced herbaceous cover (Thurow and Hester, 2001; Ueckert, 1997).

Control alternatives are available, but most are expensive and may adversely impact other desirable shrubs (Johnson *et al.*, 1999). The use of goats in a biological control system may offer a practical and environmentally friendly solution for juniper control. However, both juniper species contain monoterpenes that limit intake by goats (Riddle *et al.*, 1999). The concentration and composition of monoterpenes differs between the two species and creates a selective preference for ashe juniper over redberry juniper (Riddle *et al.*, 1996). Goats will consume some redberry juniper especially during the dormant season, but intake is limited because of aversive postingestive feedback from the monoterpenes in redberry juniper (Launchbaugh *et al.*, 1997). Collaborative research between Angelo State University, Texas Agricultural Experiment Station and Texas A&M College of Veterinary Medicine have assessed: (1) toxicological effects of juniper and (2) methods of increasing juniper consumption. This paper presents a review of the current state of knowledge regarding biological control of juniper using goats.

### Toxic Effect of Juniper

Consumption of redberry juniper affects rumen metabolism (Straka, 2000). Goats dosed with redberry juniper at 30% of their diet for 10 days, exhibited an increase in total volatile fatty acid (VFA) production along with changes in proportional VFA concentrations. At levels above 30% of the diet, total mM VFA production

dropped and proportional VFA concentrations shifted indicating changes in the microbial population (Table 65.1).

When intact leaves were administered to goats, microbial function was negatively affected. Most likely, the high concentration present within the leaves may have impaired the ability of cellulolytic species to adhere to and degrade the leaves.

Intraruminal dosing for 9 days with redberry juniper oil ( $0.18 \text{ g oil kg}^{-1}$  bodyweight (BW)) resulted in cachexia and mild hepatic injury at low dose levels in the form of lipid vacuolization. At higher dose levels ( $0.36 \text{ g oil kg}^{-1}$  BW), cellular necrosis and lobular encapsulation were evident after 9 days. Hepatic injury overall due to vacuolization was not severe and was most likely attributable to fasting. Feed consumption and bodyweights declined in both Angora and Spanish goats, and aspartate aminotransferase (AST) levels increased in Spanish goats, most likely because of skeletal muscle catabolism. The decrease in serum glucose in all goats and the presence of some ketones in pooled urine samples from Angora goats after 9 days provide further evidence of a catabolic state.

Juniper consumption by goats not only serves a management objective by controlling plant proliferation but moderate consumption can be beneficial to goats as well. Juniper is moderately nutritious (dry matter digestion (DMD) 57-66%; crude protein (CP) 6-9%) (Launchbaugh *et al.*, 1997; Riddle *et al.*, 1999). Consumption of juniper throughout the year may increase the likelihood of goats meeting nutritional requirements especially during dormant seasons or droughts. Juniper consumption within 30% of the diet may also result in a favourable shift of VFA production towards lower acetate:propionate ratios, thus improving feed efficiency. Finally, low levels of juniper intake (i.e. at or below 30% diet) may induce both stage I and stage II detoxification enzymes. Induction of mixed function oxidase (MFO) enzymes (De-Oliveira *et al.*, 1997; Pass *et al.*, 1999) and glutathione-S-transferases (Elegbede *et al.*, 1993) by consumption of monoterpenes would provide a protective benefit to goats exposed to other dietary toxins.

Goats consume higher levels of juniper in the dormant seasons when alternative forage is unavailable. Strategies to improve consumption need to be employed during other seasons. We have investigated three approaches to improving juniper consumption: (1) exposure to low levels of juniper after weaning to induce liver detoxification, (2) genetic selection and (3) strategic supplementation of nutrient and non-nutrients.

## Increasing Juniper Consumption

In the first study, we determined if: (1) dosing goats with the adsorptive compound activated charcoal or (2) exposing goats to juniper immediately after weaning would increase juniper consumption. Goats dosed with activated charcoal initially consumed more redberry juniper, but after 10 days intake was

**Table 65.1.** Changes in % mM concentration of individual VFAs in *in vitro* fermentation tubes after dosing goats with juniper for 10 days at four different dose levels.

VFA	Juniper in the diet							
	30%		40%		50%		60%	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Acetic %	55 <sup>a</sup>	53 <sup>a</sup>	53 <sup>a</sup>	4 <sup>b</sup>	47 <sup>a</sup>	40 <sup>b</sup>	43 <sup>a</sup>	25 <sup>b</sup>
Propionic %	23 <sup>a</sup>	22 <sup>a</sup>	21 <sup>a</sup>	30 <sup>b</sup>	29 <sup>a</sup>	34 <sup>b</sup>	32 <sup>a</sup>	44 <sup>b</sup>
Isobutyric %	4 <sup>a</sup>	3 <sup>a</sup>	3 <sup>a</sup>	5 <sup>a</sup>	3 <sup>a</sup>	3 <sup>a</sup>	3 <sup>a</sup>	3 <sup>a</sup>
Butyric %	12 <sup>a</sup>	14 <sup>b</sup>	15 <sup>a</sup>	13 <sup>a</sup>	14 <sup>a</sup>	15 <sup>b</sup>	13 <sup>a</sup>	13 <sup>a</sup>
Isovaleric %	5 <sup>a</sup>	3 <sup>b</sup>	5 <sup>a</sup>	8 <sup>a</sup>	4 <sup>a</sup>	5 <sup>a</sup>	6 <sup>a</sup>	5 <sup>a</sup>
Valeric %	3 <sup>a</sup>	4 <sup>b</sup>	3 <sup>a</sup>	3 <sup>a</sup>	3 <sup>a</sup>	3 <sup>a</sup>	3 <sup>a</sup>	6 <sup>a</sup>
Total VFA (mM)	7.90 <sup>a</sup>	12.87 <sup>b</sup>	4.39 <sup>a</sup>	2.04 <sup>b</sup>	4.99 <sup>a</sup>	2.65 <sup>b</sup>	2.08 <sup>a</sup>	2.01 <sup>b</sup>
DMD %	62 <sup>a</sup>	58 <sup>b</sup>	61 <sup>a</sup>	61 <sup>a</sup>	56 <sup>a</sup>	54 <sup>b</sup>	62 <sup>a</sup>	64 <sup>b</sup>

\* Sig. at ( $P = 0.0526$ )

<sup>a,b</sup> Numbers with the same letter are not significantly different ( $P = 0.05$ ).

similar among goats dosed with activated charcoal or not dosed (Fig. 65.1). Activated charcoal did not affect consumption of ashe juniper (Fig. 65.2). Goats increased intake daily over 10 days of feeding each species, apparently because controlled exposure to juniper resulted in adaptive physiological changes that allow goats to reduce aversive feedback (Bisson *et al.*, 2001).

Goat producers in Texas typically select female replacements for the flock at weaning. This would be an opportunistic time to introduce goats to juniper. During weaning, goats are separated from the flock and placed in confinement. Producers could harvest juniper and feed it to replacements during weaning to increase subsequent intake.

## Selective Breeding

Inherited physiological, neurological or morphological characteristics influence diet selection and can serve as a basis for genetic selection (Launchbaugh *et al.*, 1999). Animals within a population that could be identified as selecting greater than average amounts of a particular plant species could be bred to create successive generations with exceptional preferences. Intake patterns of some toxic plants like basin big sagebrush (*Artemisia tridentata*) are heritable traits (Snowder *et al.*, 2001). However, the estimated heritability of juniper ranged from near 0% (P. Johnson, San Angelo, personal communication) to highs of 26% (C.A. Taylor, Sonora, personal communication). Breed differences in consumption of juniper exist: in voluntary intake studies Spanish goats ate more

redberry juniper than Angora goats (Pritz *et al.*, 1997). Angora goats have been selected almost exclusively for mohair production and have received intensive care because of the profitability of mohair production in the past. Selection for a single trait and providing supplementation and additional care may have reduced their ability to consume toxic plants like juniper. Conversely, Spanish goats have received little selection pressure, and survival of the species probably depended on evolving characteristics for survival under a wide variety of foraging conditions including consumption of toxic plants.

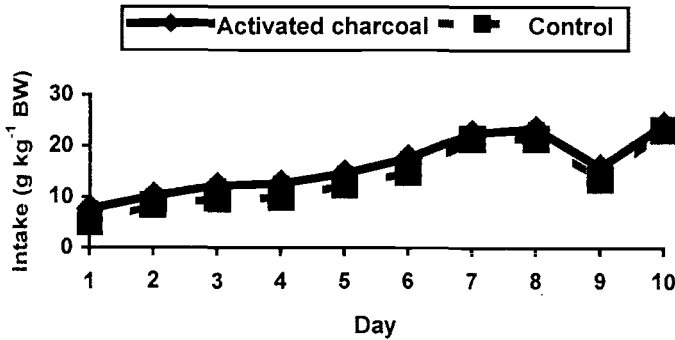


Fig. 65.1. Voluntary intake of goats of redberry juniper after dosing with activated charcoal for 10 days (Bisson *et al.*, 2001).

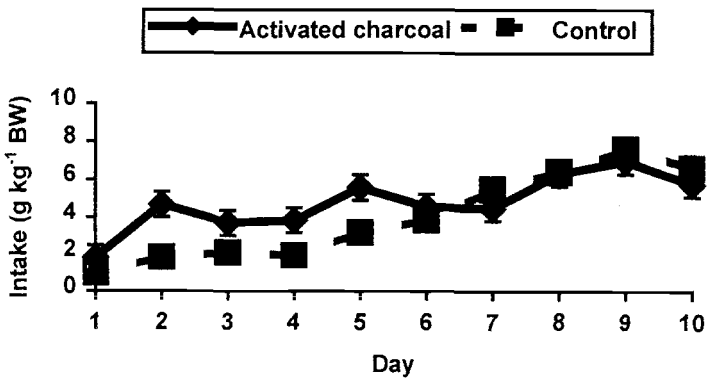


Fig. 65.2. Voluntary intake of ashe juniper by goats after daily dosing of activated charcoal (Bisson *et al.*, 2001).

## Supplementation

Nutrient intake can affect the ability of ruminants to detoxify some compounds (Illius and Jessop, 1996). In order to increase the tolerance of goats by providing adequate nutrition to meet the demands of detoxification of monoterpenoids, cottonseed meal, lucerne and maize were tested as supplemental feeds. A protein rather than energy supplement appears to be more beneficial to goats consuming juniper. Feeding goats cottonseed meal and lucerne as a supplement increased redberry juniper intake 40% compared to goats fed a maize supplement and 30% compared to goats receiving no supplement (Taylor *et al.*, 1997).

Polyethylene glycol (PEG) increases intake of several toxic plants that contain stannins (Silanikove *et al.*, 1996; Provenza *et al.*, 2000). Supplementation with PEG did not affect juniper intake (Taylor *et al.*, 1997). Monensin improves rumen efficiency and reduces the likelihood of lactic acidosis (Phy and Provenza, 1998a, 1998b) but had no effect on juniper consumption.

## Reducing Monoterpene Levels in Juniper

Top-kill of ashe juniper by fire results in plant death, but redberry juniper will resprout from basal buds after burning. Redberry juniper resprouts are lower than mature juniper in total monoterpenes. Following a prescribed burn, total monoterpenoid concentration in redberry resprouts was measured for 3- and 11-month regrowth and mature juniper. Juniper samples were collected from the same pasture to reduce geographic variability. Samples were collected from resprouts of redberry trees which had been top-killed during two different burns and a control group of non-burned plants. Ten trees per treatment were collected for sampling. Fifty grams of leaf and small stem tissue was collected from each tree and placed in liquid nitrogen to halt physiological activity and prevent volatilization. Samples were steam distilled and analysed by gas chromatography (Owens *et al.*, 1998). Monoterpenoid concentrations averaged 5.1<sup>a</sup>, 10.1<sup>b</sup> and 12.2<sup>b</sup> g mg<sup>-1</sup>, respectively (numbers with the same superscript letter are not significantly different at  $P = 0.05$ ). These data indicate that the initial response of redberry juniper after fire is to regrow shoots capable of photosynthesis rather than invest in terpene products. Following the initial growth, is a period of differentiation where glandular secretory cells are developed and monoterpene synthesis is initiated. This period creates a window of opportunity when goats can consume juniper and avoid the adverse effects from monoterpene ingestion.

## Biological Control

Advances in understanding how animals coexist with plant defence chemicals and in biotechnology and molecular genetics, provide much promise in developing goats for management of juniper. Combining strategies that increase consumption

of juniper such as selective breeding, protein supplementation and early exposure, along with burning programmes that reduce monoterpene availability, provide promise in developing sustainable juniper management programmes for the future.

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## Chapter 66

# The Effect on Quail of Feed Contaminated with the Mycotoxin Aurofusarin

J.E. Dvorska<sup>1</sup>, P.F. Surai<sup>2</sup> and N.H.C. Sparks<sup>2</sup>

<sup>1</sup>Sumy State Agrarian University, Sumy, Ukraine; <sup>2</sup>Avian Science Research Centre, SAC, Ayr, KA6 5HW, Scotland, UK

### Introduction

The fungus *Fusarium* produces a range of mycotoxins. Among them are trichothecenes (mainly T-2 toxin and deoxynivalenol), zeralenone and fumonisins, which have received substantial attention in relation to poultry production for the last few years. However, there is a group of pigments that are also produced by *Fusarium* species that have, until recently, been ignored. Seminal studies by Kotyk (1999) at the Ukrainian Poultry Research Institute over the last 10 years with the pigment aurofusarin demonstrate why this compound should be included in the list of important *Fusarium* mycotoxins. The aim of the present work was to evaluate effects of aurofusarin on quail. In this respect, quail are regarded as more resistant to ochratoxicosis than the chicken or turkey (Prior *et al.*, 1976) but more susceptible to aflatoxin B1 than chicken (Arafa *et al.*, 1981).

### Materials and Methods

Thirty eight 45-day-old Japanese quails (*Coturnix japonica*, egg-type reared in Ukraine) were divided into two groups (experimental and control, 15 females + four males in each group) and were fed *ad libitum* on a maize–soya diet balanced in all nutrients. Aurofusarin was added at the level of 26.4 mg kg<sup>-1</sup> feed in the form of *Fusarium graminearum* culture enriched with aurofusarin. The culture was a gift from A.N. Kotyk (Poultry Research Institute, Borky, Ukraine) and was produced by growing *F. graminearum* B-5 on millet. The millet was steamed, dried and ground to a fine powder and analysed for aurofusarin content by a bioautographic method (Kotyk and Trufanova, 1998) confirming that the culture contained exclusively aurofusarin. Spectral analyses of aurofusarin revealed three major peaks at 248, 265 and 345 nm which are characteristic for aurofusarin (Kotyk, 1999). Eggs were collected, the egg yolk colour was evaluated visually and the eggs were incubated in the commercial conditions of 37.5°C/55% RH. To assess the effect of aurofusarin on the immune system, quails were vaccinated



against Newcastle disease and 6 and 8 weeks after vaccination antibody titres were evaluated. After 8 weeks of experimental feeding five quails from each group were sacrificed and spleen, liver, kidney and ovary were collected for histological analyses.

## Results and Discussion

The results of this study show that aurofusarin consumption did not affect the bodyweight of the quails and that egg morphology was not changed. In fact, there was no difference between control and experimental group in egg weight, proportions of yolk, white and shell. As in a previous report (Kotyk, 1999) there were no clinical signs of mycotoxicosis. However, egg production was decreased (Table 66.1) and there were pathological changes in kidney and liver. For example, in the liver de-pigmentation of hepatocytes with granular dystrophy were observed. In general the liver had a tendency to enlargement with local haemorrhages and vessel hyperaemia. Histochemical studies revealed degenerative changes in the kidney: a decreased size, different degrees of degeneration and resorption of the glomeruli. The ovary was brown in colour with a decreased weight and number of follicles. There were also significant affects on the immune system with antibody titres to Newcastle disease after vaccination being decreased significantly. The spleen was also reduced in size as a result of aurofusarin consumption.

The major effect of aurofusarin was observed on egg yolk colour, which was changed from yellow-orange to brown and, in some instances, to greenish-brown. However, quails were more resistant to changes in egg yolk colour in comparison to chickens. For example, the first changes in egg yolk colour in quail were observed at day 12 while in another experiment it was shown that in laying hens similar changes took place after 5-6 days of aurofusarin consumption (Dvorska, 2001). After removing aurofusarin from the diet egg yolk colour was restored in quail after 17-18 days and in chickens after 18-20 days.

**Table 66.1.** Effect of aurofusarin on quail productive characteristics.

Traits	Control	Experimental
Mortality, %	0	0
Egg production, %	82.6	76.1
Egg weight, g	11.0 ± 1.0	10.8 ± 0.9
Female weight, g	138.0 ± 10.3	135.7 ± 11.0
Male weight, g	120.1 ± 9.2	116.0 ± 10.2
Antibody titre, log <sub>2</sub>		
6 weeks	10.3 ± 0.33	8.8 ± 0.46*
8 weeks	9.6 ± 0.41	7.6 ± 0.35*

\*Significant difference from the control group at  $P < 0.05$ .

While there are no data available on the molecular mechanisms involved in yolk colour changes, we suggest that it is associated with the ability of aurofusarin to change colour depending on pH (Kotyk, 1999). In our preliminary study it has been shown that at acidic pH aurofusarin is yellow-orange in colour and this colour changes to pink or beyond, to violet, in alkali conditions. HPLC separation of aurofusarin from extracts from control and experimental eggs confirmed the presence of aurofusarin in the egg yolk of quail fed an aurofusarin-supplemented diet.

Aurofusarin accumulation in the egg yolk had a detrimental effect on the egg yolk composition. Concentrations of vitamins E, A and carotenoids were significantly ( $P < 0.05$ ) decreased (Dvorska *et al.*, 2001) and the proportion of docosahexaenoic acid (DHA) in the yolk was also significantly ( $P < 0.05$ ) reduced. These changes in egg yolk composition probably were most likely to be responsible for increased quail mortality during embryonic development (Table 66.2).

**Table 66.2.** Effect of aurofusarin on quail reproduction.

Traits	Control	Experimental
Fertility, %	97.7	85.3
Embryonic mortality, %:		
1-14 days of development	1.1	3.4
15-18 days of development	5.6	25.9
Hatchability of fertile eggs, %	94.3	70.4
Hatch of eggs set, %	92.2	60.0

It is especially important to mention that increased mortality in quail from the experimental group was observed at the late stages of embryonic development. Indeed, analyses of antioxidant composition of embryonic tissues clearly showed a decrease in vitamins E, A and carotenoid concentration in the embryonic liver and other tissues (Dvorska *et al.*, 2002). Taking into account our data (Surai, 1999) indicating that the last days of chicken embryo development are extremely vulnerable to oxidative stress we concluded that aurofusarin can increase embryonic mortality by decreasing the efficacy of the embryo's antioxidant defence system. This suggestion was further supported by studying the susceptibility to lipid peroxidation of tissues from newly hatched quails. In fact, experimental quails were characterized by significantly ( $P < 0.05$ ) increased susceptibility to lipid peroxidation (Dvorska *et al.*, 2002).

It is interesting to note that other mycotoxins have also been shown to have pro-oxidant properties, including T-2 toxin, ochratoxin A, fumonisin B1, aflatoxin B1 and citrinin (reviewed by Surai *et al.*, Chapter 75 in this symposium volume). In many cases antioxidant systems were also shown to be compromised as a result of mycotoxicoses.

As can be seen from Table 66.2, quail fertility was decreased as a result of aurofusarin consumption. Since avian spermatozoa are characterized by

comparatively high levels of polyunsaturated fatty acids, they are vulnerable to the damaging effects of free radicals and toxic products of their metabolism (Surai *et al.*, 2001). Therefore pro-oxidant properties of aurofusarin shown for female quail could be responsible for the compromised antioxidant system of the spermatozoa leading to decreased fertilizing ability. However this suggestion needs to be validated by further studies.

In conclusion, our results showed that mycotoxin aurofusarin is accumulated in quail egg yolk changing its colour, antioxidant and fatty acid composition. As a result the antioxidant system of the embryo was substantially compromised and increased mortality at late stages of the embryonic development were observed. Clearly, more work should be done to understand molecular mechanisms of aurofusarin action.

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## Chapter 67

# Effect of Tunicamycins on GlcNAc-1-P Transferase Activity in Rat Tissues and Toxic Effects during Pregnancy and Lactation

P.L. Stewart<sup>1</sup>, P.T. Hooper<sup>1</sup>, C. Lenghaus<sup>1</sup>, M.F. Raisbeck<sup>2</sup>, J.A. Edgar<sup>1</sup> and S.M. Colegate<sup>1\*</sup>

<sup>1</sup>*Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Australia 3220;* <sup>2</sup>*Veterinary Science Dept., University of Wyoming, Laramie, Wyoming, USA*

\*Author to whom correspondence should be addressed.

The tunicamycins (TMs) are a family of nucleoside antibiotics structurally related to the corynetoxins (CTs) responsible for Annual Ryegrass Toxicity, Floodplain Staggers and Stewart's Range Syndrome, which cause high stock losses in Australia (Bryden *et al.*, 1994; Anderton *et al.*, Chapter 9 this volume; Than *et al.*, Chapter 59 this volume). As potent, irreversible, transition state analogue inhibitors of *N*-acetylglucosamine-1-phosphate transferase (GPT), the enzyme which catalyses the initial step in the biosynthesis of the dolichol-linked oligosaccharide chains destined for N-linking to proteins, both families of compounds block N-linked glycoprotein synthesis (Jago *et al.*, 1983). The TMs and CTs have high mammalian toxicity and very similar chemical and biological properties (Edgar *et al.*, 1982; Jago *et al.*, 1983; Finnie and Jago, 1985).

Whilst the effects of large doses of CTs or TMs have been well described (Jago and Culvenor, 1987), the effects of long-term, low level exposure to these toxins in the diet or environment are unknown. However, because the CTs and TMs are cumulative in their action there is reason to suspect this type of exposure may pose a risk to human and animal health (Colegate *et al.*, 1998).

The studies briefly described herein relate to the inhibition of GPT from different tissue sources within exposed rats, the potential for CTs and TMs to transfer to the mammary gland, contaminate milk and adversely affect the nurslings, and the toxic effects on the pregnant rat and on the developing fetuses. All studies were conducted strictly according to requirements of the Australian Animal Health Laboratory's animal ethics committee.

## Tissue GlcNAc-1-P Transferase Activity

Inhibition of GlcNAc-1-P transferase in liver rough microsomes is currently the most sensitive indicator of exposure to the tunicamyluracil compounds and, as such, has been used experimentally to measure subclinical toxicity (Stewart and May, 1994; Stewart, 1998).

In order to investigate whether systemic CTs and TMs can be distributed to tissues other than the liver and adversely affect the microsomal GPT, it was first necessary to determine the native GPT activity for rough and total microsomal fractions from various tissues. The assay was optimized for each tissue (P.L. Stewart, unpublished) and it was ascertained that the rough or total microsomal GPT activity in all tissues was sensitive to tunicamycins *in vitro*. Subsequently, groups of four male rats were parenterally injected with a single dose of TMs at one of two dose rates (75 or 300  $\mu\text{g kg}^{-1}$  bodyweight (BW) or approximately 20% and 80% of the  $\text{LD}_{50}$  respectively) and tissues (liver, kidney, heart, lung, muscle and brain) taken for GPT assay after 72 hours.

The results (Table 67.1) suggest that at the higher dose rate there was moderate to substantial inhibition of GPT activity in microsomes from all tissues, whilst at the lower dose rate there was clear inhibition only in liver, kidney and lung microsomes.

## Transfer Across the Mammary Membrane

Because of the known sensitivity of liver microsomal GPT to exposure to TMs or CTs, a study was completed in which lactating rats were dosed with TMs and the mammary tissue of the dams and the liver tissue of the suckling young were examined for GPT activity. These were compared to untreated controls.

Lactating rats (eight) were injected subcutaneously with subclinical doses ( $\text{LD}_{50}$  c. 400  $\mu\text{g kg}^{-1}$  BW) of TMs three times  $\text{week}^{-1}$  for 3 weeks (i.e. nine doses of 40  $\mu\text{g kg}^{-1}$  BW  $\text{day}^{-1}$ ). Equal-numbered control groups included an *ad libitum*-fed group as well as a group pair-fed according to the feed intake of the treated animals.

The group mean bodyweights at the commencement of the study were all similar. At the completion of the study, the average bodyweight of nurslings of

**Table 67.1.** Microsomal GPT activity (means of four rats  $\pm$  standard deviation) in different tissues from TM-treated rats as a percentage of the activity of non-treated controls. (R): rough microsomal fraction. (T): total microsomal fraction.

TM conc. ( $\mu\text{g kg}^{-1}$ )	Liver (R)	Kidney (R)	Heart (T)	Lung (T)	Muscle (T)	Brain (T)
75	3 $\pm$ 0.3	59 $\pm$ 9	132 $\pm$ 50	55 $\pm$ 4	97 $\pm$ 9	89 $\pm$ 41
300	1 $\pm$ 0.2	7 $\pm$ 1	10 $\pm$ 2	2 $\pm$ 0.4	24 $\pm$ 15	71 $\pm$ 10

**Table 67.2.** Effects of tunicamycins on bodyweight (BW) and on tissue GPT after 22 days of lactation. Means ( $\pm$  standard deviation) of eight rats per group.

Treatment	Dam BW (g)	Nursling BW (g)	GPT activity (cpm mg <sup>-1</sup> protein)		
			Dam liver	Nursling liver	Mammary gland
Control	272 $\pm$ 15	41.1 $\pm$ 2.3	38,070 $\pm$ 11,100	45,160 $\pm$ 12,980	81,140 $\pm$ 18,110
TM-treated	252 $\pm$ 27	34.3 $\pm$ 5.3	2,520 $\pm$ 810	45,060 $\pm$ 6,330	89,900 $\pm$ 11,650
Pair-fed	241 $\pm$ 24	35.1 $\pm$ 3.1	37,480 $\pm$ 11,660	45,110 $\pm$ 8,820	92,650 $\pm$ 20,980

TM-treated dams was similar to that of the nurslings from the pair-fed controls but about 17% lower than of nurslings from the *ad libitum*-fed controls (Table 67.2). While the GPT activity of TM-treated dams was decreased by more than 90% compared to both groups of controls, there were no differences in enzyme activity of the mammary tissue of the dams or the liver rough microsomes of the suckling young. Histopathologically, the mammary tissue of treated and control rats looked similar.

The failure to gain weight exhibited by the suckling young of dams exposed to TMs was ascribed to an adverse effect of TMs on the appetite of the dams.

### Effects of Tunicamycins during Pregnancy

Because N-linked glycoproteins are essential to many of the stages of reproduction, it is important to determine whether underglycosylation of reproduction-related proteins has a functional effect during the reproductive cycle. In addition, because of the cumulative toxicity of the TMs and CTs, it is necessary to determine whether the toxins can cross the placental barrier and adversely affect the fetus *in utero*, as determined by assessing the liver rough microsomal GPT activity in the new born young.

Consequently, using four animals per group, TMs were administered to pregnant rats as a single parenteral dose at one of three dose rates (150, 75 and 37.5  $\mu\text{g kg}^{-1}$  BW or approximately 30, 15 and 7.5% of an LD<sub>50</sub> respectively) on day 15 of gestation. As a negative control, pregnant rats were administered saline solution on the same day of gestation whilst (as positive controls) non-pregnant rats (groups of three to six) were treated with the same doses of TMs.

Sixteen hours after dosing all the TM-treated pregnant rats were showing moderate to extensive vaginal bleeding and one of four at each of the two top doses died within 26 hours, presumably from loss of blood. The more severely affected surviving pregnant rats from the high dose group were euthanased 26-28 hours following administration of the TMs, i.e. on day 16 of gestation. Each of

**Table 67.3.** Effects of tunicamycins on biochemical and haematological parameters in pregnant and non-pregnant rats.

Dose rate ( $\mu\text{g TM kg}^{-1} \text{ BW}$ )	Liver GPT activity (%)	Serum chol ( $\text{mmol l}^{-1}$ )	Serum protein ( $\text{g l}^{-1}$ )	RCC ( $\times 10^{12} \text{ l}^{-1}$ )	Hb ( $\text{g l}^{-1}$ )	PCV	Plasma protein ( $\text{g l}^{-1}$ )
Pregnant							
0	100	3.2	64	6.23	117	0.36	72
37.5	33	2.1	52	5.58	105	0.33	58
75	6	1.6	51	4.62	85	0.26	59
150	2	0.6	37	2.90	53	0.16	43
Non-pregnant							
0	100	3.8	65	7.42	136	0.44	77
37.5	33	2.7	55	7.07	129	0.41	65
75	10	2.5	57	7.37	132	0.42	62
150	5	1.3	53	7.83	142	0.45	60

these rats had free blood in the uterus and an associated decreased red cell count (RCC), haemoglobin (Hb) and packed cell volume (PCV) (Table 67.3). At post-mortem examination, the amniotic sacs were easily detached from the maternal placenta by gentle manipulation.

The less severely affected rats were euthanased on day 17 of gestation, i.e. 2 days after receiving the TMs. There were treatment-related (but not dose-related) decreases in serum and plasma proteins in both pregnant and non-pregnant rats, but there was no evidence of vaginal bleeding or overt clinical signs in the latter (Table 67.3). The liver rough microsomal GPT activity was severely depressed for both the TM-treated pregnant and non-pregnant rats (Table 67.3). Serum cholesterol (chol) levels were also reduced.

Histopathologically the only changes observed in the TM-treated rats were consistent with the gross observations, i.e. haemorrhage, venous thrombosis and necrosis, particularly in the maternal placenta. There was haemorrhage in the placental labyrinth, spongy zone and subplacenta and extensive thrombosis in vessels in the uterine wall.

Whilst the clinical effects, gross post-mortem observations, histopathological differences and effects on GPT activity were unambiguous, the small numbers of animals used in this study (affected also by unexpected deaths in higher dose groups) means that the statistical significance of the trends indicated by the clinical chemistry data will need follow-up studies.

## Conclusions

Inhibitory effects of tunicamycins on GPT activity have been demonstrated in rat liver, kidney, heart, lung, muscle and brain microsomes, although the effect on the latter is marginal and would require further investigation. The results show



that liver rough microsomal GPT is the most sensitive indicator of *in vivo* exposure to TMs.

No evidence of toxin transfer into milk was found, using nursing rat liver GPT enzyme activity as an indicator and there was no evidence of a direct effect of TMs on the mammary gland. However, a significant reduction in weight gain of nurslings of toxin-treated dams, compared to the nurslings of untreated dams, was observed.

Due to the rapid onset of haemorrhage, requiring euthanasia 1 or 2 days after dosing, the question of whether TMs can cross the placental barrier remains unresolved. However, substantial maternal toxicity has been demonstrated at doses less than 10% of a lethal dose in non-pregnant rats.

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## Chapter 68

# Experiences with the Quantitative Trace Analysis of Pyrrolizidine Alkaloids using GCMS and LCMS

K. Beales, S.M. Colegate\* and J.A. Edgar

*CSIRO Livestock Industries, Plant Toxins Research Group, Private Bag 24, Geelong, Victoria 3220, Australia*

\*Author to whom correspondence should be addressed.

The hepatotoxic pyrrolizidine alkaloids (PAs) are monoesters, diesters or macrocyclic diesters of the unsaturated necine or otonecine bases. In addition to being toxic to liver, some PAs can cause pulmonary arterial hypertension, neurotoxicity and right ventricular hypertrophy and can be carcinogenic and genotoxic. The major reported clinical symptoms of PA intoxication in humans result from a veno-occlusive disease of the liver (Prakash *et al.*, 1999). However, other effects of long-term, low-level exposure to PAs via the diet (grain, milk, meat, honey and related products) (Colegate *et al.*, 1998) are unknown. The German Federal Health Bureau established regulations after a comprehensive risk assessment of toxic PAs. These restrict oral exposure to PAs or their N-oxides (PANOs) in herbal preparations to  $0.1 \mu\text{g day}^{-1}$ , with the exclusion of pregnant and lactating women for whom zero exposure is recommended (German Federal Health Bureau, 1992). Exposure levels such as these provide a challenge to the accurate identification and quantitation of PAs and their N-oxides.

This report describes the advantages and disadvantages associated with the application of gas chromatography/mass spectrometry (GCMS) and liquid chromatography/electrospray and atmospheric pressure chemical ionisation mass spectrometry (LCesiMS and LCapciMS) to the trace analysis of PAs and PANOs.

### Gas Chromatography/Mass Spectrometry

GCMS has been shown to be a useful analytical procedure for the PAs but there is a need to reduce the PANOs prior to analysis and, for trace analysis, sensitivity and chromatographic considerations related to the potential requirement for derivatization need to be considered. The observations reported here were made using a Finnigan GCQ Ion Trap mass spectrometer and various fused silica capillary columns (5, 35 and 70% phenyl substitution on dimethylpolysiloxane phase).

## Reduction of N-oxides

Methods of N-oxide reduction include the use of sodium dithionite, redox reagents such as indigocarmine adsorbed on to anion exchange resin and zinc powder in the presence of an acid.

The efficiency of sodium dithionite as a reducing agent was investigated using solutions of senecionine N-oxide, with heliotrine as an internal standard, in water at pH values of approximately 2, 7 or 9. The acidic and basic samples failed to produce any senecionine on treatment with sodium dithionite, whilst the samples of senecionine N-oxide in deionized water only yielded 35% of the calculated recoverable senecionine. However, only 40-50% of the heliotrine internal standard was recovered from the dithionite reduction mixture. This contrasts with the total recovery of heliotrine from solutions that were not treated with dithionite thereby indicating that some of the PA material has probably been destroyed by the dithionite solution with potential adverse ramifications for trace PA analysis.

The use of a redox resin prepared by adsorbing indigocarmine on to an anion exchange resin was shown to be efficient and useful for the analysis of grain samples using an ELISA (Cavallaro *et al.*, Chapter 17 this volume). However, its applicability to different samples (plants, honey, meat, dairy products, etc.) will need to be individually tested to ensure compatibility.

A significant variation in reduction efficiency using zinc/sulphuric acid ( $\text{Zn}/\text{H}_2\text{SO}_4$ ) was found to occur between different batches of zinc dust. For example, in our experience, one batch yielded 60% of the possible senecionine and 26% of the heliotrine expected from the respective N-oxides treated. Another batch however yielded 60-90% of the senecionine and 90-100% of the heliotrine expected. The length of reduction time was also important. Thirty min to 4 h stirring gave the best results whereas if samples were stirred overnight, only 36% of the expected senecionine and 70% of the expected heliotrine was recovered.

At a macro level, it is usually assumed that N-oxide reduction to the parent PA is quantitative. However, experience with the N-oxides of lasiocarpine and sarracine has shown that this is not necessarily the case and that, at trace levels, the formation of products other than the parent PA will adversely affect the analysis. For example, three by-products were observed following reduction of lasiocarpine-N-oxide. At concentrations of 4.8-100  $\mu\text{g ml}^{-1}$  no parent lasiocarpine was observed at all. Analysis of the two major by-products using carbon-13 nuclear magnetic resonance spectroscopy and liquid chromatography/mass spectrometry (LCMS) indicated hydrogenation or complete loss of the angelic acid side chain of lasiocarpine forming dihydro-lasiocarpine and europine respectively. The third, and minor, by-product was tentatively identified by LCMS as a demethyl-lasiocarpine. Based on work with various PANOs so far, it is possible that this observation is restricted to those PAs with angelic acid moieties.

Reduction using zinc/citric acid was also investigated, however this gave extremely variable results with recoveries from 30-100%, the reason for which has not been determined. An associated problem is that the resultant citrate salt is

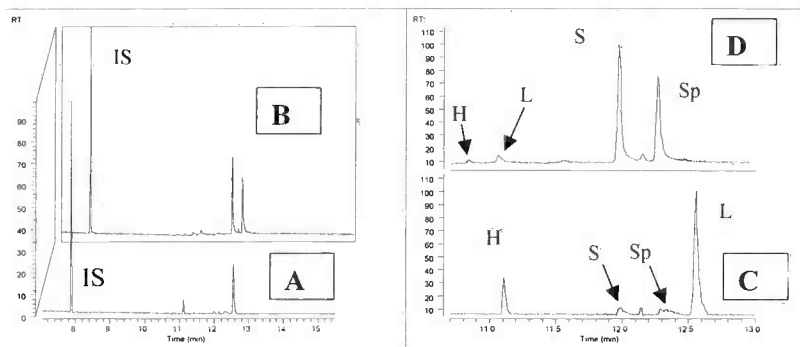
relatively insoluble in aqueous solution and eventually produces a thick white precipitate detrimental to further processing of the sample.

### Derivatization

Methods for derivatization include the alkylboronation of vicinal diols and the trimethylsilylation or trifluoroacetylation of hydroxyls (Edgar, 1985). Trimethylsilylation is not very satisfactory for use with vicinal, tertiary hydroxyls whereas trifluoroacetylation requires post-derivatization clean-up to remove the column-damaging trifluoroacetic acid formed.

In general terms, the advent of the use of fused silica capillary columns in gas chromatography has allowed the analysis of PAs without the derivatization usually required to improve volatility, stability and chromatography on the older, packed columns. However, such a general approach is not foolproof with PAs from some plant sources still requiring derivatization to improve chromatography or to enhance their detectability when trace amounts are being analysed and, conversely, derivatization can be detrimental to the chromatography of PAs from other plant sources. For example, the resultant chromatograms when a mixture of heliotrine, lasiocarpine, senecionine and seneciophylline is analysed with and without derivatization are shown in Fig. 68.1.

The detection of the macrocyclic diesters, senecionine and seneciophylline is adversely affected by derivatization whereas detection of heliotrine and lasiocarpine, 9-monoester and 7,9-open ring diester respectively, is enhanced under the derivatization conditions.



**Fig. 68.1.** GCMS analysis of a mixture of heliotrine (H), lasiocarpine (L), senecionine (S) and seneciophylline (Sp) along with an internal standard (IS). (A) derivatized by methylboronation and trifluoroacetylation; (B) underivatized; (C) expanded A; (D) expanded B.

These considerations would therefore necessitate multiple GCMS analyses of the same sample, derivatized in different ways and underivatized, if the source of PAs were unknown or if PAs of various plants were present in the sample.

## Liquid Chromatography/Mass Spectrometry

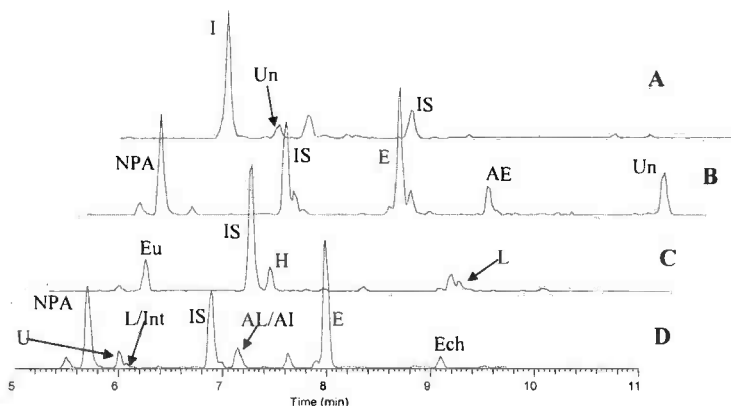
Direct, solution infusion and the high pressure liquid chromatography (HPLC) modes of presenting sample to the electrospray ionization (esi) source of a Finnigan LCQ Ion Trap mass spectrometer are particularly suitable for the analysis of PAs in combination with their N-oxides without the need for prior reduction of the N-oxides back to the parent PA or derivatization of the PAs. However the ionization of PANOs was markedly lower in the atmospheric pressure chemical ionization (apci) mode compared to the esi mode to the extent that apci would not be applicable to trace analysis of some PANOs. The resolution of individual PAs using HPLC is often poorer compared to GC but is compensated for by software data management to reconstruct specific ion chromatograms (RICs) for individual PAs. Whilst RICs do not help in the differentiation of co-eluting stereoisomers such as lycopsamine and intermedine, in cases where total PA content is required this disadvantage does not impact on the results.

For analysis of PAs, the choice of apci rather than esi was due to the significantly enhanced stability of the apci MS response (Relative Standard Deviation (RSD) 5%), compared to the esi MS response (RSD 25%), over an extended time period (24 h) required to complete an analytical sequence. Quantitative analysis of the PANOs is potentially complicated by their strong tendency to form dimer adducts.

PAs and their N-oxides could be readily identified by their elution time combined with the molecular weight obtained from observation of the very strong molecular ion adducts (M+H, M+Na, M+K etc.). Further confirmation of the PA or, indeed, confirmation of PA character in an unknown sample could be obtained by MS/MS procedures.

### HPLC/MS analysis of honeys

The occurrence of PAs in honeys, as a result of bees foraging on PA-containing plants is well documented (Colegate *et al.*, 1998 and references therein). Crews *et al.* (1997) captured the PAs and PANOs from honeys by trapping them on solid phase liquid-liquid partitioning Extralut columns and then followed the sodium dithionite reduction of the PANOs with an LCapciMS analysis. In our hands, it was found that this procedure resulted in complex extracts that required RIC manipulation and could impede the recognition of trace levels of PAs, especially unexpected or unknown PAs. Therefore a method relying on cation exchange capture of the PAs and PANOs was developed and optimized. Samples (25 g) of pre-processed honeys were diluted with water (50 ml), centrifuged to remove particulates and then applied to a strong cation exchange resin cartridge (Alltech,



**Fig. 68.2.** HPLC/MS of extracts of honey, showing PAs characteristic of (A) *Heliotropium amplexicaule*; (B) *Echium vulgare*; (C) *Heliotropium europaeum*; and (D) *Echium plantagineum*. PAs identified by retention time and mass spectra are: echimidine (E), acetylechimidine (AE), echiumine (Ech), europine (Eu), heliotrine (H), lasiocarpine (L), indicine (I), lycopsamine/intermediate (L/Int), acetyllycopsamine/acetylintermediate (AL/AI) and uplandicine (U). Unidentified PAs (Un) and non-PA material (NPA) were also observed and the internal standard (IS) is shown.

500 mg SCX). After washing with water (20 ml) the captured PAs and PANOs were eluted with acidified methanol (20 ml, methanol:water:conc.HCl 40:135:25, 0.5-1.0 ml min<sup>-1</sup>). Zinc powder (300 mg) was added to the eluate, which was stirred for up to 3 h to reduce the PANOs. Conventional base/chloroform treatment yielded a clean chloroform solution of the parent PAs. After evaporation to dryness and reconstitution in aqueous methanol (50% v/v, 0.5 ml), the sample was analysed using LCapiMS.

Pyrrrolizidine alkaloid profiles characteristic of the different plant sources were observed from some honey samples examined (Fig. 68.2).

## Conclusions

Experience with the analysis of low levels of PAs and their N-oxides has highlighted advantages and disadvantages in applying GCMS and LCMS analytical methods. The LCMS method developed had a limit of detection of about 2–3 ng on column in the full scan mode and about 0.2 ng on column in the MS/MS mode. This latter detection limit is comparable to those limits of detection by GCMS but without the added problems that derivatization requirements present for GCMS. The LCMS method has proved useful in the analysis of honeys for low levels of PAs.

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## Chapter 69

# Perinatal Study of *Senna occidentalis* Intoxication in Rabbits

A.C. Tasaka<sup>1</sup>, I.L. Sinhorini<sup>1</sup>, M.L.Z. Dagli<sup>1</sup>, M. Haraguchi<sup>2</sup> and S.L. Górnaiak<sup>1</sup>

<sup>1</sup>Research Center for Veterinary Toxicology (CEPTOX), Department of Pathology, Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil; <sup>2</sup>Biological Institute, São Paulo, Brazil

### Introduction

*Senna occidentalis* is one of the most important toxic plants of veterinary interest as regards contamination of animal rations. This plant grows vigorously amidst cereals crops such as soybean, sorghum and maize. Thus, it may be a contaminant of the ration and produce intoxication in domestic animals. In fact, many studies have reported *S. occidentalis* toxicosis in livestock (Henson *et al.*, 1965; Barros *et al.*, 1990). Experimental studies have also been conducted using several animal species, such as cattle (Barros *et al.*, 1990), swine (Colvin *et al.*, 1986), horses (Irigoyen *et al.*, 1991), sheep (Dollahite and Henson, 1965), goats (El-Sayed *et al.*, 1983), chickens (Calore *et al.*, 1997, 1998; Cavaliere *et al.*, 1997; Haraguchi *et al.*, 1998a, b), rats (Calore *et al.*, 2000) and rabbits (O'Hara and Pierce, 1974a, b; Tasaka *et al.*, 2000).

Signs of intoxication with large amounts of *S. occidentalis* include diarrhoea, myoglobinuria, ataxia and sternal recumbency, eventually leading to death. In cattle, swine and poultry, degenerative myopathy of the skeletal and myocardial musculature is the main feature (Dollahite and Henson, 1965; Barros *et al.*, 1990; Calore *et al.*, 1997). In addition, alterations in small intestine, liver, kidneys, lungs and heart have been observed in goats (El Sayed *et al.*, 1983). In rabbits, cardiac (O'Hara and Pierce, 1974a, b; Tasaka *et al.*, 2000) and hepatic alterations (Tasaka *et al.*, 2000) are more frequently observed. The mechanism of *S. occidentalis* toxicity has been proposed to involve impairment of mitochondrial function (O'Hara and Pierce, 1974b; Calore *et al.*, 1997), including swelling, loss of mitochondrial matrix, fragmented mitochondrial cristae and glycogen depletion.

Previous studies conducted in our laboratory (Tasaka *et al.*, 2000) have shown that the administration of *S. occidentalis* seeds to rabbits can produce severe pathological changes. Thus, the purpose of the present study was to ascertain if *S. occidentalis* seed consumption during pregnancy causes the same pattern of lesions in the litter as observed in adult animals.



## Materials and Methods

Ripe *S. occidentalis* (So) seeds, collected from a culture at the Biological Institute of São Paulo, São Paulo State, were dried and ground with a hammer mill and next incorporated into a rabbit diet at three different concentrations: So 1%, So 2% and So 3%. The mixture (seeds + the ingredients of the rabbit diet) was homogenized and pelleted.

Thirty-two virgin New Zealand rabbits (5- to 6-months-old) were bred naturally to proven males. The time of mating was considered as 0 h of pregnancy, and the ensuing 24 h were considered as day 1. After mating, the animals were housed separately in metal cages measuring 80 x 60 x 40 cm, equipped with an automatic watering system. Three experimental groups of eight animals each received So 1%, So 2% or So 3% in their ration on days 6 to 30 of gestation. Eight control animals received the pelleted feed without *S. occidentalis* seed throughout gestation. All animals received food and water *ad libitum*. The experiments were carried out in accordance with the ethical principles in animal research adopted by the bioethics commission of the Faculty of Veterinary Medicine and Zootechny of the University of São Paulo.

The does' bodyweights and consumption of ration were measured weekly during gestation and lactation and young rabbits of each litter (eight per doe) were weighed weekly from birth to weaning. Four weeks after parturition, the does and their litters were anaesthetized and blood samples were obtained by cardiac puncture for the determination of serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), creatine kinase (Ckmb) and aldolase (ALD), using a commercially available kit (Merck®). Next, the animals were euthanized. For histopathological examination, representative samples of skeletal muscles (diaphragm, tibialis anterior), heart and liver were harvested, fixed in 10% formalin and routinely embedded in paraffin. Sections (5 µm) were stained with haematoxylin and eosin.

For the electron microscopy, small portions of liver and tibialis anterior muscle tissue were pre-fixed in 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, for 2 h and then post-fixed in 1% osmium tetroxide for 60 min. The tissue blocks were dehydrated in alcohol and embedded in araldite resin. Ultra thin sections of samples were cut with a microtome (Reichert-Jung®) and stained with uranyl acetate and lead citrate, and examined with an EM-201 Phillips transmission electron microscope (Phillips, Holland).

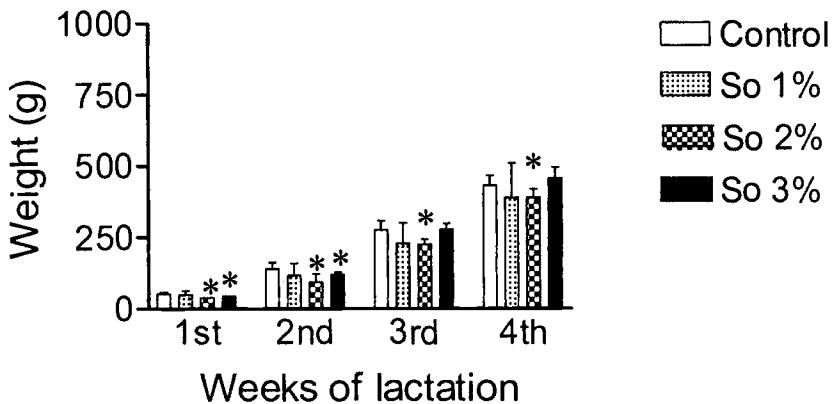
Data were expressed as the mean  $\pm$  SD and were analysed statistically by ANOVA followed by the Tukey-Kramer test. Results were considered to differ significantly from the control group when  $P < 0.05$ .

## Results

There were no significant differences ( $P < 0.05$ ) in feed intake or bodyweight of any experimental groups of does compared to the control group throughout gestation (data not shown). On the other hand, a decrease in bodyweight was observed during the first 4 weeks after birth in pups from dams fed So 2% throughout gestation, and during the first and second weeks after birth in pups from dams fed So 3% (Fig. 69.1).

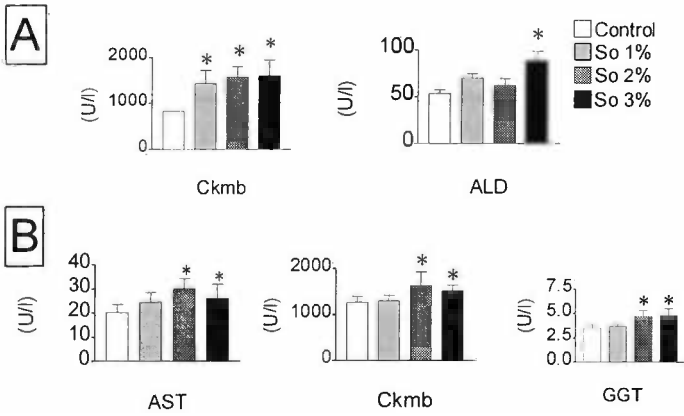
Biochemical evaluation revealed increased Ckmb and ALD levels in females fed So 1% to So 3% and So 3%, respectively, when compared to the control group. The pups from dams in the So 2% and So 3% groups also showed increased serum levels of AST, GGT and Ckmb (Fig. 69.2).

The histopathological study showed alterations in almost all mothers that received rations containing the three different concentrations of *S. occidentalis*. These lesions were more prominent in those females that consumed larger seed amounts. Pups from experimental mothers also presented the same pattern of lesions. The heart was the most affected organ showing intense vacuolization of myocardial fibres, with a characteristic inflammatory process and necrosis. The experimental rabbits, dams and their pups, also showed vacuolar degeneration in hepatocytes with cells presenting foamy cytoplasm. Electron-microscopic study of the liver from the experimental animals revealed dilated mitochondria, with destruction of the internal cristae (Fig. 69.3).

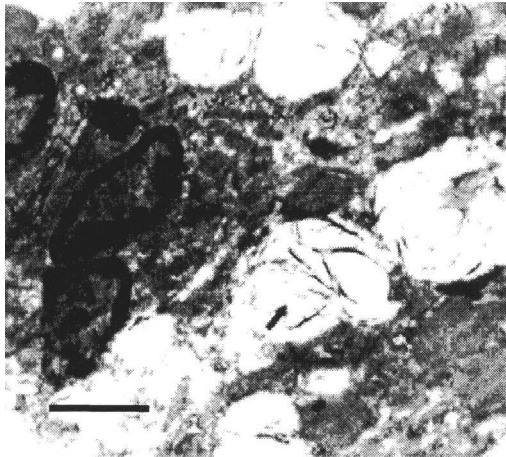


**Fig. 69.1.** Semanal weight from pups whose dams received *Senna occidentalis* (So) during the gestation period.

\* (ANOVA,  $P < 0.05$ ) significantly different from control animals.



**Fig. 69.2.** Creatine kinase (Ckmb) and aldolase (ALD) from does that received ration containing 1%, 2% or 3% of *S. occidentalis* (So) seeds during the gestation period (A) and aspartate aminotransferase (AST), Ckmb and gamma-glutamyltransferase (GGT) serum levels from its respective litter (B). \*(ANOVA,  $P < 0.05$ ) significantly different from control animals.



**Fig. 69.3.** Pup rabbit liver electron microscopy whose mother received 2% of *Senna occidentalis* in ration during gestation period, showing internal mitochondria cristae destruction (arrow). Bar marker 5000  $\mu\text{m}$ .

## Discussion

Previous work conducted in our laboratory (Tasaka *et al.*, 2000) clearly demonstrated that *S. occidentalis* seeds cause toxicity in growing rabbits;

however, contrary to other animal species such as chicks (Haraguchi *et al.*, 1998b), cattle (Barros *et al.*, 1990) and swine (Colvin *et al.*, 1986), whose main lesions were found in skeletal muscle, rabbits showed the most conspicuous lesions in the liver and heart. In the present evaluation, histopathological changes were also predominantly observed in the heart and liver of does from all experimental groups, with the animals receiving the high dose the most severely affected. Thus, we may conclude that pregnancy does not affect the susceptibility of rabbits to these seeds.

Considering that the presence of *S. occidentalis* seeds in ration could cause natural intoxication in domestic animals (Barros *et al.*, 1990), we may assume that ingestion of the toxic ration could cause harm to the fetuses during pregnancy. However, while many studies have pointed out the toxic effects of plants on embryo development (James *et al.*, 1994), no reports on the effects of *S. occidentalis* on fetuses were found in the literature. Therefore, the present study represents the first attempt to evaluate the toxicosis of *S. occidentalis* during the gestation period.

It is well known that nutritional status may affect embryonic and fetal development (Khera, 1984; Keen, 1992); however, the present study showed that neither dam feed intake nor bodyweight differed from those of the control group, which permits us to rule out a toxic effect of *S. occidentalis* on mothers, indirectly producing injury to the fetuses. In addition, pups from experimental dams showed cardiac and hepatic lesions of the same pattern as verified in their mothers. Thus, it could be theorized that the impairment of development shown by the lesions verified in pups may be caused by the toxin of *S. occidentalis* seeds that passed through the placenta, with a toxic effect on the fetuses. However, further teratogenicity studies should be conducted in order to better understand the present results.

## Acknowledgements

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## Chapter 70

# Fetal Effects of Maternal Ingestion of *Solanum malacoxylon*: Evaluation in Rats

S.L. Górnjak, M. Barbosa-Ferreira, B. Schumacher-Henrique, P.C. Raspantini, R.Z. Hosomi, A.P. Moraes, P.C. Maiorka and M.L.Z. Dagli

*Research Center for Veterinary Toxicology (CEPTOX), Department of Pathology, School of Veterinary Medicine, University of São Paulo, São Paulo, Brazil*

### Introduction

A disease of grazing livestock, known as 'espichamento' in Brazil and 'enteque seco' in Argentina characterized by deposition of calcium phosphate in soft tissues, has been diagnosed for many years. The disorder occurs following the chronic ingestion of a toxic plant called *Solanum malacoxylon*, which contains a glycoside conjugated with vitamin D<sub>3</sub> (1,25 [OH]<sub>2</sub>D<sub>3</sub>) (Haussler *et al.*, 1976; Wasserman *et al.*, 1976). *Solanum malacoxylon* causes hypercalcaemia, hyperphosphataemia and extensive mineralization of soft tissues (Collier, 1926; Worker and Carrillo, 1967; Carrillo *et al.*, 1971; Dobereiner *et al.*, 1975).

Many studies showing the calcinogenic effects of *S. malacoxylon* have been conducted on different animal species (Carrillo and Worker, 1967; Matualen, 1972; Uribe *et al.*, 1974; Done *et al.*, 1976). There is, however, little information on the effects of *S. malacoxylon* during pregnancy. Recently, we verified that administration of *S. malacoxylon* during gestation to rats (Górnjak *et al.*, 1998) and rabbits (Górnjak *et al.*, 1999) produces litters with alterations of soft tissue and increased phosphorus and calcium levels. The purpose of the present study was to provide further information on the teratological features of *S. malacoxylon* when administered to rats.

### Materials and Methods

Leaves of *S. malacoxylon* (Sm) were collected from the Pantanal region, Mato Grosso do Sul, Brazil. They were air-dried, powdered and incorporated into a rat diet at the following four concentrations: Sm 0.1%, Sm 0.2%, Sm 0.5% and Sm 1%. The mixture (leaves + the ingredients of the rat diet) was homogenized and pelleted.

Female Wistar rats (50) were paired overnight with proven sires. Females showing evidence of mating (a vaginal plug or vaginal smear with sperm cells) were assigned in rotation to each group up to the day when the required ten positive females had been allotted to each group. During gestation, the dams were housed individually. The experimental groups received Sm 0.1%, Sm 0.2%, Sm 0.5% and Sm 1% on days 6 to 21 of gestation. Ten control animals were fed pelleted food containing no plant material throughout gestation. Food consumption and bodyweights were measured every other day.

On the 21st day of gestation the dams were anaesthetized and blood samples were taken from of the hepatic vein for evaluation of serum calcium (Ca), inorganic phosphorus (P), magnesium (Mg) and alkaline phosphatase (AP). After this procedure, all females were euthanized and the uterine horns were immediately removed. The number of corpora lutea, implantations, resorptions, live and dead fetuses were recorded. The fetuses were weighed and examined for macroscopic external malformations. Half of each litter was stained with alizarin red according to the technique of Staples and Schnell (1964) to detect alterations of the skeleton, and representative samples of lungs, aorta, heart and kidneys of the other half of the litters were fixed in 10% formalin and stained with haematoxylin and eosin (H&E) or Von Kossa stain for histopathological study.

Data were expressed as the mean values and the standard error of the mean and were analysed statistically by ANOVA followed by Duncan's test. Results were considered significant when  $P < 0.05$ .

## Results

Maternal food consumption during gestation was similar in all groups (data not shown). No alteration was also detected on bodyweight gain between experimental and control groups (Table 70.1).

The biochemical data are outlined in Fig. 70.1. Females from all experimental groups showed an increase in serum Ca levels, and inorganic P levels were significantly higher in dams from the Sm 1% group. An increase in serum Mg was observed only in females that received the highest concentration of Sm. No differences in AP levels were observed between control and experimental groups.

Histopathological evaluation revealed alterations in almost all dams fed the ration containing Sm, with the lesions being much more conspicuous in females treated with a larger amount of Sm. Pups from treated mothers also showed several alterations. Lungs were the most affected organs, showing intense inflammatory infiltrates of mostly mononuclear cells around the bronchi, bronchioles and blood vessels. Hyperplasia of bronchial epithelium was also observed.

The reproductive data summarized in Table 70.1 show that Sm administration did not cause any change in the number of corpora lutea, implantations, or pre- and post-implantation loss, the same occurring with fetal and placental weight

**Table 70.1.** Reproductive performance of rats fed rations containing 0.1%, 0.2%, 0.5% or 1% *S. malacoxylon* (Sm) leaves.

	Groups				
	Control	Sm 0.1%	Sm 0.2%	Sm 0.5%	Sm 1%
Pregnant at term with total resorption	0	0	0	0	0
Dams' bodyweight gain	88.0 ± 18.4	77.1 ± 18.4	74.8 ± 21.5	64.3 ± 19.1	61.0 ± 19.3
Number of corpora lutea (mean ± SD)	12.1 ± 2.1	12.5 ± 3.8	13.3 ± 2.2	13.8 ± 4.7	11.1 ± 2.1
Number of implantations (mean ± SD)	10.6 ± 1.9	10.5 ± 1.3	11.5 ± 1.8	10.1 ± 1.6	9.2 ± 1.1
Number of resorptions (mean ± SD)	1.0 ± 1.0	1.2 ± 1.2	1.7 ± 2.0	1.3 ± 1.3	2.3 ± 1.1 <sup>a</sup>
Number of live fetuses (mean ± SD)	9.6 ± 1.8	9.4 ± 1.6	9.8 ± 2.2	8.7 ± 2.1	6.9 ± 1.7 <sup>a</sup>
Gravid uterus weight (g ± SD)	62.0 ± 11.9	60.8 ± 12.3	61.5 ± 14.6	56.5 ± 11.4	43.3 ± 12.3 <sup>a</sup>
Fetal weight (g ± SD)	4.6 ± 0.6	4.6 ± 0.5	4.4 ± 0.6	4.6 ± 0.5	4.1 ± 0.9
Placental weight (g ± SD)	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Fetal length (cm ± SD)	4.0 ± 0.1	4.0 ± 0.2	3.9 ± 0.2	4.0 ± 0.2	4.1 ± 0.5
Pre-implantation loss (%)	12.4	16.0	13.5	26.8	17.1
Post-implantation loss (%)	9.4	10.4	14.8	13.8	25.0

<sup>a</sup>Significant vs. control group,  $P < 0.05$  (Duncan's test).

and fetal length. However, the number of resorptions, live fetuses and gravid uterus weight were lower in the Sm 1% group.

No skeletal malformations were found in the experimental groups (Table 70.2). Nevertheless, the teratological examinations revealed differences in the incidence of fetal variations between the control and experimental groups. A significantly higher incidence of fetuses having sternal anomalies was detected in the Sm 0.1% and Sm 0.2% groups, and vertebral and rib anomalies were higher in the Sm 0.2% and Sm 1% groups. Fetuses from dams treated with Sm 0.2% to Sm 1% showed a higher incidence of reduced cranial ossification.

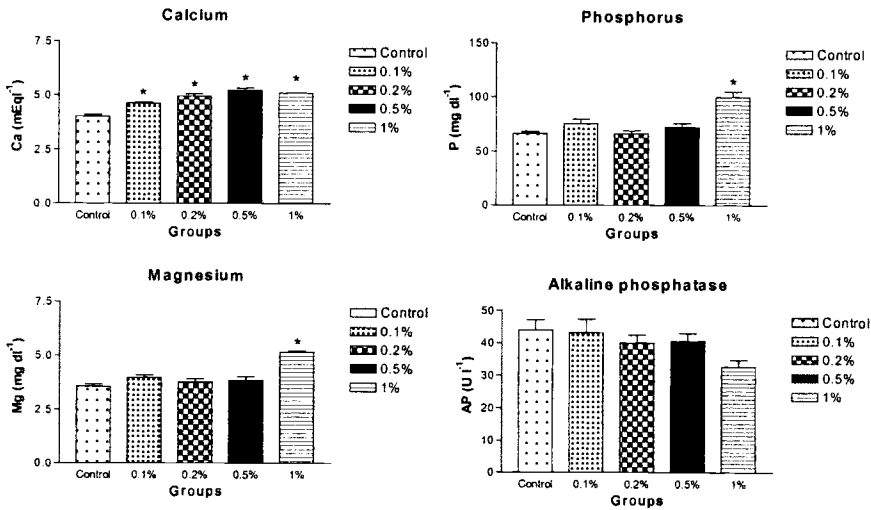


**Table 70.2.** Incidence of external skeletal malformations and/or variations in fetuses from mothers fed ration containing 0.1%, 0.2%, 0.5% or 1% *S. malacoxyton* (Sm) leaves.

	Control	0.1%	0.2%	0.5%	1.0%
<b>Skeletal malformation</b>					
Affected litters	0/10	0/10	0/11	0/10	0/9
Affected fetuses	0/59	0/64	0/85	0/65	0/55
<b>Skeletal variations</b>					
Affected litters	10/10	0/10	11/11	10/10	9/9
Affected fetuses	37/59	55/64 <sup>a</sup>	79/85 <sup>b</sup>	52/65 <sup>a</sup>	50/55 <sup>a</sup>
Sternal anomalies	34/59	50/64 <sup>a</sup>		40/65	36/55
Vertebral anomalies	5/59	4/64	23/85 <sup>a</sup>	7/65	24/55 <sup>b</sup>
Rib anomalies	3/59	9/64	16/85 <sup>a</sup>	7/65	11/55 <sup>a</sup>
14th rib	0/59	1/64	0/85	0/65	0/55
Reduced cranial ossification	2/59	7/64	12/85 <sup>a</sup>	15/65 <sup>a</sup>	24/55 <sup>b</sup>

<sup>a</sup>Significant vs. control group,  $P < 0.05$  (Fisher's Exact Test).

<sup>b</sup>Significant vs. control group,  $P < 0.0001$  (Fisher's Exact Test).



**Fig. 70.1.** Serum levels of calcium, phosphorus, magnesium and alkaline phosphatase, in control and experimental dams treated during the 6th to 21st day of gestation with rations containing 0.1%, 0.2%, 0.5% and 1% of *S. malacoxyton* leaves; \* $P < 0.05$  (Duncan's test), compared to control.

## Discussion

The histopathological findings obtained here confirmed our previous studies in both rats (Górniak *et al.*, 1998) and rabbits (Górniak *et al.*, 1999), showing that litters from dams treated with *S. malacoxylon* during pregnancy presented alterations in soft tissue, which support our assumption that the vitamin D<sub>3</sub>-glycoside passes through the placental barrier to the fetus.

Anorexia and rapid weight loss are initial and prominent symptoms of *S. malacoxylon* toxicity in rabbits, guinea-pigs and cattle (Worker and Carrillo, 1967; Moraña *et al.*, 1994; Górniak *et al.*, 1999). In addition, it is known that maternal nutritional status may affect embryonic and fetal development (Khera, 1985; Keen, 1992). However, we did not observe changes in feed consumption or bodyweight gain in experimental dams throughout the gestational period. Thus, the decreased number of live fetuses and the lower weight of the gravid uterus from female rats treated with the highest concentration of *S. malacoxylon* in the ration could be attributed to a direct toxic effect.

An important finding of the present study was the significant incidence of skeletal variations observed in fetuses from the experimental groups. To date, there are no studies in the literature showing the effects of *S. malacoxylon* on skeletal development; on the other hand, some reports have pointed out that high doses of vitamin D during gestation can produce teratogenic effects, particularly bone defects (Ornoy *et al.*, 1969, 1972; Zane, 1976; Ariyuki, 1986). Thus, it could be assumed unequivocally that *S. malacoxylon* possesses a teratogenic effect produced by its toxic principle, a glycoside conjugated with vitamin D<sub>3</sub>.

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## Chapter 71

# **A Survey of Venezuelan Plants Toxic to Livestock and their Potential as Anti-insect Materials**

M.E. Alonso-Amelot, E. Arellano, J.L. Avila, L. Aubert, R. Romero, M.P. Calcagno, M. Avendaño, M. Perez and L.D. Otero  
*Grupo de Química Ecológica, Departamento de Química, Facultad de Ciencias, Universidad de Los Andes, Mérida 5101, Venezuela*

### **Evolution of Poisonous Plant Risk Potential in the Neotropics**

Classical ecological theories explain the production of plant xenobiotics as a response to insect pressure over evolutionary time, not only because the number of arthropod species is large but also they continuously and rapidly evolve detoxification strategies. Mammals, however less species-rich and numerous, also constitute a major threat to plant development. For example, a single bull weighing 1000 kg may devour in excess of 100 kg of plant-based fodder each day while 50,000 grasshoppers of average size would be required to cause similar damage. It is to be expected, therefore, that many range plants potentially exposed to attack by large vertebrate herbivores also synthesize xenobiotic materials (Gronin, 1978; Huxtable, 1990) capable of deterring the animal from feeding, poisoning it, or even causing its demise (Laycock, 1978). The phenomenon is of worldwide importance and is particularly critical in the tropics (Nielsen, 1978; Sperry *et al.*, 1986; Gonzalez Stuart, 1989; Huxtable, 1990) where plant biodiversity and nearly year-round conditions for growth exist. The ensuing toxicosis may not be restricted to animal health since it has been shown that some key range plant xenobiotics find their way into the human food chain with potentially severe consequences (Deinzer *et al.*, 1977; Roitman, 1983; Molyneux and James, 1990; Alonso-Amelot *et al.*, 1996; Alonso-Amelot and Castillo, 1997; Alonso-Amelot, 2001).

### **Establishing Plant Poisoning Risks: Survey of Poisonous Plants in Venezuela**

Establishing solid correlations between a given pathological scenario in individual animals, and particular poisonous plant species of the tropics is difficult. This is because: animals may develop symptoms stemming from various

poisonous plants simultaneously in view of the diversity of toxic plants; a variety of prevalent bacterial or viral-based tropical diseases may be confounding factors; and symptoms may develop several days or weeks after ingestion of the obnoxious plants. Therefore, the task of assessing the risk of the poisoning of animals by plants in tropical grazelands cannot rely solely on case studies of poisoning outbreaks unless the pathological profiles are very well defined. Instead, we resorted to inspect the floristic composition of typical rangelands of western Venezuela and determine which species of acknowledged toxicity were of common occurrence there. We found over 140 poisonous plant species distributed in 37 families (Table 71.1). Many more, still undefined, undergrowth species may grow mingled with edible grasses and forbs in rangelands of the neotropics (Aristiguieta, 1964; Steyermark and Huber, 1978; Ricardi, 1992), so their consumption may be accidental, overriding the ability of the animals to select plants to their liking only.

**Table 71.1.** List of plant species deemed as toxic to farm animals found in the west of Venezuela where most of the cattle business is settled. Recollection by the authors. Symptoms are mostly those described by ranchers and in a few instances by professional veterinarians.

Genus/species	Affected livestock	Reported effects
<i>Agave lecheguilla</i>	Caprines	Jaundice, severe nasal-eye discharges
<i>Amaranthus retroflexus</i>	Bovines, caprines	Those of nitrate poisoning
<i>Toxicodendron striatum</i>	Bovines, caprines, equines	Severe mucosal irritation, mouth oedema, death
<i>Asclepia subverticilata</i>	Bovines, caprines, equines	Heart disease and consequences, paralysis
<i>Asclepia latifolia</i>	Bovines, caprines, equines	<i>Idem</i>
<i>Asclepia curassavica</i>	Bovines, caprines, equines	<i>Idem</i>
<i>Calotropis gigantea</i>	Bovines, caprines, equines	Heart arrhythmia, depression, emesis, digestion stoppage, death
<i>Calotropis procera</i>	Bovines, caprines, equines	<i>Idem</i> , condition developed by ingestion of cardiac glycosides
<i>Gomphocarpus fruticosus</i>	Bovines, caprines, equines	<i>Idem</i>
<i>Nerium oleander</i>	Bovines, caprines, equines	Paralysis, fever, bloody diarrhoea
<i>Thevetia thevetioides</i>	Caprines	Emesis, gastrointestinal irritation
<i>Ageratum conyzoides</i>	Equines	Loss of appetite and weight
<i>Artemisia filifolia</i>	Horse	Restlessness, front leg paralysis
<i>Baccharis pterionoides</i>	Bovines	Restlessness, front leg paralysis, death
<i>Conyza coulteri</i>	Ovines	General intoxication
<i>Eupatorium wrightii</i>	Bovines	General intoxication, death
<i>Parthenium hysterophorus</i>	Bovines	Thrombocytopenia, bleeding
<i>Senecio longilobus</i>	Bovines, equines	Those of PA <sup>a</sup> intoxication

Table 71.1. (cont.)

Genus/species	Affected livestock	Reported effects
<i>Verbesina encelioides</i>	Bovines, caprines, equines	Those of nitrate poisoning
<i>Viguiera annua</i>	Bovines	Dyspnea, convulsions
<i>V. leptodonta</i>	Bovines	<i>Idem</i>
<i>V. mucronata</i>	Bovines	<i>Idem</i>
<i>Xanthium strumarium</i>	Bovines	Ataxia, hypothermia, neck contraction, death
<i>Cassia</i> spp.	Bovines	Dark urine, diarrhoea, anorexia,
<i>Isotoma longiflora</i>	Equines	General undefined intoxication, death
<i>Drymaria arenarioides</i>	Bovines, caprines, equines	Those of ergot alkaloidosis, < 0.1% in feed causes acute intoxication
<i>D. cordata</i>	Bovines, caprines, equines	<i>Idem</i>
<i>Chenopodium album</i>	Bovines, caprines	Those of nitrate poisoning
<i>Convolvulus</i> spp.	Equines, pig	Gastrointestinal irritation, diarrhoea
<i>Cuscuta squamata</i>	Bovines, equines	Digestive disorders
<i>C. campestris</i>	Bovines, equines	Digestive disorders
<i>Ipomoea</i> spp.	Bovines, equines	Acute intoxication, death
<i>Pteridium aquilinum</i>	Bovines, caprines, equines	Severe haematuria, cancer, death
<i>Equisetum arvense</i>	Bovines, equines	Those of avitaminosis B1
<i>E. giganteum</i>	Bovines, equines	<i>Idem</i>
<i>Leucothoe mexicana</i>	Bovines, caprines, equines	Gastroenteritis, nephritis with necrosis, liver degradation
<i>Croton texensis</i>	Bovines	General intoxication from mild to acute symptoms and death
<i>C. bredemeyeri</i>	Bovines	<i>Idem</i>
<i>C. caracasanus</i>	Bovines	<i>Idem</i>
<i>C. curranii</i>	Bovines	<i>Idem</i>
<i>C. gossypifolius</i>	Bovines	<i>Idem</i>
<i>C. hirtus</i>	Bovines	<i>Idem</i>
<i>C. huberi</i>	Bovines	<i>Idem</i>
<i>C. ovalifolius</i>	Bovines	<i>Idem</i>
<i>C. populifolius</i>	Bovines	<i>Idem</i>
<i>C. pungens</i>	Bovines	<i>Idem</i>
<i>C. ruizianus</i>	Bovines	<i>Idem</i>
<i>C. speciosus</i>	Bovines	<i>Idem</i>
<i>C. xanthoschloros</i>	Bovines	<i>Idem</i>
<i>Euphorbia brasiliensis</i>	Bovines	Gastrointestinal irritation, diarrhoea, photosensitivity, hair loss, some examples of HCN toxicosis
<i>E. caracasana</i>	Bovines	<i>Idem</i>
<i>E. chamaesyce</i>	Bovines	<i>Idem</i>
<i>E. cotinifolia</i>	Bovines	<i>Idem</i>

Table 71.1. (cont.)

Genus/species	Affected livestock	Reported effects
<i>E. glomerifera</i>	Bovines	Hair loss, some examples of HCN toxicosis
<i>E. gollmeriana</i>	Bovines	<i>Idem</i>
<i>E. graminea</i>	Bovines	<i>Idem</i>
<i>E. heterophylla</i>	Bovines	<i>Idem</i>
<i>E. hirta</i>	Bovines	<i>Idem</i>
<i>E. hyssopilia</i>	Bovines	<i>Idem</i>
<i>E. insulata</i>	Bovines	<i>Idem</i>
<i>E. prostata</i>	Bovines	<i>Idem</i>
<i>Phyllanthus lathyroides</i>	Bovines	General intoxication, undefined specific symptoms
<i>P. micrandrus</i>	Bovines	<i>Idem</i>
<i>Sapium aubletianum</i>	Bovines	General intoxication, undefined specific symptoms
<i>S. biglandosu</i>	Bovines	<i>Idem</i>
<i>S. stylare</i>	Bovines	<i>Idem</i>
<i>Hura polyandra</i>	Fish, caprines	Severe gastrointestinal irritation, death
<i>Hura crepitans</i>	Fish, caprines	Severe gastrointestinal irritation, death
<i>Jatropha spathulata</i>	Bovines	Gastrointestinal irritation
<i>J. kunthiana</i>	Bovines	<i>Idem</i>
<i>Ricinus communis</i>	All	LD50 = 0.1 µg kg <sup>-1</sup> of ricin in seed
<i>Andira inermis</i>	Fish, caprines, human	General, unspecified toxicity, death
<i>Crotalaria retusa</i>	Chicken	Those of monocrotalin and other PA toxicosis
<i>C. sagittalis</i>	Horse	< 1% of daily diet caused death
<i>C. spectabilis</i>	Bovines, caprines, equines	Acute intoxication and death
<i>Erythrina herbacea</i>	Bovines, caprines, equines	Undefined intoxication
<i>E. glauca</i>	Bovines, caprines, equines	<i>Idem</i>
<i>E. poeppigiana</i>	Bovines, caprines, equines	<i>Idem</i>
<i>Lupinus</i> spp.	Bovines, caprines, equines	Convulsions, paralysis, death
<i>Robinia pseudoacacia</i>	Bovines, caprines, equines	Weakness, paralysis of rear legs, 'cold feet disease'
<i>Humboldtiella ferruginea</i>	Bovines, caprines, equines	Similar to <i>R. pseudoacacia</i>
<i>Sesbania</i> spp.	Ovines	Abdominal pain, diarrhoea, irregular heartbeat
<i>Sophora secundiflora</i>	Caprines, ovines	Muscle incoordination, rarely fatal
<i>Trifolium repens</i>	Bovines, caprines, equines	Diarrhoea, profuse insalivation, liver damage, HCN intoxication
<i>Salvia reflexa</i>	Bovines, ovines, equines	Nitrate intoxication, intense inflammation of gastrointestinal,

Table 71.1. (cont.)

Genus/species	Affected livestock	Reported effects
<i>Salvia reflexa</i> (continued)		necrosal lesions and death
<i>S. angulata</i>	Bovines, ovines, equines	Nitrate intoxication-like symptoms
<i>S. axilliflora</i>	Bovines, ovines, equines	<i>Idem</i>
<i>S. coccinea</i>	Bovines, ovines, equines	<i>Idem</i>
<i>S. occidentalis</i>	Bovines, ovines, equines	<i>Idem</i>
<i>S. rubescens</i>	Bovines, ovines, equines	<i>Idem</i>
<i>S. tilliaefolia</i>	Bovines, ovines, equines	<i>Idem</i>
<i>Linum usitatissimum</i>	Bovines, ovines, equines	Uncharacterized intoxication
<i>Phoradendron</i> spp.	Bovines, ovines, equines	Uncharacterized intoxication
<i>Gossypium hirsutum</i>	Bovines, caprines, equines	Heart muscle oedema, kidney, lung and liver congestion and death
<i>G. barbadense</i>	Bovines, ovines, equines	<i>Idem</i>
<i>Acacia berlandieri</i>	Bovines, caprines, equines	Ataxia and death
<i>A. constricta</i>	Bovines, caprines, equines	Weakness, muscle incoordination, death, HCN toxicosis
<i>A. vernicosa</i>	Bovines, caprines, equines	HCN toxicosis
<i>A. greggi</i>	Bovines, caprines, equines	<i>Idem</i>
<i>Leucaena glauca</i>	Bovines	Excess in fodder causes weight and hair loss
<i>L. thricodes</i>	Bovines	<i>Idem</i>
<i>Prosopis juliflora</i>	Bovines, caprines, equines	Chronic intoxication by extended feeding leads to death
<i>Ligustrum vulgare</i>	Bovines, equines, human	Abdominal pain, diarrhoea, muscle incoordination, death in horse
<i>Argemone mexicana</i>	Bovines, poultry	Generalized oedema, infrequent death
<i>Phytolacca icosandra</i>	Bovines	Emesis, prostration
<i>Petiveria alliacea</i>	Bovines	Muscle atrophy, glomerulonephritis
<i>Polygonum punctatum</i>	Bovines, fish	Photosensitivity, death in fish
<i>Driopteris filix-mas</i>	Equines	Thiamine deficiency, muscle incoordination, paralysis
<i>Portulaca oleracea</i>	Ovines	Profuse insalivation, rumen stasis, prostration, oxalate intoxication
<i>Clematis drummondii</i>	'Farm animals'	Undefined intoxication
<i>Blighia sapida</i>	Equines, undefined others	Severe hypoglycemia
<i>Hydrangea hortenses</i>	Equines	Abdominal pain, diarrhoea, prostration and death
<i>H. macrophylla</i>	Equines	<i>Idem</i>



**Table 71.1. (cont.)**

Genus/species	Affected livestock	Reported effects
<i>Cestrum</i> spp.	Bovines, equines	Central nervous system affected
<i>Ipomoea batatas</i>	Bovines, undefined others	Uncharacterized intoxication
<i>Physalis peruviana</i>	Caprines, ovines, pig	Uncharacterized intoxication
<i>Solanum</i> spp. (48 spp. in Venezuela)	Bovines	Tremors, prostration, bloody diarrhoea, toxic > 0.1% animal weight
<i>Duranta repens</i>	Bovines, caprines	Uncharacterized intoxication
<i>D. mutisii</i>	Bovines, caprines	Uncharacterized intoxication
<i>Lantana camara</i>	Bovines, caprines	Photosensitivity, blindness, skin erosion
<i>L. achyranthifolia</i>	Bovines	<i>Idem</i>
<i>L. armata</i>	Bovines	<i>Idem</i>
<i>L. canescens</i>	Bovines	<i>Idem</i>
<i>L. caracasana</i>	Bovines	<i>Idem</i>
<i>Aloysia lycioides</i>	Equines	Ataxia, nervousness, muscle incoordination
<i>Kallstroemia hirsutissima</i>	Bovines	Rear leg paralysis, prostration, death
<i>K. maxima</i>	Bovines	<i>Idem</i>
<i>Tribulus terrestris</i>	Ovines	Photosensitivity, blindness, loss of soft tissue, death

<sup>a</sup> PA, pyrrolizidine alkaloids.

## Poisonous Plants and Anti-insect Chemistry

If some benefit is to be gained from these injurious plants, they may be exploited as a quarry from which new natural anti-insect compounds and preparations may be discovered. Up to now, we have screened over 70 plant species selected partly from this source using specially designed bioassays (Alonso-Amelot *et al.*, 1994; Alonso-Amelot, 1996) and results at the extract level are promising. Several active compounds have been obtained and are presently under additional evaluation.

## Acknowledgements

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## Chapter 72

# Dietary Butylated Hydroxytoluene Protects Against Aflatoxicosis in Turkeys

P.J. Klein, T.R. Van Vleet, J.O. Hall and R.A. Coulombe Jr  
*Graduate Program in Toxicology, and Department of Veterinary Sciences, Utah State University, Logan, UT 84322-4620, USA*

### Introduction

Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), a hepatotoxin produced by the ubiquitous fungi *Aspergillus flavus* and *Aspergillus parasiticus*, is a nearly universal contaminant of poultry feeds (Klein *et al.*, 2000). Avoidance of contaminated feeds is rarely possible, and feed that contains relatively low concentrations of AFB<sub>1</sub> may still have deleterious effects on sensitive species, such as poultry (Giambrone *et al.*, 1985). In poultry, AFB<sub>1</sub> causes a reduction in growth rate, feed efficiency, hatchability, increased susceptibility to bacterial and viral diseases, and severe hepatotoxicosis (Kubena *et al.*, 1995).

In order to exert its toxic effects, AFB<sub>1</sub> must be activated to the *exo*-AFB<sub>1</sub>-8,9-epoxide (AFBO) by cytochrome P450s (CYPs) (Guengerich *et al.*, 1998), and AFBO detoxification occurs via glutathione *S*-transferases (GSTs), which utilize glutathione (GSH) to effectively 'trap' the highly reactive epoxide (Raney *et al.*, 1992). The affinity of phase II enzymes, such as GSTs, toward AFBO can often determine a species' resistance or susceptibility to this toxin (Hayes *et al.*, 1991).

The food antioxidant butylated hydroxytoluene (BHT) has been shown to induce phase II enzymes as well as protect chickens from aflatoxicosis (Larsen *et al.*, 1985; Ehrich *et al.*, 1986, 1988). We previously have shown that turkeys are extremely sensitive to the effects of AFB<sub>1</sub> due to a deficiency of AFB<sub>1</sub> detoxifying GSTs, a condition intensified by efficient AFB<sub>1</sub> activating CYPs (Klein *et al.*, 2000). In this study, we report that dietary BHT protects against many of the deleterious effects caused by AFB<sub>1</sub>, and that this antioxidant may prove to be a viable feed additive for the reduction of adverse health effects in poultry related to AFB<sub>1</sub> contamination.

### Materials and Methods

Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), butylated hydroxytoluene (BHT), acetone and all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO). Day-old male poult (Orlopp strain) were obtained from Moroni Feed Co. (Moroni, UT),

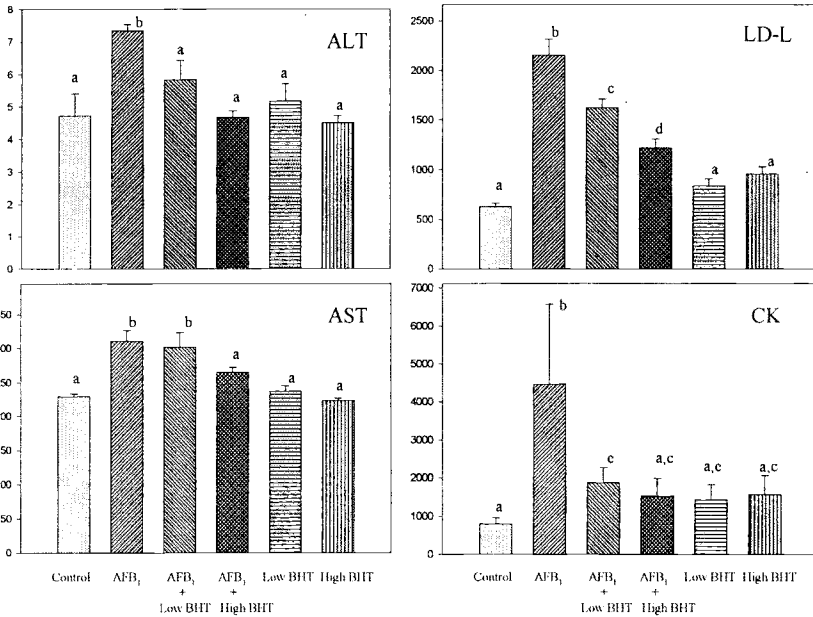
and maintained on a maize-based commercial diet (Moroni Feed Co., Moroni, UT). Turkeys at 10 days of age ( $n = 7$ ) were placed on the following antioxidant treatments: control, low (1000 ppm) and high (4000 ppm) BHT. After 10 days of BHT pretreatment, 1 ppm AFB<sub>1</sub> was added to the diets of two groups of BHT treated poults and these BHT + AFB<sub>1</sub> diets were continued for 10 more days. The birds were then killed and samples collected for analysis. Serum was analysed by a Synchron CX 5 Clinical System (Beckman, Fullerton, CA) for alanine aminotransferase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LD-L), alkaline phosphatase (ALP), albumin (ALB), creatine phosphokinase (CK), total protein (TP), total bilirubin (TBIL) and direct bilirubin (DBIL). Livers were fixed in neutral buffered formalin immediately after removal, then embedded in paraffin, thin sectioned and stained with haematoxylin and eosin (H E). Frozen sections were thin-sectioned and stained with oil red O stain for lipid (McKinney and Riley, 1967). Groups were compared, for differences, using One Way ANOVA and *post-hoc* Tukey test. Significant differences were set at  $P < 0.05$ .

## Results

Weight gain in the AFB<sub>1</sub> group was significantly lower compared to control (data not shown). However, that of the AFB<sub>1</sub> + BHT (low and high) groups were not different from control values and significantly higher than the AFB<sub>1</sub> group.

Serum ALT, AST, LD-L and CK, marker enzymes for hepatocellular necrosis, were significantly elevated, compared to control, in the AFB<sub>1</sub> group (Fig. 72.1), but there was, in many cases, a dose-related decrease in these indicators in groups receiving both AFB<sub>1</sub> + BHT (Fig. 72.1). In contrast to these markers, serum bilirubin was not significantly affected among treatment groups (data not shown).

Pathologic examination of livers revealed several differences among the livers of the treatment groups. Gross post-mortem examinations of the livers identified increased tissue firmness and haemorrhaging associated with AFB<sub>1</sub> treatment alone, whereas these changes were reduced or absent in AFB<sub>1</sub> + BHT treated birds and were completely absent in all other groups (data not shown). Histopathological examination of liver tissue revealed biliary hyperplasia, as well as hepatocellular necrosis and loss of hepatic cords in turkeys that had received only AFB<sub>1</sub>, with these lesions not identified in control birds. These lesions are depicted in photomicrographs from a representative individual from control (Fig. 72.2A) and AFB<sub>1</sub> only groups (Fig. 72.2B).

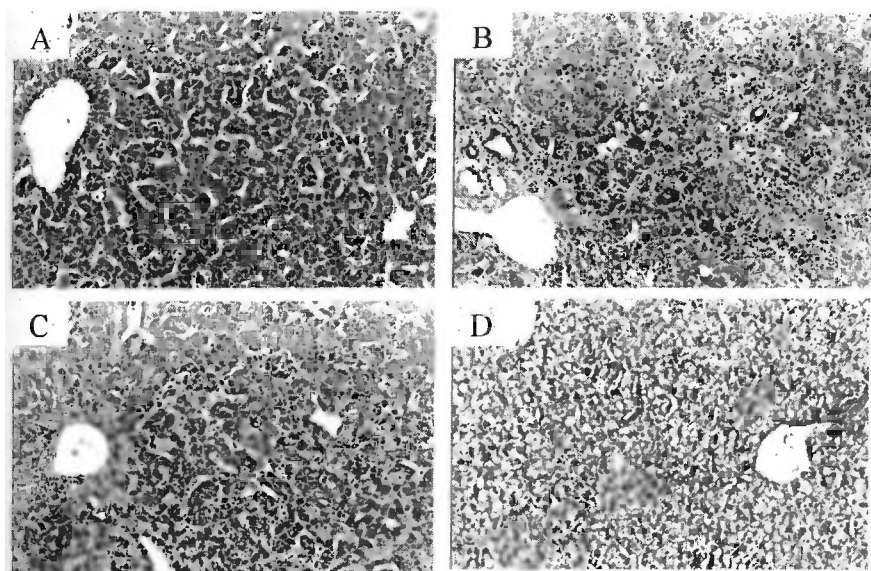


**Fig. 72.1.** The chemoprotective effect of BHT on AFB<sub>1</sub>-induced hepatotoxicity as indicated by altered concentrations of alanine aminotransferase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LD-L) and creatine phosphokinase (CK) in IU/L. Turkeys fed low (1000 ppm) and high (4000 ppm) BHT for 10 days, after which time 1 ppm AFB<sub>1</sub> was added to the diets, and continued for 10 more days. Each bar represents the mean ( $\pm$  SD;  $n = 7$ ). Different superscripts indicate a significant difference among groups ( $P < 0.05$ ).

The AFB<sub>1</sub>-associated hepatic pathology was significantly reduced in AFB<sub>1</sub> + BHT turkeys (Fig. 72.2C). Although BHT alone did not result in this type of lesion, a distinct hepatic lesion was associated with turkeys that had received BHT only (Fig. 72.2D). This lesion was identified as hepatocellular hydropic degeneration, as characterized by vacuolar cytoplasm of the cells. A lack of oil red O stain uptake further confirmed that this condition was not associated with lipid accumulation. This pathology was noticeably absent, or reduced, in other groups, including those that received a diet including BHT and AFB<sub>1</sub> (Fig. 72.2C).

## Discussion

This study has demonstrated strong protective effects of BHT against AFB<sub>1</sub>-induced toxicosis in turkeys. The mechanism of this protection is not yet known.



**Fig. 72.2.** Haematoxylin and eosin stained liver from turkeys dosed for 20 days with (A) control diet, (B) 1 ppm AFB<sub>1</sub>, (C) 1 ppm AFB<sub>1</sub> plus 4000 ppm BHT or (D) 4000 ppm BHT (X 120).

However, we have previously shown that the extreme sensitivity of turkeys to AFB<sub>1</sub> is likely due to a combination of highly efficient CYP-mediated activation and low GST-mediated AFB<sub>1</sub> detoxification (Klein *et al.*, 2000). Thus, it is possible that BHT treatment may inhibit CYP activation and/or increase GST-mediated detoxification of AFB<sub>1</sub> in these turkeys. Several other mechanisms are possible, many of which are the subject of current investigations. Our preliminary studies indicate that BHT directly inhibits the *in vitro* activation of AFB<sub>1</sub> by turkey liver microsomes.

Such protection conferred by these concentrations of BHT has precedent in other animal models. For example, in many mammalian species, compounds such as phenolic antioxidants have been shown to be strongly protective against the deleterious effects of AFB<sub>1</sub> (Kensler, 1994). In fact, such models have formed the basis for ongoing human cancer intervention trials.

Hepatic damage associated with AFB<sub>1</sub> treatment was exemplified by significant elevations of ALT, AST, LD-L and CK activities, all of which were significantly lower in groups receiving AFB<sub>1</sub> + high BHT. These trends were reflected in the histological analysis of the livers. AFB<sub>1</sub> causes both biliary hyperplasia as well as hepatocellular necrosis in sensitive species, such as turkeys (Coulombe, 1993). There was marked biliary hyperplasia and hepatocellular

necrosis found in turkeys receiving AFB<sub>1</sub> alone, while the severity of these lesions was reduced significantly in AFB<sub>1</sub> + BHT birds. This was a clear indication of protection associated with BHT treatments. The histopathological observations we made are consistent with those seen in other animal models using BHT as an AFB<sub>1</sub> chemoprotectant (Kensler *et al.*, 1994). The hydropic degeneration observed in the livers of the BHT-only groups has also been observed in other species (Safer and al-Nughamish, 1999). Because this hepatic lesion was not observed in the AFB<sub>1</sub> + BHT group, a shared metabolic pathway between these two compounds is possible. An additional trial has shown that long-term dietary BHT results in no apparent ill effects other than this hepatic lesion (data not shown).

Importantly, other than transient hydropic degeneration, we detected no adverse effects caused by BHT in these turkeys. Serum enzymes in both the low and the high BHT group were statistically identical to those of the control. This indicates that BHT may be a safe management method of reducing aflatoxicosis in poultry. In total, these results show that BHT counteracts many of the deleterious effects caused by AFB<sub>1</sub>. It is possible that antioxidant may prove to be a viable feed additive for the reduction of adverse health effects in poultry related to AFB<sub>1</sub> contamination.

## Acknowledgements

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## Chapter 73

# Mycotoxins in Agricultural Crops

G.K. Mortensen

*Risø National Laboratory, Plant Research Department, PRD-124, PO Box 49,  
DK-4000 Roskilde, Denmark*

This research project focuses on natural fungal toxins. It is well known that *Fusarium* species are plant pathogenic fungi, which attack wheat, maize and other cereals and produce different toxic metabolites such as nivalenol, deoxynivalenol and zearalenone. These toxins are studied together with ochratoxin A and citrinin produced by *Penicillium* species (Fig. 73.1).

Many toxigenic species of *Fusarium* are common pathogens of cereal plants, causing diseases such as head blight of wheat and barley and ear rot of maize. Consequently, when cereal plants are infected with these fungi, there is a risk that grain may become contaminated with *Fusarium* mycotoxins. *Penicillium* species are generally regarded as storage fungi but studies have shown that *Penicillium verrucosum* can survive in Danish fields and become an integral part of the soil ecosystem (Elmholt and Hestbjerg, 1999).

The production of these different toxins may cause human exposure to serious danger either directly or as residues in animal tissues. A review of the contamination of cereal grains with *Fusarium* mycotoxins showed different crops from many countries were highly contaminated with these toxins (Placinta *et al.*, 1999). *Fusarium* toxins were measured in wheat grown in Germany 1987 and mean concentrations found were in the range from 7 to 1632  $\mu\text{g kg}^{-1}$  (Müller and Schwadorf, 1993). Many contents were below 100  $\mu\text{g kg}^{-1}$  but high concentrations were also measured: maximum values at 20538 and 8036  $\mu\text{g kg}^{-1}$  for deoxynivalenol and zearalenone respectively were measured. These two toxins were often measured in the same samples. In 140 samples of maize imported into the UK zearalenone and fumonisins were measured in nearly all samples (Scudamore and Patel, 2000). Of the zearalenone contents 41.7% was above 100  $\mu\text{g kg}^{-1}$  and the maximum value was 584  $\mu\text{g kg}^{-1}$ . In 10% of the samples the concentrations of ochratoxin A were above the detection limit at 0.1  $\mu\text{g kg}^{-1}$  and the maximum content was 1.5  $\mu\text{g kg}^{-1}$ . Investigations in various maize fractions showed great variations (di Menna *et al.*, 1997). The highest levels of nivalenol and deoxynivalenol were measured in the rachis and peduncle and generally nivalenol was measured at higher concentrations compared to deoxynivalenol. Zearalenone was measured most consistently in the leaf axis but the highest concentrations were measured in the rachis (417  $\text{mg kg}^{-1}$ ). There was a tendency for concentrations to increase with plant age.

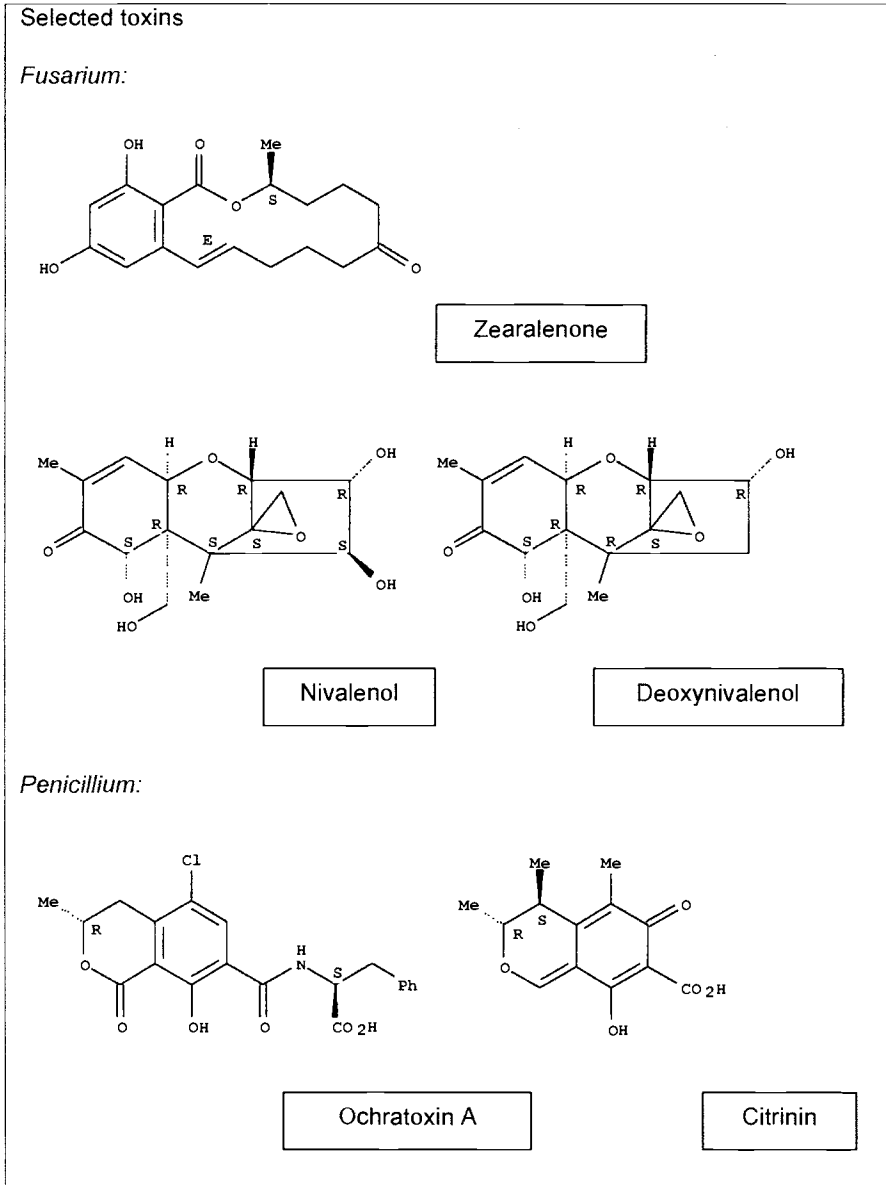


Fig. 73.1. Natural mycotoxins investigated in this project.

On the basis of the toxicological evaluations, a Nordic working group on food toxicology and risk assessment has established temporary tolerable daily intakes (tTDIs) for different toxins (Eriksen and Alexander, 1998). In the Nordic

countries the major dietary source is wheat and barley. The contribution from rice and maize is of little significance due to low consumption. Comparisons of estimated average intakes of the toxins with the recommended tTDIs showed that for example deoxynivalenol and zearalenone are close to the tTDI.

Therefore the fate of these toxins in soil-plant systems will be investigated in more detail in the period 2000-2002. The amounts of toxins produced, the availability and concentrations in different plant species are the major aspects that will be studied. Special consideration will be given to how organic farmers, who do not use fungicides in their farming, can prevent contamination by toxins. Adsorption experiments are being conducted in the laboratory with a variety of soil types. Batch experiments with zearalenone, nivalenol and deoxynivalenol are conducted with well-known soil types from Danish experimental stations. The same soils are used in pot experiments where the degradation of these toxins is investigated. Experiments with the addition of toxins as spiked solutions as well as experiments with toxin infected grain or stalk will be established to assess the plant leaf, root and soil interactions. These different laboratory experiments will be conducted in cooperation with Danish organic farmers in order to measure the toxins in soil and agricultural crops, when there are occurrences of for example *Fusarium*.

Different analytical methods have been used to determine mycotoxins as reviewed by Krska and Josephs (2001). Although the toxins consist of aromatic structures, the hydroxyl and carboxylic groups confer strong hydrophylic properties to these compounds. Therefore, the extraction procedures generally use mixtures of water and methanol or acetonitrile. The clean-up process is very important too for accuracy and low detection limits. Different SPE columns and also immuno-affinity columns are now available for the clean-up (Krska and Josephs, 2001).

The analytical methods are now developed for determination of the toxins at low levels in both soil and plant material in order to determine both the available and the total concentrations in soil and plant material. The methods are based on different extraction procedures and purification procedures with solid phase extraction techniques combined with the use of immuno-affinity columns.

Ochratoxin A, citrinin and zearalenone are extracted with methanol and water as described by Seidel *et al.* (1993) and separated using HPLC with fluorescence detection where a phenyl-hexyl column (LUNA; 5  $\mu$ m, 250 mm, 4.6 mm). Acetonitrile and water with addition of trifluoroacetic acid (TFA) is used as the mobile phase and the wavelengths are optimized for each compound.

Nivalenol and deoxynivalenol are extracted using the method described by Tanaka *et al.* (1985) and determined by GC/MS with chemical ionisation (Schwadorf and Müller, 1991) with few modifications. Isobutane is used as the ionization gas (column is from Restek XTI-5; 30 m, 0.25 mm ID, 0.25  $\mu$ m). The detection limits vary by orders of magnitude and are shown in Table 73.1.

The aim of the project in Denmark is to increase the understanding, knowledge and mechanisms of the environmental fate of these mycotoxins with a view to preventing mycotoxicosis in man.

**Table 73.1.** Detection limits for the investigated mycotoxins.

Toxin	Response area ( $\mu\text{g l}^{-1}$ )	Detection limit ( $\mu\text{g kg}^{-1}$ )
Ochratoxin A	4700	0.1
Citrinin	1350	0.5
Zearalenone	710	1.0
Nivalenol	53	1.0
Deoxynivalenol	43	1.0

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## Chapter 74

# Intoxication by *Ramaria flavo-brunnescens* in Domestic Animals

F. Riet-Correa<sup>1</sup>, C.S.L. Barros<sup>2</sup> and A.L. Schild<sup>1</sup>

<sup>1</sup>Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Pelotas University, 96010-900, Pelotas RS, Brazil; <sup>2</sup>Departamento de Patologia, UFSM, 97119-900, Santa Maria RS, Brazil

*Ramaria flavo-brunnescens*, from the Clavariaceae family, is a yellow cauliflower-like mushroom, up to 11 cm high, which grows in eucalyptus woods (Fidalgo and Fidalgo, 1970). It is found in southern and southeastern Brazil (Bauer *et al.*, 1966; Prucoli and Camargo, 1966; Santos *et al.*, 1975; Paschoal *et al.*, 1983), Uruguay (Freitas *et al.*, 1966; Quiñones-Sowerby, 1973), Argentina (Perusia and Rodriguez Armesto, 1992), North America, Australia and China (Fidalgo and Fidalgo, 1970). It has been also reported from India in an oak forest (Fidalgo and Fidalgo, 1970). Fidalgo and Fidalgo (1970) gave a detailed description of *R. flavo-brunnescens*.

Barros (1958), in southern Brazil, reported disease associated with this mushroom for the first time and suggested the possibility that it was caused by selenium toxicity. Later the intoxication was produced experimentally in cattle (Bauer *et al.*, 1966) and guinea pigs (Bauer *et al.*, 1966; Freitas *et al.*, 1966) by the administration of *R. flavo-brunnescens*. The object of this paper is to review intoxication by this mushroom.

### Epidemiology

In Brazil, Uruguay and Argentina, intoxication occurs from February to June (end of summer and autumn), when the mushroom is normally found in eucalyptus forests. Occasionally *R. flavo-brunnescens* appears also in spring (Quiñones-Sowerby, 1973), but intoxication has not been observed at this time. Santos (1993) reports that the mushroom is palatable and cattle look for it for food.

The spontaneous intoxication by *R. flavo-brunnescens* is reported in cattle in Brazil in the states of Rio Grande do Sul (Bauer *et al.*, 1966; Santos *et al.*, 1975; Riet-Correa *et al.*, 1983, 1985; Schild *et al.*, 1996) and São Paulo (Paschoal *et al.*, 1983), in Uruguay (Freitas *et al.*, 1966; Quiñones-Sowerby, 1973), and in Argentina (Perusia and Rodriguez Armesto, 1992). Morbidity rates in cattle vary from 10% to 100%, and case fatality rates are from 10% to 90%. Cattle of different ages and breeds are affected (Bauer *et al.*, 1966; Freitas *et al.*, 1966;

Quiñones-Sowerby, 1973; Paschoal *et al.*, 1983; Riet-Correa *et al.*, 1985; Schild *et al.*, 1996).

In sheep the disease has been reported in Uruguay (Freitas *et al.*, 1966; Riet-Correa *et al.*, 1996) and in the Brazilian states of Rio Grande do Sul (Riet-Correa *et al.*, 1983) and São Paulo (Prucoli and Camargo, 1966). Morbidity rates in sheep vary from 1% to 100% and case fatality rates from 0% to 100% (Quiñones-Sowerby, 1973; Riet-Correa *et al.*, 1996). Sheep of different ages are affected (Quiñones-Sowerby, 1973; Riet-Correa *et al.*, 1996).

Spontaneous intoxication also occurs in horses (Santos *et al.*, 1975) and swine (Freitas *et al.*, 1966), but clinical signs and pathology had not been described in these species.

Intoxication was frequent in Uruguay and southern Brazil during the 1950s, but after 1966, when its aetiology was established, there was a decrease in the incidence of the disease. However, in Uruguay the frequency of intoxication in cattle and sheep increased in the last years, due to the increase of the forestation area with eucalyptus, which are also used for breeding livestock (Rivero *et al.*, 2000).

## Clinical Signs

In cattle the main clinical signs are anorexia, depression, lameness, drooling of saliva, lingual ulcers and loss of hairs from the tip of the tail. Due to the lameness and salivation, farmers and practitioners usually confuse the intoxication with foot and mouth disease.

Lesions of the tongue are characterized by smoothness of the dorsal surface due to atrophy of lingual papillae, ulcerations on the border of the anterior portion, and occasionally sloughing of epithelium. Lameness is severe. There are hoof abnormalities characterized by hypersensitivity, and swelling and reddening of the coronet, coronary skin and proximal interdigital space. Occasionally, these lesions are later covered by crusts. There is separation of the hoof-skin junctions followed, sometimes, by separation and sloughing of the hooves. The animals remain recumbent most of the time. Softening of the base of the horns followed by separation and sloughing of the horn sheath is frequent. Loss of long hairs from the tail switch is characteristic of the disease. Loss of hairs from the dorsum, mainly by pulling, is also observed. Haemorrhages of the anterior chamber, hyperemia of the conjunctiva and corneal opacity are observed in the eyes. These lesions frequently cause blindness (Barros, 1958; Bauer *et al.*, 1966; Freitas *et al.*, 1966; Quiñones-Sowerby, 1973; Santos *et al.*, 1975; Paschoal *et al.*, 1983; Riet-Correa *et al.*, 1985; Schild *et al.*, 1996).

Decreased ruminal movements or ruminal paralysis (Bauer *et al.*, 1966), nasal serous discharge, sloughing of the skin of the muzzle (Schild *et al.*, 1996), subcutaneous oedema of the brisket and intermandibular space, photosensitization (Riet-Correa *et al.*, 1985), diarrhoea (Paschoal *et al.*, 1983) and abortion in

pregnant cows (Barros, 1958; Bauer *et al.*, 1966; Schild *et al.*, 1996) are also reported. There is a progressive weight loss and clinical course, until death or recovery, ranges from 8 to 30 days (Freitas *et al.*, 1966; Santos *et al.*, 1975). Animals affected for long periods take 2-3 months or more to regain the weight lost. When the animals lose the hooves full recovery takes 6-12 months (Freitas *et al.*, 1966).

Clinical signs in sheep in field cases are markedly different from cattle. They are characterized mainly by nervous disorders with convulsions, muscle tremors, ataxia, hypermetria, nystagmus and opisthotonos (Freitas *et al.*, 1966; Riet-Correa *et al.*, 1996). Some animals remain recumbent and die in 20-30 days, but recover if food is given (Freitas *et al.*, 1966). Hypertemia, polyuria, hyperemia of the conjunctiva and corneal opacity, ulcers in the tongue and necrotic lesions in the extremities characterized by a hyperemic line with crusts at the coronary band were also observed in experimental intoxications (Prucoli and Camargo, 1966; Sallis *et al.*, 2000).

## Pathology

In cattle, the tongue lesions mentioned before and ulceration of the oesophagus covered by fibrinous exudate are almost always present (Barros, 1958; Bauer *et al.*, 1966; Freitas *et al.*, 1966; Quiñones-Sowerby, 1973; Santos *et al.*, 1975; Paschoal *et al.*, 1983; Riet-Correa *et al.*, 1985; Schild *et al.*, 1996). Reddening and ulceration of the abomasum and rumen (Santos *et al.*, 1975), reddening of the large and small intestine, oedema of the subcutaneous tissues, peritoneum and walls of the gallbladder and abomasum (Riet-Correa *et al.*, 1985), and red or white discoloration of the liver (Quiñones-Sowerby, 1973) are also reported.

In cattle, histological lesions in the hooves are vacuolation of keratinocytes and irregular keratinization of the periople and laminar region. These lesions are more marked in the laminar region where most keratinocytes are vacuolated and keratinization is lacking or discontinuous, occurring in small floccular structures. Vacuolated keratinocytes are also observed in the sole (Kommers and Santos, 1995). In sheep, lesions in the hooves are characterized by areas of coagulative necrosis, followed by ulceration with crusts in the epithelium of the coronary band and laminar epithelium of the periople. The corium is congested and haemorrhagic and the arterioles have a thickened muscular wall with swollen endothelial cells. Thrombosis is occasionally observed (Sallis *et al.*, 2000). Vacuolation of keratinocytes is also observed in the horns (Kommers and Santos, 1995).

Histological lesions of the skin of the tip of the tail of cattle are characterized by a decreased number of hair follicles, with disorganization in the structure of the hair shaft and irregular arrangement of the cuticular hair cells. The follicles are either partially closed with trichilemmal keratinization in the central area or

completely closed, having few scattered apoptotic keratinocytes and a more conspicuous fibrous root sheath (Kommers and Santos, 1995).

In cattle the dorsal epithelium of the tongue is flattened with shortened or absent papillae and decrease in the number of keratinocytes. Parakeratotic hyperkeratosis, epithelial necrosis, ulceration and separation of the epithelium are observed (Santos *et al.*, 1975; Schild *et al.*, 1996). Thrombosis of the blood vessels is also reported (Quiñones-Sowerby, 1973). On histological examination of the tongue in sheep the stratum spinosum has hydropic degeneration, dyskeratosis, spongiosis, epithelial necrosis and acantholysis, leading to intraepithelial clefts and vesicles with infiltration of neutrophils in the epithelium. In some areas the epithelium is lost and the submucosa is oedematous and severely infiltrated by neutrophils. In areas close to the ulceration the outer layers of the stratum spinosum are severely vacuolized and necrotic. Blood vessels of the submucosa are congested and the arterioles have a thickened muscular wall and endothelial swelling (Sallis *et al.*, 2000).

Histological lesions in the oesophagus of cattle are characterized by epithelial necrosis and ulceration, and occasionally thrombosis of blood vessels (Quiñones-Sowerby, 1973; Santos *et al.*, 1975; Schild *et al.*, 1996).

Epithelial necrosis in the omasum and intestine (Santos *et al.*, 1975) are observed. Degeneration (Santos *et al.*, 1975) and necrosis (Quiñones-Sowerby, 1973) of hepatocytes, and centrilobular necrosis (Riet-Correa *et al.*, 1985) are reported in the liver. Epithelial necrosis and thrombosis of the blood vessels of the submucosa are occasionally found in the nose (Santos *et al.*, 1975). Infiltration by neutrophils, eosinophils and/or lymphocytes are observed in the submucosa of the tongue, nose, abomasum and gut (Quiñones-Sowerby, 1973; Santos *et al.*, 1975; Schild *et al.*, 1996).

## Toxicity, Active Principle and Pathogenesis

In cattle the administration of 5 g kg<sup>-1</sup> bodyweight, daily during 5 days, causes clinical signs. Daily doses of 20-25 g kg<sup>-1</sup> bodyweight cause clinical signs in 3-6 days and death in 15-40 days (Santos *et al.*, 1975; Kommers and Santos, 1995). A single administration of 36 g kg<sup>-1</sup> caused death in 13 days (Santos *et al.*, 1975). Sheep are more resistant to intoxication than cattle. Prucoli and Camargo (1966) produced intoxication in sheep fed with 250 g kg<sup>-1</sup> bodyweight of the fungus, daily, during 30 days. In other experiments the smallest amount of *R. flavo-brunnescens* that causes intoxication was of 167 g kg<sup>-1</sup> bodyweight administered in 9 days; the administration of 200 g kg<sup>-1</sup> in 3-10 days caused death after 5-13 days (Sallis *et al.*, 2000). Intoxication was produced in guinea pigs by giving the mushroom *ad libitum* (Bauer *et al.*, 1966; Freitas *et al.*, 1966).

Variations in the toxicity of *R. flavo-brunnescens* collected in different years in the same place have been demonstrated, and in some years the mushroom was not toxic (Sallis *et al.*, 2000). The mushroom rapidly lost its toxicity after



collection, was not toxic when dry, and lost considerable toxicity after freezing (Sallis *et al.*, 1993; Kommers and Santos, 1995). It is edible with no risk for humans after cooking (Fidalgo and Fidalgo, 1970). The toxic principle of the mushroom is unknown, but a volatile thermolabile alkaloid was detected in it (Bauer *et al.*, 1966).

Kommers and Santos (1995) suggest that the lesions caused by *R. flavo-brunnescens* are due to the interference within the keratinocytes of the metabolism of the sulphurous amino acids, mainly cystine, that results in changes in the molecular structure in the hard keratin. Clinical and pathological similarities between the different forms of ergotism and the intoxication by *R. flavo-brunnescens* suggest the presence, in the latter, of vasoactive constrictive substances with biologic action similar to the ergoalkaloids (Sallis *et al.*, 2000).

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## Chapter 75

# Natural Antioxidants and Mycotoxins: Theoretical Considerations and Practical Applications

P.F. Surai<sup>1</sup>, J.E. Dvorska<sup>2</sup> and N.H.C. Sparks<sup>1</sup>

<sup>1</sup>*Avian Science Research Centre, SAC, Ayr, KA6 5HW, Scotland, UK;* <sup>2</sup>*Sumy State Agrarian University, Sumy, Ukraine*

### Introduction

Natural antioxidants have received substantial attention in recent years in relation to human health and animal production. Modern animal and particularly poultry production heavily rely on the dietary supplementation of various synthetic (ethoxyquin, BHT and BHA) and natural (vitamins E and C) antioxidants. As our understanding of the molecular mechanisms associated with oxidative stress and how oxidative stress contributes to the development of various diseases, so it becomes increasingly apparent why antioxidants are such an important component of the diet.

Among the many stress factors associated with food and feed, mycotoxins are considered to be the most important group of stressors. With mycotoxin contamination of grain affecting 25% of the world production this is a global problem. In this context the elucidation of the protective mechanisms against such toxicants as mycotoxins as well as how antioxidants may be used to decrease the toxic effects of mycotoxins are considered to be important.

### Antioxidant–Pro-oxidant Balance in the Cell

It is well recognized that a delicate balance between antioxidants and pro-oxidants in the body is responsible for the regulation of many physiological processes. When free radical production overwhelms the defensive systems oxidative stress takes place. In such conditions many biological molecules can be damaged and biological membranes in particular are considered as an important target for free radical attack. For example, the peroxidation of the poly-unsaturated fatty acids (PUFA) in membranes can cause membrane fluidity to decrease and altered signal transmission (Halliwell and Gutteridge, 1999). Peroxidized PUFAs can also form clusters, creating pores in the membrane disturbing ionic balance (Halliwell and Gutteridge, 1999). Free radicals and lipid

peroxides can also inactivate a range of enzymes, which disrupts important metabolic pathways in the cell. This in turn can lead to the development of various degenerative conditions and diseases.

## Mycotoxins and Lipid Peroxidation

The mycotoxins aflatoxin B1 (AFB1), T-2 toxin, ochratoxin A (OA), fumonisin B1 (FB1), zeralenone (ZEN) and deoxynivalenol (DON) have been studied extensively (Galvano *et al.*, 2001) and all have been implicated in lipid peroxidation.

### Ochratoxin A

Of the mycotoxins studied, ochratoxin A (OA) is probably characterized as having the most pronounced pro-oxidant properties. The results of Rahimtula *et al.* (1988) showed that OA possessed the ability to enhance lipid peroxidation when added to liver or kidney microsomes *in vitro* or when administered to rats *in vivo*. Furthermore, lipid peroxidation, measured as malondialdehyde (MDA) formation or by oxygen uptake, was stimulated markedly by OA in a reconstituted system consisting of phospholipid vesicles (Omar *et al.*, 1990). After 24 h incubation OA increased by 50.5% lipid peroxidation (MDA production) in Vero cells in a concentration dependent manner (Baudrimont *et al.*, 1997). Similarly, after 48 h *in vitro* incubation, OA induced MDA accumulation and lactate dehydrogenase release from astrocytes and neurones (Belmadani *et al.*, 1999). Acute (24 h) treatment of brain cells in culture with high OA concentrations (10 to 50  $\mu\text{M}$ ) caused a significant increase in ROS formation, as measured by the intracellular oxidation of 2',7'-dichlorofluorescein (Monnet-Tschudi *et al.*, 1997).

There are species- and tissue-specific differences in animal susceptibility to OA-stimulated lipid peroxidation. For example, OA supplementation of the chicken diet (2.5 ppm for 14 days) increased MDA accumulation in the liver (Hoehler and Marquardt, 1996; Hoehler *et al.*, 1996) and plasma (Hoehler *et al.*, 1996). In contrast, MDA accumulation in chicken muscles decreased following treatment with OA (Hoehler *et al.*, 1996). Furthermore, there was no increase of MDA formation in the plasma, kidney and liver of rats treated with OA at level of 5 ppm in the feed (Hoehler *et al.*, 1996). Lipid peroxidation could be a result of a compromised antioxidant system or reflect alterations in general cell metabolism causing an increase in free radical production. For example, rats treated with low doses of OA exhibited a reduction in the activities of the membrane bound enzymes, most probably by inducing their release, as a result of the impairment of the functional integrity of cell membranes (Zanic-Grubisic *et al.*, 1995).

In spite of the stimulating effect of OA on lipid peroxidation *in vitro* and *in vivo*, the molecular mechanisms of such stimulation remain unclear. For example, Baudrimont *et al.* (1994) suggested that the effects associated with OA resulted

from the activity of superoxide radicals and hydrogen peroxide. It has been proposed that hydroxyl radical production is the major mechanism of such stimulation, Omar *et al.* (1990) suggesting that OA may complex with  $\text{Fe}^{3+}$  thereby facilitating its reduction. Hasinoff *et al.* (1990) showed that the  $\text{Fe}^{3+}$  complex of OA produced hydroxyl radicals in the presence of NADPH and NADPH cytochrome-P-450 reductase. However other mechanisms of free radical production as a result of mycotoxicosis have been proposed. In particular, it was also suggested (Hoehler *et al.*, 1996) that OA increases the permeability of the cell to  $\text{Ca}^{2+}$ . The increased cellular concentration of  $\text{Ca}^{2+}$  and pro-oxidant properties of OA may then be responsible for uncoupling oxidative phosphorylation and an increased leakage of electrons from the respiratory chain. This is associated with superoxide and  $\text{H}_2\text{O}_2$  production and ultimately leads to hydroxyl radical formation via the Fenton reaction. It is notable that the production of hydroxyl radicals by OA does not require a dissociable phenolate group or the prior formation of an OA-Fe complex (Hoehler *et al.*, 1997).

### T-2 toxin

The mechanisms of T-2 toxicity include effects on various metabolic functions of the body. For example, the chemical structure of T-2 toxin makes it fat soluble, allowing it to be incorporated into cell membranes and potentially changing the structural and functional properties of the membrane (Coulombe, 1993). Lipid peroxidation by T-2 toxin in the liver has also been identified as an important underlying mechanism of T-2 toxin-induced cell injury (Leeson *et al.*, 1995; Hoehler and Marquardt, 1996). Similarly, damage to DNA has been recorded (Atroschi *et al.*, 1997). Therefore, oxidative damage caused by T-2 toxin may be one of the underlying mechanisms for T-2 toxin-induced cell injury and DNA damage, eventually leading to tumorigenesis.

Lipid peroxidation was implicated in trichothecene poisoning (Karppanen *et al.*, 1989). A single oral dose of T-2 toxin ( $3.6 \text{ mg kg}^{-1}$  bodyweight (bw)) enhanced conjugated diene formation in rat liver, spleen, kidney, thymus and bone marrow (Chang and Mar, 1988). A lower single dose of T-2 toxin ( $2 \text{ mg kg}^{-1}$  bw) caused significant increase in liver lipid peroxidation in rats at 8, 16 and 24 h post-treatment (Suneja *et al.*, 1989). Treatment of fasted mice with a single dose of T-2 toxin ( $1.8$  or  $2.8 \text{ mg kg}^{-1}$  bw) by oral gavage led to 76% hepatic DNA fragmentation (Atroschi *et al.*, 1997) suggesting that oxidative damage may be an important mechanism for T-2 toxin-induced cell injury and DNA damage. A single oral dose of T-2 toxin ( $3.6 \text{ mg kg}^{-1}$  bw) given to vitamin C and tocopherol-deficient rats more than doubled the thiobarbituric acid reactive substances (TBARS) levels in the liver cells (Rizzo *et al.*, 1994).

The consumption by rats of T-2 toxin over several days was also implicated in lipid peroxidation. For example, lipid peroxidation was increased in rat liver as a result of T-2 toxin feeding ( $0.75 \text{ mg kg}^{-1}$  daily) for 14 and 21 days. Similarly, oral administration to rats of T-2 toxin for five days ( $1.25 \text{ mg kg}^{-1}$ ) causes an increase in ascorbate-stimulated as well as NADPH-dependent lipid peroxidation in hepatic nuclei (Ahmed and Ram, 1986). Furthermore, the MDA content in yeast

increased as a result of increased concentrations of T-2 toxin in the growth medium (Hoehler *et al.*, 1998).

Rizzo *et al.* (1992) suggested that T-2 toxin exerts its toxicity on cells by penetrating the phospholipid bilayer, interacting with the cellular membranes and as a result causing free radical mediated phospholipid peroxidation. Similarly, alteration of the membrane structure by T-2 toxin was also considered (Tsuchida *et al.*, 1984) to stimulate lipid peroxidation *in situ*. It has been also suggested (Segal *et al.*, 1983; Rizzo *et al.*, 1992) that the haemolytic activity of T-2 toxin is associated with lipid peroxidation stimulation by this mycotoxin. Indeed T-2 toxin affected the permeability of cell membranes *in vitro* (Bunner and Morris, 1988) and caused changes in the phospholipid turnover in bovine platelets (Grandoni *et al.*, 1992). Furthermore, T-2 toxin can inhibit electron transport system in yeast by inhibiting succinic dehydrogenase (Khachatourians, 1990) as well as gap-junctional intracellular communication in Chinese hamster cells (IARC, 1993).

However, results on T-2 toxin effect on lipid peroxidation are not consistent and are sometimes controversial. For example, in spite of the fact that T-2 toxin caused an increase (up to 50% over the controls) in the amount of TBARS in rat liver homogenates (Schuster *et al.*, 1987), in the same experiment T-2 toxin used *in vivo* or *in vitro* failed to stimulate lipid peroxidation in liver hepatocytes. Furthermore, ethane exhalation did not differ from the controls. When fed to chickens T-2 toxin (4 ppm) failed to stimulate lipid peroxidation in the liver (Hoehler and Marquardt, 1996).

### **Fumonisin B1**

Dietary fumonisin B1 (FB1) (250 and 500 mg FB1 kg<sup>-1</sup> diet for 21 days) significantly increased the TBARS concentration in the rat liver (Abel and Gelderblom, 1998). This was associated with increased lipid peroxidation in the plasma membrane. In the same study, *in vitro* experiments with primary rat hepatocytes showed that the level of TBARS increased in a dose dependent manner associated with an increase in cytotoxicity. FB1 was also found to be a potent inducer of lipid peroxidation in monkey kidney cells (Abado-Becognee *et al.*, 1998). In this study FB1 stimulated lipid peroxidation at concentrations lower than that required to inhibit cellular synthesis of protein and DNA.

A model system of isolated rat liver nuclei was used by Sahu *et al.* (1998) to study the effects of FB1 on membrane lipids and DNA. They showed that FB1 induced lipid peroxidation concurrently with DNA strand breaks suggesting that hydroxyl radicals may be the initiators of the lipid peroxidation with the resultant peroxy radicals being responsible for the DNA strand breaks. The authors also considered the possibility that hydroxyl radicals induced site-specific strand breaks. FB1 has been shown to have a potentiating effect on iron-induced lipid peroxidation in the rat liver (Lemmer *et al.*, 1999). Stimulating effects of FB1 on lipid peroxidation *in vivo* could be intermediated by changes in membrane fatty acid profiles (Wu *et al.*, 1995; Gelderblom *et al.*, 1997). In fact, altered fatty acid composition of the hepatocytes as a result of FB1 action is considered to be a key

event in explaining the cytotoxic effects and altered growth responses induced by fumonisins in primary hepatocytes (Gelderblom *et al.*, 1996).

As for other mycotoxins, it has been postulated that cellular membranes are a principal target for the fumonisins *in vivo* (Riley *et al.*, 1994). The presence of FB1 has been shown to increase oxygen transport and decrease the ordering of the hydrocarbon chains near the surface of the membranes (Yin *et al.*, 1996a). In the membrane's fluid phase, FB1 increases the fluidities of spin labels and in the gel phase, FB1 imparts rigidifying effects on membrane fluidity (Yin *et al.*, 1996b). This could lead to acceleration of oxidative reactions involving molecular oxygen and ROS and as a result to stimulation of free radical production and lipid peroxidation (Yin *et al.*, 1998). In general, FB1 perturbs a complex interrelationship between membrane structure and its susceptibility to lipid peroxidation. In particular, the combination of membrane structure disruption, with enhanced membrane permeability and increased oxygen concentration could accelerate the chain reaction associated with lipid peroxidation (Yin *et al.*, 1998).

### Other mycotoxins

The effect of aflatoxin B1 (AFB1) on lipid peroxidation is discussed elsewhere in this symposium volume (Dvorska *et al.*, Chapter 66 this volume). Other mycotoxins are also involved in free radical generation and lipid peroxidation. For example, after oral administration of a single dose of deoxynivalenol (DON; 28 mg kg<sup>-1</sup> bw) to male rats the TBARS value in the liver was increased by 21% (Rizzo *et al.*, 1994). Interestingly, in laying hens consuming feed contaminated with DON (0.35 or 0.7 ppm for 86 or 135 days) resulted in the total lipid and triglyceride content of livers being increased significantly.

After intravenous injection rats with zeralenone MDA concentrations in the mitochondrial and microsomal fractions of the liver were increased (Karagezian *et al.*, 1995) and activity of phospholipase A2 was elevated (Karagezian, 2000). Treatment of Vero cells with zeralenone substantially (by 45-87%) increased MDA accumulation (Ghedira-Chekir *et al.*, 1999). Zeralenone also led to DNA adduct formation in mice organs (Grosse *et al.*, 1997). This was confirmed later, when treatment of mice with zeralenone (2 mg kg<sup>-1</sup> bw i.p. or orally) caused the formation of DNA adducts in the liver and kidney (Pfohl-Leskowicz *et al.*, 1995). It is interesting that zeralenone also induces an SOS-DNA repair response in lysogenic bacteria (Ghedira-Chekir *et al.*, 1998, 1999). The mycotoxin citrinin increased the generation of reactive oxygen species, stimulating the production of the superoxide anion in the respiratory chain (Ribeiro *et al.*, 1997). Inclusion of *Fusarium* pigment aurofusarin in the quail diet was shown to increase lipid peroxidation in egg yolk (Dvorska *et al.*, 2001) and in the liver of newly hatched quails (Surai and Dvorska, 2001).

## Conclusion

The results of recent studies clearly show that in many cases it is the membrane-active properties of various mycotoxins that determine their toxicity, indeed, incorporation of mycotoxins into membranes causing detrimental changes. These changes are associated with alteration of fatty acid composition of the membrane structures with peroxidation of long chain PUFAs inside membranes. This ultimately leads to effects that range from damage of membrane receptors, causing alterations in the secondary messenger system; to inactivation of a range of membrane-binding enzymes, responsible for regulation of important pathways; to alteration in membrane permeability, flexibility and other important characteristics determining membrane functions. As a result functional alterations in many biochemical pathways and changes in physiological functions including growth, development, reproduction, etc. occur. The importance of lipid peroxidation in these processes is confirmed by the protective effects of natural antioxidants against mycotoxin toxicity. While the molecular mechanisms associated with mycotoxin-antioxidant interactions *in vivo* await investigation, we can, taking into account data analysed above, suggest a hypothetical scheme of mycotoxin-antioxidant interactions.

- Further research is required to clarify if mycotoxins in the feed (at least OA, T-2 toxin and AFB1) cause malabsorption in the intestine which results in impaired absorption and decreased concentrations of vitamin E, C and carotenoids in tissues.
- Mycotoxins promote free radical formation ( $O_2^-$  and  $OH^-$ ) in the intestine, which results in antioxidant depletion, oxidative stress, enterocyte apoptosis and contribute to the development of malabsorption and decreased antioxidant absorption and accumulation.
- Mycotoxins and their active metabolites are absorbed from the intestine and accumulated in target tissues.
- Mycotoxin in tissues can generate free radicals, further decreasing antioxidant protection, causing lipid peroxidation and damage to other biological molecules, including proteins and DNA. This could lead to antioxidant-pro-oxidant imbalance causing oxidative stress, which leads to apoptosis and other cytotoxic effects.
- Increased antioxidant supplementation protects against the toxic action of mycotoxins by interfering with one or several steps described above, including gastrointestinal tract, plasma and tissue membranes.

Further research is required to clarify important points of this scheme.

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## Chapter 76

# ***Rumex obtusifolius*: Its Costs and the Benefits of Control**

A.D. Bailey

*Dow AgroSciences Ltd, Latchmore Court, Brand Street, Hitchin, Herts SG5 1NH, UK*

### **Introduction**

*Rumex* species are considered as weeds and contain oxalates which have been implicated in a number of poisoning events including the death of sheep where 10% of ewes died in one flock in New Zealand (Connor, 1977). Death in most cases has been attributed to acute renal failure (Panciera *et al.*, 1990). In addition to their toxic properties, Courtney (1985) found that *Rumex obtusifolius* grown in artificial monoculture had a lower herbage potential than perennial ryegrass, and the presence of *R. obtusifolius* in a ryegrass sward reduced the yield of dry matter (DM) by approximately 1%. This paper discusses work carried out at SAC (Scottish Agricultural College) in Scotland and IGER (Institute of Grassland and Environmental Research) in England over 2 years on behalf of Dow AgroSciences to evaluate the loss of grassland productivity as a function of varying levels of *R. obtusifolius* infestation.

### **Materials and Methods**

#### **SAC trial**

A small plot trial was carried out at SAC Crichton Royal Farm in Dumfries, Scotland. Areas of a ryegrass sward naturally infested with *R. obtusifolius* were selected on the basis of the level of infestation in April 1999. Four levels of infestation were identified: no infestation, 2% infestation, 5% infestation and 10% infestation. Each of these areas was replicated four times in the same field. Three silage cuts were taken at normal commercial timings, total fresh weight per plot was recorded along with the fresh weight of the *R. obtusifolius* fraction. Grab samples were taken for DM assessment.

#### **IGER trial**

Two small-plot field experiments were carried out at IGER North Wyke in 1999

and 2000. The experimental site used for both experiments was at 185 m above sea level on a silty clay loam soil over clay (Halstow series). The sward had been sown in 1992 with early flowering perennial ryegrass (*Lolium perenne* L.; cv. Cropper) and managed by frequent mowing during 1993–95. The sward was free of *R. obtusifolius* until it was introduced as spaced plants in 1995 and 1996 for experimental purposes (Hopkins *et al.*, 1997).

*Previous management.* The site had the following characteristics in March 1999 when experimentation on investigating the effects on herbage production of herbicidal *R. obtusifolius* control began. There were two adjacent experimental areas of 33 m x 33 m each of which contained 64 *R. obtusifolius* infested micro-plots of 1 m x 1 m. Each micro-plot was surrounded by an undisturbed *R. obtusifolius* free area of the existing sward, also 1 m x 1 m, which separated it from the next plot in each direction. Detailed measurements of *R. obtusifolius* plant density and morphology were recorded in September of the second year of each experiment. On the area occupied by Experiment 1 (recorded in 1996/97 and planted in 1995 with *R. obtusifolius* root transplants at 13 plants m<sup>-2</sup>) the mean *R. obtusifolius* ramet density was 11.9 m<sup>-2</sup> in September 1997. On the area occupied by Experiment 2 (recorded in 1997/98 and planted with *R. obtusifolius* plug plants raised from seed and transplanted in 1996 at 25 plants m<sup>-2</sup>) the mean *R. obtusifolius* ramet density was 16.2 m<sup>-2</sup> in September 1998. During 1998 the area that had been used for Experiment 1 was mown uniformly three times but received no fertilizer inputs or assessments. In the winter of 1998/99 sheep grazed the whole experimental site. The two experimental areas were environmentally similar but provided different situations in terms of *R. obtusifolius* density and history. The *R. obtusifolius* plant density was lower on Experiment 1 than on Experiment 2, but the *R. obtusifolius* plants on Experiment 1 were more vigorous in growth, having been established for one year longer.

On each of the two experimental areas a randomized split-block design was used to investigate the effects of herbicidal control of *R. obtusifolius* on swards of different *R. obtusifolius* density. Each experiment consisted of two herbicide treatments (i.e. with and without herbicide) and three sward types, viz. (i) no *R. obtusifolius*, (ii) high *R. obtusifolius* density, and (iii) lower *R. obtusifolius* density. There were four replicates of each treatment. The no *R. obtusifolius* treatments utilized the between-plot *R. obtusifolius* free discard areas, the high *R. obtusifolius* density utilized four 1 m x 1 m micro-plots, and the lower dock density utilized a contiguous strip of four *R. obtusifolius* micro-plots plus the inter-plot discard area. Plot size was 1 m x 7 m (maximum).

The experimental areas were laid out to these allocated treatments in March 1999. Herbicide (Pastor™ at the recommended rate of 4 l ha<sup>-1</sup> in 400 l of water, using a gas pressurized, small-plot, pedestrian-controlled applicator) was applied on 30 April 1999 to the 'with-herbicide' treated plots. From this point the experimental areas were managed uniformly throughout the 1999 growing season. In 2000 an additional application of Pastor was applied to the herbicide treated plots of Experiment 1 only. The dose was 2 l ha<sup>-1</sup> in 400 l of water, applied with a knapsack applicator uniformly to the treated plots (calibrated with deflector AN2.0 nozzle for a 1-metre band spray and a walking speed of 50 m

min<sup>-1</sup>). This second application was made on 13 June 2000, which was 3 weeks after harvest 1.

Fertilizer N (as ammonium nitrate) was applied at a rate of 320 kg N ha<sup>-1</sup> year<sup>-1</sup> (120 kg ha<sup>-1</sup> in March/early April, followed by 120 kg ha<sup>-1</sup> after the first harvest and 80 kg ha<sup>-1</sup> after the second harvest). Supporting PK fertilizer was applied as 0-24-24 in April to supply 120 kg ha<sup>-1</sup> of each of P<sub>2</sub>O<sub>5</sub> and K<sub>2</sub>O, and additional K (as muriate of potash) was supplied with the second and third N applications so that the total annual K<sub>2</sub>O input was 248 kg ha<sup>-1</sup>. Fertilizers were weighed and applied on a per-plot basis to ensure uniformity.

The plots were harvested with a Resant autoscythe mower, and herbage production determined using standard procedures for small-plot trials. Harvesting was carried out on three occasions at c. 8-week intervals (25 May, 6 July and 7 September in 1999 and 23 May, 5 July and 21 September in 2000), the management simulating a three-cut silage system, and the date of first harvest coinciding with local practice for first-cut silage. Swards were mown to a residual sward height of 5–7 cm, and the mown herbage from each plot was immediately weighed in the field. From each plot sub-samples of c. 500 g of fresh herbage were taken, one of which was dried in a forced draught oven at 85°C to determine the sample DM content (%DM), enabling calculation of the DM yield at each harvest.

Representative dried samples of herbage from each plot at each harvest were ground and prepared for digestibility analyses. The second sample from each plot was subsequently hand sorted to determine the proportions of *R. obtusifolius* (both live and dead fractions) and other herbage (i.e. predominantly grass) in the total sample, these proportions being determined on an oven-dry basis. In 1999 representative samples of the non-*R. obtusifolius* herbage at each harvest were taken to determine the stage of digestibility (% digestible organic matter in the dry matter (DOMD)). In 2000 a more detailed sampling was carried out to determine, for each of the three harvests of each of the two experiments, the DOMD of the *R. obtusifolius* fraction, and of the mainly grass fraction, from both the herbicide-treated and the untreated swards. These chemical analyses were done at IGER's Aberystwyth laboratories.

In addition to determination of the relative proportions of *R. obtusifolius* and grass in harvested samples, assessments were made of *R. obtusifolius* ramet densities on a per plot basis. This was done during the period 28 February to 7 March, 2000. Similar assessments had been made prior to the commencement of this work in February 1999.

Analysis of the treatment effects on total DM yield and the separate *R. obtusifolius* and grass component yields followed a split-plot analysis of variance using GENSTAT. In 1999 and 2000 analyses were carried out on the total annual yield and on the yields at each harvest. In 2000 additional analyses were made of DOMD yields for total herbage and the separate *R. obtusifolius* and grass components of yields, based on %DOMD and proportions of *R. obtusifolius* and grass herbage in the hand sorted samples.

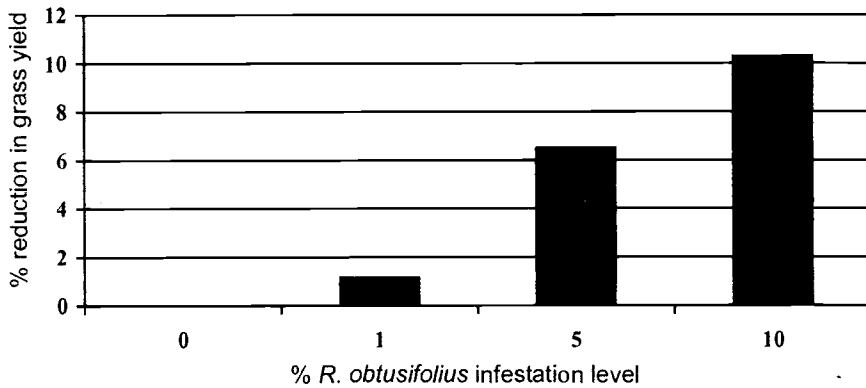
## Results

The results of production of forage and contamination with *R. obtusifolius* are presented in Tables 76.1, 76.2 and 76.3. It is clear (Table 76.1) that the expected contamination level of the forage with *R. obtusifolius* was obtained in practice but that overall yield of DM tended to increase with *R. obtusifolius* contamination.

### SAC trial

**Table 76.1.** Total dry matter (DM) and *R. obtusifolius* (*R. o*) DM (kg plot<sup>-1</sup>).

<i>R. o</i> infestation level (%)	Cut 1		Cut 2		Cut 3	
	Total	<i>R. o</i>	Total	<i>R. o</i>	Total	<i>R. o</i>
0	2.05	0	2.40	0	2.25	0
1	2.10	0.030	2.40	0.028	2.40	0.023
5	2.05	0.130	2.48	0.183	2.45	0.173
10	2.15	0.222	2.80	0.280	2.55	0.270



**Fig. 76.1.** Percentage reduction of grass yield as a function of *R. obtusifolius* infestation level.



## IGER trial

**Table 76.2.** Herbage dry matter (DM) yield excluding *R. obtusifolius* fraction (kg DM ha<sup>-1</sup>) from Experiment 1 (transplanted root sections) in 2000, for swards classified by initial *R. obtusifolius* density, unsprayed and sprayed with Pastor in each case, 1999 and 2000.

1999	Cut 1	Cut 2	Cut 3	Total
<i>R. o</i> free				
Unsprayed	4921	2701	3915	11538
Pastor	4424	3266	4060	11749
High density				
Unsprayed	4448	2080	3722	10250
Pastor	4678	2516	5012	12206
Low density				
Unsprayed	4048	2219	3230	9496
Pastor	4730	2448	4464	11643
S.E.D. and significance				
Herbicide	213.7 NS	210.0 <sup>a</sup>	169.5 <sup>c</sup>	305.3 <sup>c</sup>
Density	261.7 NS	171.4 <sup>b</sup>	207.6 NS	373.9 <sup>a</sup>
H x D	370.2 NS	296.9 NS	293.5 <sup>a</sup>	528.8 <sup>a</sup>

2000	Cut 1	Cut 2	Cut 3	Total
<i>R. o</i> free				
Unsprayed	7200	2089	3570	12860
Pastor	7400	1585	3690	12680
High density				
Unsprayed	8560	1654	2270	12484
Pastor	7920	1905	4010	13835
Low density				
Unsprayed	5360	1568	2250	9180
Pastor	6460	1607	3650	11720
S.E.D. and significance				
Herbicide	429.0 NS	103.4 NS	235.0 <sup>c</sup>	443.0 <sup>a</sup>
Density	525.0 <sup>c</sup>	126.6 NS	288.0 NS	543.0 <sup>c</sup>
H x D	743.0 NS	179.1 <sup>a</sup>	407.0 <sup>a</sup>	768.0 NS

NS, not significant; <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$ .

**Table 76.3.** Herbage dry matter (DM) yield excluding *R. obtusifolius* fraction (kg DM ha<sup>-1</sup>) from Experiment 2 (transplanted plug plants grown from seed), for swards classified by initial *R. obtusifolius* density, unsprayed and sprayed with Pastor in each case, 1999 and 2000.

1999	Cut 1	Cut 2	Cut 3	Total
<i>R. o</i> free				
Unsprayed	5138	2860	3902	11900
Pastor	4748	2736	3975	11459
High density				
Unsprayed	5781	2850	3563	12193
Pastor	5776	3249	4253	13279
Low density				
Unsprayed	4803	2198	3078	10079
Pastor	4946	2572	3902	11420
S.E.D. and significance				
Herbicide	177.5 NS	202.9 NS	159.5 <sup>b</sup>	259.8 <sup>a</sup>
Density	217.4 <sup>c</sup>	248.5 <sup>a</sup>	195.3 NS	318.2 <sup>c</sup>
H x D	307.5 NS	351.4 NS	276.2 NS	450.0 <sup>a</sup>

2000	Cut 1	Cut 2	Cut 3	Total
<i>R. o</i> free				
Unsprayed	7750	2154	3400	13304
Pastor	8830	2097	3230	14157
High density				
Unsprayed	7860	1544	2390	11794
Pastor	9610	2257	3390	15257
Low density				
Unsprayed	7020	1200	1610	9830
Pastor	8960	1949	3130	14039
S.E.D. and significance				
Herbicide	389.0 <sup>c</sup>	140.3 <sup>b</sup>	229.0 <sup>b</sup>	446.0 <sup>c</sup>
Density	476.0 NS	171.9 <sup>a</sup>	280.0 <sup>a</sup>	537.0 <sup>b</sup>
H x D	673.0 NS	243.1 NS	397.0 <sup>a</sup>	761.0 <sup>a</sup>

NS, not significant; <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$ .

The mean proportion of the total DM yield contributed by *R. obtusifolius* over the two studies in 1999 and 2000 were 5.08 and 28.15% respectively for the areas contaminated at a high density and sprayed and not sprayed, respectively, with the herbicide Pastor. A similar trend was observed with the pastures containing the low density *R. obtusifolius* which were sprayed (4.25%) and unsprayed (24.88%). It was clear that the use of Pastor increased grass DM yields by 1.97% and 2.56% respectively in the plots with high and low contamination of *R. obtusifolius*. It was noted that the %DOMD of *R. obtusifolius* decreased from about 61.3 at first cut to 44.1 at third cut suggesting that substantial contamination of grass sward by *R. obtusifolius* at the later cut would reduce the

overall DOMD value. The forage treated with Pastor tended to have slightly higher mean overall DOMD values (62.27%) compared to forage from untreated pasture (61.26%). Combined with the increased herbage yields (Tables 76.2, 76.3) the total DOMD produced from treated pasture was higher for treated pasture compared to untreated pasture.

## Discussion

### SAC trial

The trial conducted at SAC was designed to examine the hypothesis that the area occupied by *R. obtusifolius* plants was directly related to the yield loss, i.e. that 5% ground cover would lead to a 5% loss in total yield. Figure 76.1 shows that this hypothesis is valid within the range of *R. obtusifolius* infestation investigated. This is an undesirable situation where grass of high nutritional value is contaminated with the nutritionally poorer, and toxic, *R. obtusifolius*.

### IGER trial

Having established that yield losses occurred, a more detailed study was carried out to investigate the effects of removing *R. obtusifolius* from the sward using a herbicide and ultimately developing a model to establish a cost benefit relationship for herbicide use.

In these trials *R. obtusifolius*-free pastures were not affected by the herbicide while the *R. obtusifolius*-contaminated pastures that received herbicide gave similar or in some cases slightly lower DM yields compared to the unsprayed treatments. However since *R. obtusifolius* contributed up to 35% of the total DM and the herbicide controlled this element, some decline in yield would not be unexpected. As the grass component of the sward exploited the area previously occupied by the *R. obtusifolius* this difference generally declined and was particularly evident in the second year of the trial (Table 76.2).

When only the grass component of the total DM was calculated, treatment receiving herbicide always gave higher yields than those which did not receive herbicides (Tables 76.2, 76.3). It is also important to note that a high proportion of this additional grass was produced at the third harvest when the digestibility of the *R. obtusifolius* was particularly low.

## Conclusions

A single application of the herbicide Pastor effectively controlled the population of *R. obtusifolius* in both experiments; however, in Experiment 2, where the plants were more established, a second low dose application was required to

eradicate the weed. This tactic may be appropriate for improving pastures contaminated with other toxic weeds.

In the trials reported here where the *R. obtusifolius* contributed 20–35% of the total DM generally the additional grass produced on the herbicide treated plots replaced the contribution made by the *R. obtusifolius* on the equivalent untreated plots. It is interesting to note that in the low density *R. obtusifolius* plots the increase in grass was the greatest suggesting that in this situation the grass was better able to exploit the area made available to it from the control of the *R. obtusifolius*.

The benefits of the additional grass DM continued and were in many cases greater in the second year after application probably due to the grass exploiting the area previously occupied by the *R. obtusifolius*, and spreading the cost of the herbicide over two and possibly more growing seasons.

Though the digestibility of the *R. obtusifolius* was similar to that of the grass in the first harvest it declined towards the end of the growing season. This meant that the additional grass had more value as the season progressed.

## Acknowledgements

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## Chapter 77

# Effect of Varying Trypsin Inhibitor Activity of Full Fat Soya on Nutritional Value for Broiler Chicks

E. Clarke and J. Wiseman

*Division of Agriculture and Horticulture, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leics LE12 5RD, UK*

### Introduction

Soybeans are of major importance worldwide as, for example, a plant protein component of diets for non-ruminant livestock. However, as inclusion levels have increased (as a consequence of increasing concerns over the safety of proteins of animal origin), the problems associated with feeding soybeans have been accentuated. It is accepted that limitations to their use are associated with comparatively modest concentrations of protein and nutritionally essential amino acids (although levels are still higher than most other plant sources) and there is considerable interest in selecting cultivars with improved nutritional quality (reviewed by Clarke and Wiseman, 1999).

Soybean proteins, like other legumes, contain low concentrations of the nutritionally essential sulphur amino acid, methionine. Cysteine, although not an essential amino acid because it can be synthesized from methionine, also influences the nutritional quality of soybean products when it is only present in low levels. A low cysteine content will also aggravate a methionine deficiency. Soybean lines deficient in 7S protein subunits have been identified. The 7S proteins contain substantially less methionine and cysteine than the 11S proteins. With the myriad of genetic null alleles for these subunits it may be possible to tailor the 7S/11S storage protein ratio and their total composition in seeds to include only those subunits with the richest sulphur amino acid composition.

The following UK example highlights the importance of small fluctuations in soybean quality on the broiler industry. Sixteen million broilers are placed in the UK per week with an average performance of reaching 2.3 kg at 42 days with FCR of 1.8. The current UK average feed cost of £157 tonne<sup>-1</sup> allows an average margin of 6 pence kg<sup>-1</sup>. Therefore a 0.01 improvement in FCR equals a feed saving of 23 g per bird. This equates to a saving of 0.36 pence per bird, which would make a total annual saving of £3 million.

If the above calculations for FCR are equated to an improvement in soya quality then the financial implications of variability in quality may be calculated. Current data shows an improvement in 3 day weight gain (days 19–21) of 10% if

trypsin inhibitor activity (TIA) moved from 7.0 mg kg<sup>-1</sup> to 2.3 mg kg<sup>-1</sup> (Clarke, 2001). If the same improvement was observed throughout the bird's life, this increase should equate to an improvement in FCR of 0.14, which is worth £42 million year<sup>-1</sup>. This is an extreme example produced in an experimental environment but it does highlight the scale of the situation. In a commercial setting, a 3% improvement is more viable and would still equate to an improvement in FCR of 0.05, that is £15 million year<sup>-1</sup>.

### **Trypsin inhibitors**

A further area of fundamental importance is the presence of a number of naturally-occurring factors which are antinutritional insofar as they interfere with nutrient digestion, absorption and assimilation in animals. Many factors are heat stable and effective means of their removal remain to be identified, although plant breeding programmes have been moderately successful. Other factors are heat labile and may be reduced below levels likely to cause problems, although necessary processing is associated with increased cost. There are many variables affecting processing but these are rarely quantified in research papers in detail.

Soybeans contain a number of antinutritional factors (ANFs) that can be divided into proteinaceous (lectins, trypsin inhibitors and antigenic proteins; predominantly heat labile) and non-proteinaceous factors (phytic acid and non-starch polysaccharides; essentially heat stable). Antigenic proteins particularly affect pre-ruminant calves and young pigs but no effects have been recorded in chickens. The ANF considered in the current paper is trypsin inhibitor (TI). The most abundant trypsin inhibitors in soybean are the Kunitz inhibitor (KSTI) and the Bowman Birk inhibitor (BBI), containing 181 and 71 amino acids respectively. They form well characterized stable enzyme-inhibitor complexes with pancreatic proteolytic enzymes on a molar 1:1 ratio.

KSTI was the first plant proteinase inhibitor to be isolated and characterized (Kunitz, 1947). It has a molecular weight of about 21,000 Da and includes two disulphide bridges. It is primarily a single-headed inhibitor of trypsin but was also shown to be weakly reactive against chymotrypsin at two reactive sites, one of them overlapping with the trypsin reactive site (Kassell, 1970). BBI has a molecular weight of approximately 8000 Da with a high content of cysteine, forming seven disulphide bridges. The increased number of disulphide bridges in BBI endow it with greater structural stability than KSTI, making it more resistant to denaturation by heat. It forms a 1:1 complex with either trypsin or chymotrypsin and a ternary complex with both enzymes. The soybean BBI consists of two domains, each containing a genetically distinct reactive site. The sequence alignment of these domains shows a large homology and it is generally assumed that these double headed inhibitors have evolved by gene duplication (Birk, 1985).

*Role of trypsin inhibitors in plants*

The roles of trypsin inhibitors in plants are diverse: they are thought to be involved in the regulation of and protection against unwanted proteolysis in plant tissues and also act as a defence mechanism against attack from diseases, insects and animals (Xavier-Filho and Campos, 1989). Accumulation of proteinase inhibitors occurs both locally, at the site of injury and systemically in other organs of the plant distal to the primary wound site. Being proteins, with high concentrations of cysteine in BBI, they are able to fulfil a secondary role; this involves recycling their constituent amino acids for use as building blocks in *de novo* protein synthesis. Proteases are influential in the mobilization of proteins in plants during germination, which seems to be achieved by an interplay of many proteases. They have also been detected as a 'cloud' that has leaked into the soil to surround the germinating seed and guard it against attack from micro-organisms (Wilson, 1980). Characterization of 11 wild perennial species of soybean revealed that seeds of all species studied contained both trypsin and chymotrypsin inhibitors (Kollipara and Hymowitz, 1992).

*Antinutritional effects of trypsin inhibitors*

Liener (1958) observed that only soybeans that had been cooked could support growth in rats and many other animal species. Initially it was assumed that this growth reduction was due to limited proteolysis in the gut due to trypsin inhibition. However, it was reported that there was still a growth reduction in rats when pre-digested proteins or free amino acids were fed together with a high antitryptic fraction prepared from soybeans (Liener and Kakade, 1980). This result indicated that the antinutritional effect of TI cannot only be explained by the inhibition of trypsin activity in the gut. In other studies it was shown that TI also influenced the secretion of other pancreatic enzymes (Schneeman *et al.*, 1977). When trypsin is inhibited by TI, cholecystokinin (CCK) production is enhanced resulting in an increased production of pancreatic digestive enzymes. Hence the growth depression observed is a combined effect of endogenous loss of essential amino acids and decreased intestinal proteolysis.

Due to the enhanced enzyme production, hypertrophy and hyperplasia of the pancreas occurs; Chernick *et al.* (1948) observed pancreatic enlargement in chicks caused by feeding raw soybeans. This finding was confirmed in several other studies, not only in chicks but also in rats, mice and young guinea-pigs (reviewed by Liener and Kakade, 1980). Subsequent work by Khalifa *et al.* (1994) suggests that control of the composition of pancreatic secretions may not only be attributable to CCK but also to other intestinal hormones together with metabolites resulting from the transformation of other nutrients. The negative feedback mechanism regulating the secretion of pancreatic enzymes found in rats also exists in pigs and calves, but without causing pancreatic hypertrophy (Gallaher and Schneeman, 1986).

### Processing to reduce antinutritional factor content

Raw soybeans normally have a TIA of between 20 and 35 mg g<sup>-1</sup>, therefore it is necessary to process them prior to feeding to denature the trypsin inhibitors and lectins so the residual TI activity is below the currently recommended threshold of 4 mg g<sup>-1</sup>. This is the level assumed to have minimum adverse effects in birds. There are three main variables to processing, which are time, temperature and moisture content. The key is to quantify these processing variables and study their impact both chemically and biologically. Recent work at Nottingham University studied the impact of varying processing conditions on the nutritional quality of both oil-extracted soybean meal and full fat soya and has established that broiler chicks are extremely sensitive to changes in the processing conditions of soya. There is concern in the UK that inconsistency in processing of full fat soybean meal (FFSB) is resulting in a product of variable quality. In order for FFSB to become a more valuable commodity in the UK feed industry, a more uniform quality product must be produced.

### Method

A trial was designed to examine the nutritional and physiological consequences of feeding young broilers on four commercially obtained products (all within the tolerated range of TIA) in a metabolism trial. Four samples of FFSB were obtained in the UK from four different European feed companies and the TIA of each sample was determined using the method of Smith *et al.* (1980) (Table 77.1). The FFSB samples were ground through a 3 mm mesh and fed at three inclusion levels of 200, 400 and 600 g kg<sup>-1</sup> in semi-synthetic diets (see Table 77.2). Diets are designed so that the FFSB under investigation provides the sole source of amino acids. An inert marker, titanium dioxide (TiO<sub>2</sub>) is included in the diet at a concentration of 5 g kg<sup>-1</sup> diet. The concentration of TiO<sub>2</sub> is measured in the digesta, which allows calculation of the concentration of amino acid present and, by deducting this figure from the amino acid concentration in the diet, the amount of amino acid digested may be determined.

**Table 77.1.** Trypsin inhibitor activity (TIA) values of four full fat soybean meal samples.

Sample	TIA (mg g <sup>-1</sup> )
A	3.4
B	1.7
C	3.6
D	1.1



**Table 77.2.** Diet formulation.

Ingredients	Dietary inclusion level (g kg <sup>-1</sup> FFSB diet)		
	200	400	600
FFSB	200	400	600
Soybean oil	50	50	50
Mineral premix	50	50	50
Titanium dioxide marker	5	5	5
50:50 starch/glucose mix	695	495	295

Each experimental diet was fed to six pairs of birds from 19 days of age for 6 days to male Ross broiler chicks. Following slaughter at 25 days of age, pancreas weight relative to bodyweight was recorded and ileal digesta samples were removed for amino acid analysis. The true and apparent digestibility of each amino acid was determined according to the method of Short *et al.* (1996). Data were analysed using Genstat version 5 for Windows release 4.1 and linear and non-linear contrasts were established for rate of inclusion of FFSB.

## Results

Table 77.3 shows that pancreas to bodyweight ratio (PBWR) was affected by FFSB ( $P = 0.028$ ) and also by increasing rate of inclusion ( $P = 0.014$ ). Chicks fed diets containing samples A and C (the higher TIA samples) showed greater pancreatic enlargement than chicks fed diets containing samples B and D. Linear and quadratic assessment showed the response of PBWR to rate of inclusion to be linear.

**Table 77.3.** Effect of processing and rate of inclusion on pancreas to bodyweight ratio (PBWR).

PBWR Sample	TIA	Rate of inclusion (g kg <sup>-1</sup> )				ANOVA		
		200	400	600	Mean	Factor	SEd	P <sup>a</sup>
A	3.4	2.34	2.39	2.62	2.45	FFSB	0.095	0.028
B	1.7	2.14	2.26	2.29	2.23	ROI	0.083	0.014
C	3.6	2.17	2.45	2.56	2.39			0.004(L)
D	1.1	2.09	2.20	2.30	2.19			0.897(Q)
Mean		2.19	2.32	2.44	2.32			

<sup>a</sup>Assessment of linear and non-linear contrasts via linear (L) and quadratic (Q) partitioning.

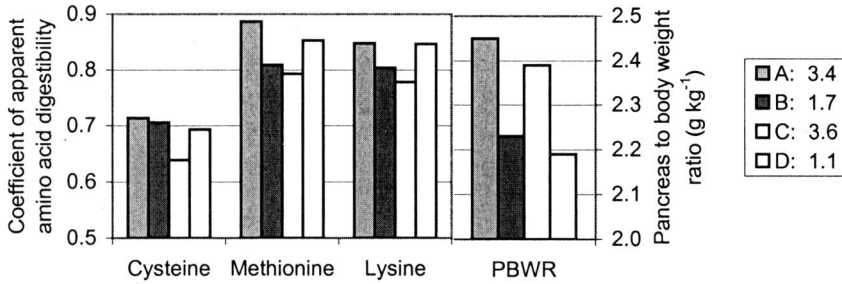


Fig. 77.1. Effect of TIA of FFSB on pancreas to bodyweight ratio (PBWR) and coefficient of apparent amino acid digestibility.

Table 77.4. Effect of processing to reduce TIA (mg g<sup>-1</sup>) on content of ileal digestible amino acids (AA) and coefficient of apparent digestibility. Data corrected to 900g kg<sup>-1</sup> DM. ROI, rate of inclusion.

AA	ROI (g kg <sup>-1</sup> )				ANOVA			Linear regression		
	200	400	600	Mean	Factor	SEd	P <sup>a</sup>	1000 g kg <sup>-1</sup> <sup>b</sup>	Total AA (g kg <sup>-1</sup> ) <sup>c</sup>	CAD <sup>d</sup>
CysA	0.61	1.36	2.02	1.33	FFSB	0.057	<0.001	3.44	4.82	0.714
B	0.70	1.21	2.22	1.38	ROI	0.050	<0.001	3.58	5.08	0.706
C	0.64	1.55	1.91	1.37			<0.001(L)	3.29	5.14	0.639
D	0.81	1.66	2.24	1.57			0.255(Q)	3.72	5.36	0.693
Mean	0.69	1.45	2.10	1.33						
MetA	0.88	1.77	2.70	1.79	FFSB	0.027	<0.001	4.52	5.09	0.887
B	0.82	1.49	2.28	1.53	ROI	0.023	<0.001	3.71	4.58	0.809
C	0.78	1.60	2.14	1.51			<0.001(L)	3.55	4.48	0.793
D	0.96	1.89	2.70	1.85			0.142(Q)	4.47	5.24	0.853
Mean	0.86	1.69	2.46	1.67						
Lys A	3.56	7.10	10.71	7.12	FFSB	0.130	<0.001	17.84	21.05	0.848
B	3.52	6.66	10.11	6.76	ROI	0.113	<0.001	20.66	20.66	0.804
C	3.68	7.69	10.19	7.19			<0.001(L)	21.86	21.86	0.778
D	3.91	7.63	10.86	7.47			0.043(Q)	21.10	21.10	0.847
Mean	3.67	7.27	10.46	7.13						

<sup>a</sup>Assessment of linear and non-linear contrasts via linear (L) and quadratic (Q) partitioning.

<sup>b</sup>1000 g kg<sup>-1</sup> = linear extrapolation to calculate content of apparent ileal digestible amino acids in FFSB. <sup>c</sup>Total individual amino acid concentration in FFSB. <sup>d</sup>CAD = coefficient of apparent digestibility.

Table 77.4 shows content of ileal digestible amino acids increased with rate of inclusion (ROI) in all samples. Assessment of linear and non-linear contrasts demonstrated there was a linear ( $P < 0.001$ ) response of content of digestible amino acids to ROI for all amino acids considered. Figure 77.1 shows the coefficient of apparent digestibility was highest in sample A for all amino acids considered and lowest in sample C.

## Conclusions

Whilst there was a trend for coefficient of amino acid digestibility to decrease with increasing TIA, sample A did not fit this trend. This indicates that digestibility of FFSB is also affected by other factors such as processing method, origin of bean and ANFs other than TIs. Data also show that there is a dose dependent response of pancreatic enlargement in chicks with increasing TIA levels in the diet. The biological model described provides a means of assessing the efficacy of processing and the nutritional/physiological consequences of variable ANF levels in soybean in poultry.

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## Chapter 78

# The Bioactivity-guided Isolation and Structural Identification of Two Novel Toxic Steroidal Glucosides, Stemodiosides B3 and B4 from *Stemodia kingii*

J.G. Allen<sup>1</sup>, S.M. Colegate<sup>2\*</sup>, A.A. Mitchell<sup>3</sup>, R. Mulder<sup>4</sup> and M.F. Raisbeck<sup>5</sup>

<sup>1</sup>Department of Agriculture, 3 Baron-Hay Court, South Perth, Western Australia 6151; <sup>2</sup>Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Victoria, Australia 3220; <sup>3</sup>Northern Australia Quarantine Strategy, Australian Quarantine Inspection Service, c/o NT DPIF, PO Box 990, Darwin, NT, Australia 0801; <sup>4</sup>NMR Laboratory, CSIRO Molecular Science, Bag 10, Clayton South, Victoria, Australia, 3169; <sup>5</sup>Department of Veterinary Sciences, University of Wyoming, Laramie, Wyoming 82070, USA

\* Author to whom correspondence should be addressed.

Ingestion of the north-west Australian shrub *Stemodia kingii* F. Muell. (Scrophulariaceae) by sheep, either in the field or in laboratory feeding trials, can cause a fatal intoxication characterized by inflammation of the entire gastrointestinal tract, dehydration resulting from watery diarrhoea, and a cardiomyopathy (Allen and Mitchell, 1998). The clinical signs of intoxication, the associated clinical chemistry and some of the pathological changes observed were consistent with the effects of cardiac bufadienolide or cardenolide steroidal glycosides (Seawright, 1989; Allen and Mitchell, 1998). Some pathological changes observed however have not previously been associated with cardiac glycoside intoxication.

A laboratory mouse model of *S. kingii* intoxication was developed and validated histopathologically (Raisbeck *et al.*, Chapter 42 this volume). This murine model was used as a bioassay to direct the fractionation and chromatographic purification of the toxins from methanolic extracts of *S. kingii*.

### Extraction and Fractionation of Toxins

*Stemodia kingii* (voucher specimen A.A. Mitchell PRP260, 2/4/95), collected at the site of poisoning in the Pilbara region of Western Australia, was air-dried and milled to a fine powder and stored, dry, at room temperature.

To confirm toxicity of the plant sample prior to extraction, aqueous slurries of the plant were administered to mice via intragastric intubation for several days until clinical signs indicated intoxication. The plant was repeatedly extracted with methanol (MeOH) at room temperature until the methanol extract was clear of colour. The dried methanol extract was shown to be toxic and then partitioned between petroleum ether and 10% of water in methanol (v/v). The toxic, aqueous methanol fraction was evaporated to dryness and the residue extracted with ethyl acetate (EtAc). Silica gel column chromatography (EtAc and MeOH in EtAc) of the toxic ethyl acetate solubles yielded several fractions that were characterized by thin layer chromatography (TLC) and assessed using the murine bioassay.

When developed TLC plates were sprayed with 2% concentrated sulphuric acid in ethanol (v/v) and warmed gently, several spots turned purple in colour. Three groups of toxins (A, B and C), varying in hydrophobicity ( $A > B > C$ ) but similar in this colour reaction, were identified with varying levels of toxicity to mice ( $B \gg C \gg \gg A$ ).

Repeated column and radial chromatography on normal phase silica gel yielded the B group of toxins free from the A and C groups. Analysis by TLC revealed at least five components (relative abundance of B3, B4  $\gg$  B1, B2  $\gg$  B5). The chromatographic similarity of the B group made purification of individual components difficult. However, the more abundant components, B3 and B4, were purified using repeated chromatography and, in the case of B4, by recrystallization in preparation for structural elucidation.

## Structure Determination

The presence of an alkaloid functional group was contraindicated by the lack of pH-induced changes in polarity by TLC and by the fact that combustion elemental analysis of B4 revealed only carbon, hydrogen and oxygen. A combination of acid hydrolysis, mass spectrometry, 1D-NMR ( $^1\text{H}$ ,  $^{13}\text{C}$ ) and 2D-NMR ( $^1\text{H}$ - $^1\text{H}$  COSY,  $^{13}\text{C}$ - $^1\text{H}$  HMQC and HMBC) was used to elucidate the structures of the stemodia toxins B3 and B4.

## Acid hydrolysis

The effect of treating (warming at 50°C for 24 h) methanolic solutions of B3 and B4 with concentrated sulphuric acid (final concentration 2% v/v) was monitored by TLC. The disappearance of the parent compound was accompanied by the appearance of a more polar spot that rapidly turned a grey colour when sprayed with the acidified ethanol. This product was isolated and shown, by TLC and nuclear magnetic resonance (NMR) to be methylglucoside thereby demonstrating the glucosidic nature of the toxins and leading to the trivial names stemodiosides B3 and B4.

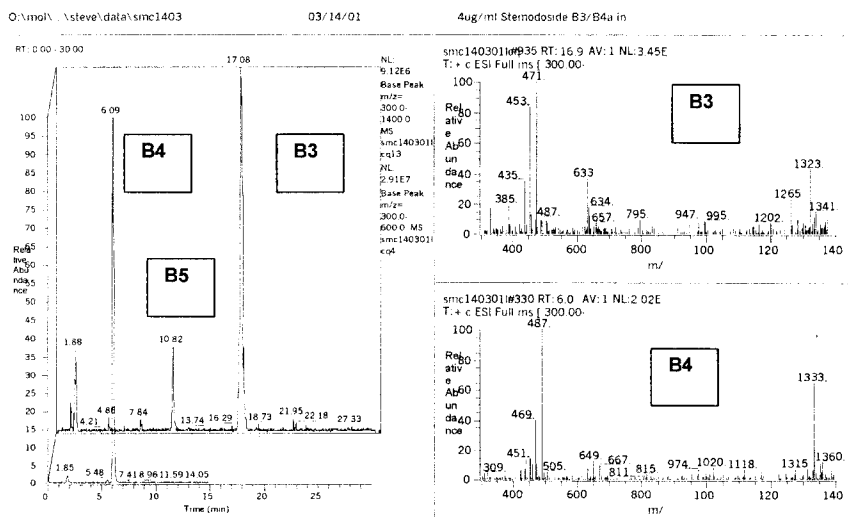
## Mass spectrometry

Solutions of the stemodiosides B3 and B4 were subjected to LCMS analysis using a Finnigan LCQ Ion Trap mass spectrometer in the electrospray ionization (esi) or atmospheric pressure chemical ionization (apci) modes. In the esi mode, abundant dimer adducts were observed from which the molecular weights could be deduced. The most abundant ions for both compounds corresponded to loss of glucose from the molecular ion and thus confirmed the molecular weights deduced from the dimer adducts. Thus, for stemodioside B3, the ions at  $m/z$  1323 (40% abundance,  $2 \times M^+ + Na$ ) and 1301 (8%,  $2 \times M^+ + H$ ) and the base peak at  $m/z$  471 (100%,  $M^+ - \text{glucose}$ ), strongly supported a MW of 650. Similarly, a molecular weight of 666 for stemodioside B4 was supported by observation of ions at  $m/z$  1355 (5% abundance,  $2 \times M^+ + Na$ ) and 1333 (50%,  $2 \times M^+ + H$ ) and the base peak at  $m/z$  487 (100%,  $M^+ - \text{glucose}$ ) (Fig. 78.1). The putative molecular weights were further confirmed when the samples were subjected to apci which increased the molecular ion abundance at the expense of the dimer adducts, albeit with an overall loss of total ionization and thus sensitivity.

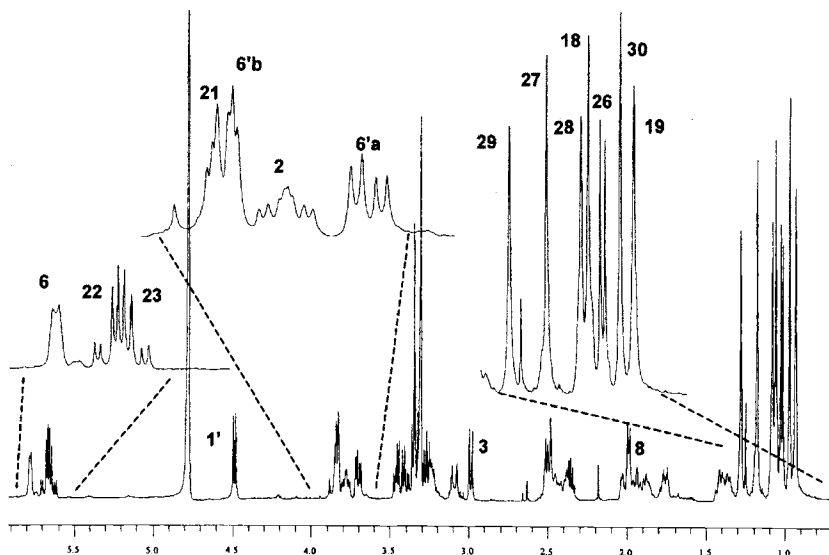
## Nuclear magnetic resonance (NMR) spectrometry

Complete mapping of the carbon and hydrogen spin systems was accomplished using 1-D and 2-D NMR experiments.

The 1-D  $^1H$  and  $^{13}C$  NMR spectra were complex (Fig. 78.2), with a degree of



**Fig. 78.1.** Ion chromatograms and mass spectra for stemodiosides B3 and B4. Stemodioside B5 is tentatively an isomer of stemodioside B4 based on MS and NMR data.



**Fig. 78.2.** Proton NMR of stemodioside B4. Some key proton assignments are indicated by the carbon number corresponding to Fig. 78.3.

overlapping that could only be fully resolved by analysis in conjunction with the 2-D homonuclear and heteronuclear experiments. Combined with the mass spectral data, analysis of the NMR data suggested the molecular formulae  $C_{36}H_{58}O_{10}$  and  $C_{36}H_{58}O_{11}$  for stemodiosides B3 and B4 respectively.

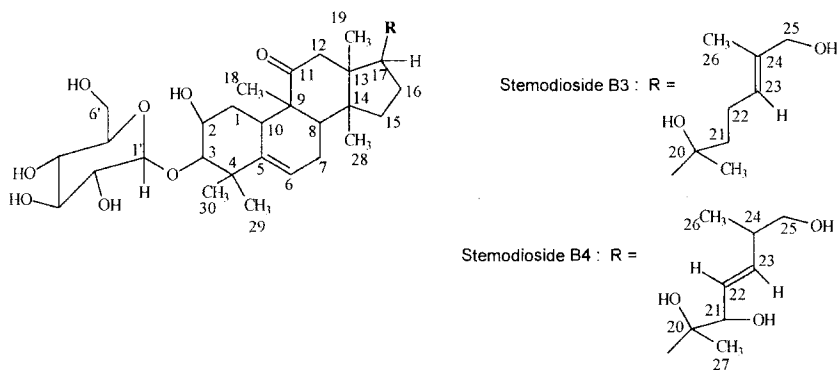
Comparison of the NMR spectra for stemodiosides B3 and B4 clearly indicated the similarity in basic structure. Both had a glucose entity, a deshielded carbonyl, seven methyls and two isolated alkenyl systems. One of these alkenyl systems (trisubstituted) appeared to be similar in both compounds whereas the others were different, with one being trisubstituted (stemodioside B3) and the other disubstituted (stemodioside B4).

Careful analysis and integrated interpretation of all NMR data provided the steroidal glucoside core structure for B3 and B4 containing the deshielded carbonyl and one of the isolated alkene moieties (Fig. 78.3). The other isolated alkene moiety and the site of difference between stemodiosides B3 and B4, were included in the side chain on C17.

## Conclusions

Stemodiosides B3 and B4 have been purified from a mixture of closely related compounds shown to be toxic to mice, producing similar clinical signs and





**Fig. 78.3.** Structures of stemodiosides B3 and B4.

pathology to the field and animal house intoxication of sheep following ingestion of *S. kingii*. Using the up and down model described by Dixon (1965), the approximate oral  $LD_{50}$ s for stemodiosides B3 and B4 in mice were estimated to be 99 and 42 mg  $kg^{-1}$  bodyweight, respectively.

Whilst the two-dimensional structures of stemodiosides B3 and B4 have been determined, the stereochemistry or three-dimensional structure remains undefined. However, by virtue of their core steroidal structure, the stemodiosides belong to the cucurbitane type triterpene steroids. Compounds of this class have been isolated from medicinal plants and have a wide range of reported bioactivities. These include a bitter or sweet taste (Kubo *et al.*, 1996), cytotoxicity and anti-tumour activity (van Dang *et al.*, 1994), analgesic and anti-inflammatory effects (Naik *et al.*, 1980) and ecdysteroid antagonism (Sarker *et al.*, 1999).

Cucurbitacins have been implicated in poisonings associated with *Cucumis* spp. and *Citrullus* spp. (Everist, 1981; McKenzie *et al.*, 1988; Jubb *et al.*, 1995). In these cases, intoxication causes gastroenteritis (particularly affecting the fore-stomachs), hepatopathy and cardiomyopathy, changes similar to those in poisoning by *S. kingii* and the stemodiosides.

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## Chapter 79

# **The Effect of Pattern of Feeding on the Development of Food Preferences in Goats in Response to Positive and Negative Post-ingestive Consequences**

A.J. Duncan and S.A. Young

*Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB15 8QH, UK*

### **Introduction**

Free-ranging ruminant herbivores generally encounter a variety of plant species that they could potentially include in their diet. Some of these plants may be toxic to various degrees due to the presence of plant secondary compounds. Plant species and plant parts also vary in their nutrient composition. Foraging herbivores are thus faced with choices as they graze which will influence the risk of toxicity as well as the nutritional rewards they gain from consuming particular plants. Previous observational studies indicate that herbivores generally select a diet richer in nutrients than the average nutritional composition of the available vegetation (Murden and Risenhoover, 1993). Similarly, although herbivores sometimes experience toxic symptoms as a result of consuming particular plants, they are generally reasonably adept at avoiding toxicity by selecting plants with low levels of secondary compounds (Foley *et al.*, 1999). Recent evidence suggests that one means by which herbivores are able to make appropriate diet choices is by learning about the nutritional and toxic consequences of consuming particular plants (Provenza, 1995). Thus, animals learn to associate positive and negative post-ingestive consequences with the flavour and other sensory properties of the plant. Such conditioned food aversions and preferences have been convincingly demonstrated in pen-fed situations with ruminants where simple, generally two-way choices, are made between foods associated with different post-ingestive effects (Zahorik *et al.*, 1990). The situation with free-ranging herbivores is more complex however, with animals often encountering a number of different plant species within a single feeding bout.

The following experiment was set up to investigate the ability of browsing herbivores to learn about post-ingestive consequences of different food plants under different feeding scenarios. In the first scenario, termed the temporal separation scenario, goats were offered the different food options singly on consecutive days. In the second scenario, the same food options were offered but

this time all food options were offered simultaneously. The objective of the experiment was to assess the ability of herbivores to make appropriate choices based on post-ingestive effects in circumstances more akin to the natural feeding situation than have been previously tested.

## Methods

Thirty-six female juvenile goats were used. The first group of 18 goats, termed the 'temporal separation' group, were individually housed and offered a basal diet of dried grass pellets at a rate calculated to maintain constant liveweight. Following a 2 week pre-experimental period, the conditioning phase commenced during which goats were offered branches of Sitka spruce (*Picea sitchensis*), Douglas fir (*Pseudotsuga menziesii*) or Scots pine (*Pinus sylvestris*), in turn, for 3 days week<sup>-1</sup> for 4 weeks. These species were chosen because they are broadly similar in terms of nutritive value and secondary compound composition. The inherent post-ingestive consequences of the experimental feeds were therefore assumed to be broadly similar. The order of offering species was randomized within weeks but was consistent between weeks. The branches were collected on the Monday of each week and offered to goats on the subsequent 3 days. Branches were hung on the gate of each individual pen and were weighed with a spring balance every hour to measure the amount consumed. During the daily feeding bouts, which lasted 4 hours, animals were dosed with either LiCl as a negative stimulus (20 mg g<sup>-1</sup> dry matter (DM) foliage consumed), sodium propionate as a positive stimulus (VFA) (90 mg g<sup>-1</sup> DM foliage consumed) or sodium chloride (54 mg g<sup>-1</sup> DM foliage consumed) as a placebo (Plac), at hourly intervals. The rates of administration were chosen based on the levels that had elicited preferences or aversions in previous studies. The chemicals to be dosed were wrapped in tissue paper to ensure rapid release in the rumen. The association of post-ingestive stimulus (Stimulus) with food type was randomized across animals but was consistent within animal. Conifer species were used because they contain similar secondary compounds and the intrinsic post-ingestive effects of different species were therefore likely to be similar. Even if each species elicited different post-ingestive consequences as a result of their intrinsic composition, the experimental design allowed the additional effects of experimentally applied post-ingestive effects to be studied since each stimulus was associated with each conifer species in a balanced fashion. After the 4 hour conditioning periods, the amount of conifer material consumed was calculated and goats were offered the remainder of their maintenance energy requirement as dried grass pellets.

Immediately prior to the experiment and at the end of each conditioning week (Week), a 20 min, three-way preference test was conducted. For the preference tests, branches of each species were hung simultaneously in the individual pens and were weighed before and after the tests to measure the relative consumption of each species. Goats sometimes dropped portions of twigs (but not individual

needles); this wastage was weighed along with the intact branches to ensure accurate measurement of food consumption.

The second group of 18 goats, termed the 'simultaneous' group, received the same experimental regime except that all food options were always simultaneously available and post-ingestive stimuli were dosed in proportion to the foods selected. Thus, on each conditioning day, branches of all three conifer species were hung in the pens and the relative consumption of each was determined by weighing every hour. Goats were then dosed with a mixture of post-ingestive stimuli depending on what they had consumed during the previous hour. Three-way preference tests were again conducted at the end of each conditioning week.

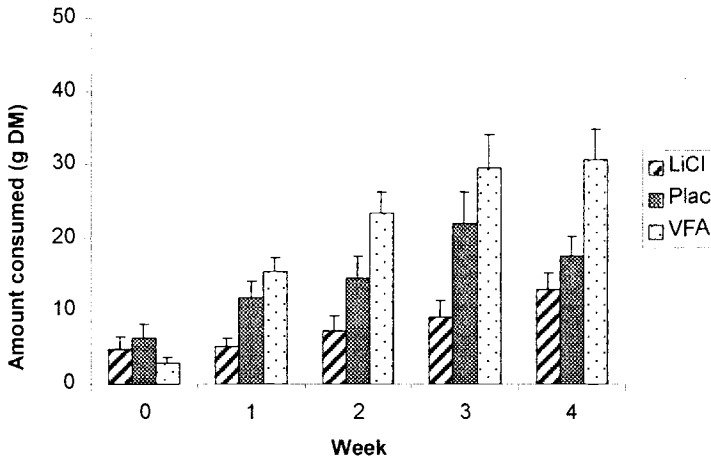
Food intake data from preference tests and from conditioning days were analysed using Residual Maximum Likelihood analysis (REML; Patterson and Thompson, 1971). Dry matter food intake of each food type was subjected to square root transformation, prior to analysis, to improve the homogeneity of the variance. Overall treatment and species effects were assessed between animals, whereas trends with time were assessed within animal.

## Results

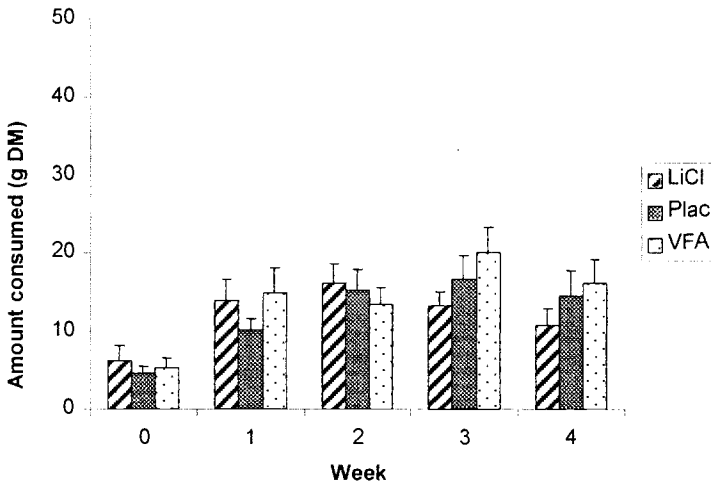
Food consumption during preference tests increased as the 4-week conditioning phase progressed in the temporal separation group (Week,  $P < 0.001$ ). A similar increase in food consumption was observed in the simultaneous group although the increase occurred primarily in the first week of the experiment and food intake thereafter was relatively stable. When foods were temporally separated during conditioning, the increase in food consumption was accentuated for VFA-associated foods and attenuated for LiCl-associated foods (Fig. 79.1; Stimulus  $\times$  week,  $P < 0.001$ ) compared to the placebo. By the end of the conditioning phase goats consumed more than twice as much of VFA-associated foods as LiCl-associated foods during preference tests. When foods were simultaneously available during conditioning, animals were less able to make appropriate associations (Fig. 79.2; Stimulus, NS). In this case, the amounts of conifer material consumed by goats were broadly similar for foods associated with the three conditioning stimuli throughout the conditioning phase. The total amount of conifer material consumed during preference tests was lower among goats in the simultaneous group than those in the temporal separation group.

## Discussion

In this experiment previous work on conditioned aversions and preferences was extended into a scenario more akin to natural feeding circumstances. The ability of goats to show learned preferences and aversions for foods associated with positive and negative post-ingestive stimuli confirmed previous findings using



**Fig. 79.1.** Influence of post-ingestive stimulus on consumption of associated conifer herbage during weekly preference tests when food options were temporally separated during conditioning periods.



**Fig. 79.2.** Influence of post-ingestive stimulus on consumption of associated conifer herbage during weekly preference tests when food options were offered simultaneously during conditioning periods.

similar conditioning stimuli (Ralphs and Cheney, 1993; Villalba and Provenza, 1996). The coupling of dose rates of conditioning stimuli to the amount of food consumed over time led to marked changes in food selection in line with expectations. The preference results in goats offered experimental foods simultaneously were quite different. In this case, animals were unable to associate particular foods with particular post-ingestive stimuli, despite the fact that the dose rates of post-ingestive stimuli were coupled to the amounts of different food plants consumed. We hypothesized that when goats were offered all three food options simultaneously they might schedule their consumption of different food types to allow them to gain information on their post-ingestive consequences. Thus, we expected that animals might consume predominantly one species on particular days and adjust their subsequent preference based on post-ingestive effects that they subsequently experienced. There appeared to be little evidence of this with animals generally consuming substantial amounts of at least two species during any one feeding bout. Further examination of the data will be required to more fully understand the scheduling of consumption of different food types within feeding bouts.

The results of this experiment suggest that goats are capable of learning about the post-ingestive consequences of foods and adjusting their diet choice accordingly. However, when foods are presented simultaneously in a manner analogous to the natural situation, the experiment indicates that goats do not appear to schedule their consumption of individual plants to learn about their post-ingestive consequences. As a result their ability to make appropriate choices is considerably reduced under this more complex, but more natural scenario.

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## Chapter 80

# Conditioned Feed Aversion as a Means of Preventing Intake of Yellow-tulp (*Homeria pallida*) by Livestock

L.D. Snyman, T.S. Kellerman, R.A. Schultz, J.P.J. Joubert, K.M. Basson and L. Labuschagne  
*Onderstepoort Veterinary Institute, P/bag X5, Onderstepoort 0110, South Africa*

### Introduction

Cardiac glycoside-containing plants, of which yellow-tulp (tulp) (*Homeria pallida*, family Iridiaceae) is the most important member, are the main cause for plant related poisonings of livestock in South Africa (Kellerman *et al.*, 1988). Tulp poisoning is characterized by posterior paresis, ruminal stasis and heart blocks (Kellerman *et al.*, 1988). Except for dosing with activated charcoal (Joubert and Schultz, 1982), which may be stressful and thus dangerous for animals with hearts about to fibrillate, no effective treatment of tulp poisoning is available.

Previous workers noticed that cattle on field can learn to avoid tulp (Steyn, 1949; Strydom and Joubert, 1983; Kellerman *et al.*, 1996). Strydom and Joubert (1983) observed that poisoning of weaner calves ceased 3 days after they were introduced on to a tulp-infested grazing because they learned to avoid tulp. If this natural aversion to tulp could be artificially induced in a controlled manner, animals would be safely averted without the risk of poisoning when newly introduced on to a tulp-infested grazing. Conditioned feed aversion (CFA) applied in this manner could be a relatively cheap and environmentally friendly way to prevent livestock from being poisoned by tulp, as chemical or mechanical eradication of the toxic plants would not be necessary. By using this method, the animal would be adapted to the environment instead of changing the environment (detrimental and costly) to suit the animal.

The objective of this study was to investigate the process of natural aversion to tulp and to apply this knowledge in an attempt to artificially avert cattle to tulp on a tulp-kikuyu grazing.



## Methods and Results

### Confirmation of aversion to tulp

As a first step, acquirement of aversion to tulp had to be confirmed under controlled conditions. Three cattle (all animals used were in agreement with the animal ethics committee of the institute) individually fed in pens were each presented with 500 g of a 1.5% tulp-maize meal mixture (the tulp was dried and milled) daily until being refused. The animals, which had been made accustomed to maize meal before the experiment, received *Eragrostis curvula* hay *ad libitum* during the day but were fasted overnight.

Two of the animals refused the tulp-maize meal mixture on Day 1 after total consumption on Day 0, while the third animal refused 84% and 98% of the mixture on Days 3 and 4, respectively, after total consumption on the preceding days. All three animals ate maize meal throughout, showing that refusal was directed specifically at tulp.

The results indicate that consumption of tulp resulted in aversion to tulp, confirming observations that stock acquires natural aversion to tulp on field.

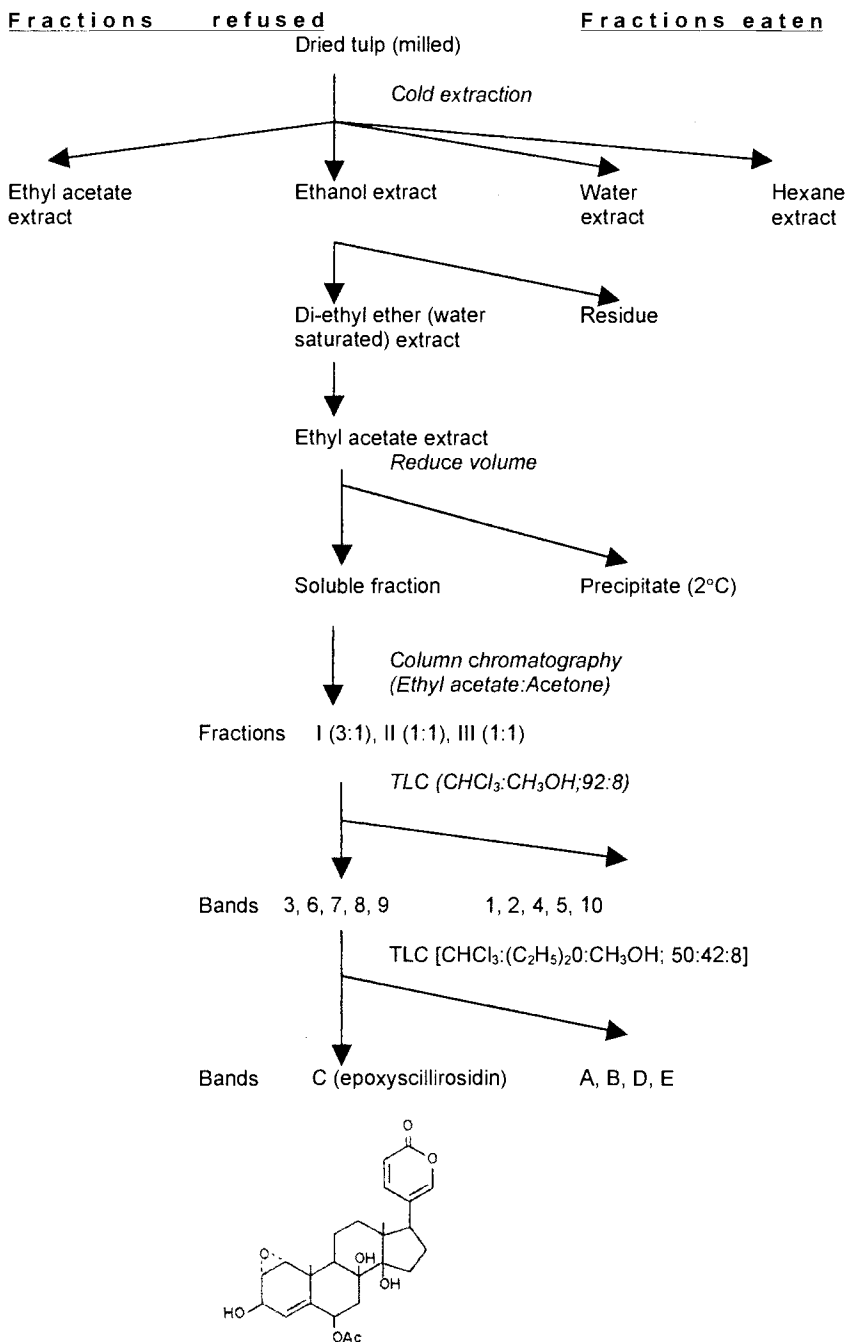
### Isolation of the aversive substance

The preceding results imply that some aversive substance(s) must be present in tulp. An attempt was made to isolate the aversive substance from tulp, as a pure aversive substance would make controlled administration and therefore safe artificial aversion of cattle to tulp possible.

A 'sniffer' sheep was used to isolate the aversive substance. This sheep, which had previously been averted to tulp, had to sense various chemical fractions of tulp, mixed with 100 g maize meal, to indicate the presence of the aversive substance. Refusal of fractions consumed by a naïve control sheep indicated the presence of an aversive substance, provided that the sniffer sheep was still willing to eat pure maize meal. By following the fractions refused as obtained by solvent extraction and chromatographic separation, epoxyscillirosidin, the main toxic principle of tulp was identified as the aversive substance of tulp. A schematic representation of the chemical procedure whereby epoxyscillirosidin was isolated, as well as the chemical structure of epoxyscillirosidin, is illustrated in Fig. 80.1.

### Confirmation of the aversion-inducing property of epoxyscillirosidin

As the isolation of epoxyscillirosidin was based on the refusal of fractions by an animal averted to tulp, the aversion-inducing property of epoxyscillirosidin *per se* still had to be confirmed. This was performed by dosing each of six steers *per os* with 22.5, 7.5, 5.0 and 2.5 mg epoxyscillirosidin on Days 0, 14, 22 and 24, respectively. The steers were individually challenged for intake of an epoxyscillirosidin-maize meal mixture (150 g), containing 5 mg



**Fig. 80.1.** Schematic representation of the procedure followed to isolate the aversive substance (epoxyscillirosidin) of tulp.

epoxyscillirosidin in 100 g maize meal on Days 1, 10, 13, 14, 15, 22 and 27. Except for those consumed by Steers 1, 5 and 6 on Day 13, all the epoxyscillirosidin-maize meal mixtures presented were totally refused, showing that intake of epoxyscillirosidin resulted in aversion to epoxyscillirosidin. However, when the averted steers were exposed together with six untreated controls to a tulip-infested kikuyu grazing on Day 28, all the steers were poisoned, showing that treatment with epoxyscillirosidin did not induce aversion to tulip. It seems that the epoxyscillirosidin-averted steers were not able to sense epoxyscillirosidin in the intact plant. Social influence by non-averted steers and unfamiliarity with the environment might also have played a role (Ralphs and Olsen, 1990).

### Tulp hexane extract as identification factor for tulp

According to the mechanism of learning in diet selection (Provenza *et al.*, 1992), avoidance of tulp on veld presumably takes place due to the sensing of easily perceptible identification factors in the plant formerly associated with the adverse effect of epoxyscillirosidin. An attempt therefore was made to extract these identification factors from tulp in order to use them in association with epoxyscillirosidin in trying to artificially avert cattle to tulp.

In a first trial, the tulp-related identification properties of a tulp-hexane extract were investigated. A steer fed on *E. curvula* hay was dosed with 0.1 mg kg<sup>-1</sup> bodyweight (BW) epoxyscillirosidin on Day 0 and tested for intake of 1.5% dried milled tulp in forage meal (500 g) the next day, as shown in Table 80.1. All the tulp forage meal mixture was consumed, showing again that intake of epoxyscillirosidin does not induce aversion to tulp. When the steer was presented with a tulp hexane extract (equivalent to 10 g tulp dry matter) mixed with forage meal (500 g) on Day 14, the mixture was refused but consumed by a naïve control steer.

The results suggest the presence of identification factor(s) in the hexane extract, resembling those in tulp to which the steer became averted on Day 1. The identification factors in tulp, therefore, seemed to be successfully extracted from the plant with hexane.

Use of the tulp-hexane extract as identification factor to induce aversion to tulp with epoxyscillirosidin as aversive agent was investigated in a next trial as

**Table 80.1.** Effect of dosing with epoxyscillirosidin followed by intake of a tulp-forage meal mixture on consumption of a tulp-hexane extract (equivalent to 10 g tulp DM) mixed with forage meal by a steer.

Day	Epoxyscillirosidin (mg kg <sup>-1</sup> BW)	Intake of 500 g forage meal mixture (g) with:	
		1.5% tulp	tulp-hexane extract
0	0.1		
1		500	
14			0

**Table 80.2.** Intake of a tulp-forage meal mixture by a steer after dosing with epoxyscillirosidin plus a tulp-hexane extract.

Day	Dosing with		Intake of 500g forage meal mixture	
	Epoxyscilli rosidin (mg kg <sup>-1</sup> BW)	Hexane extract (tulp equivalent) (g)	Tulp-hexane extract <sup>a</sup> (g)	Tulp (1.5%) (g)
0			500	
1			500	
5	0.125	5		
6				0

<sup>a</sup>Hexane extract equivalent to 10 g tulp DM

indicated in Table 80.2. A steer fed on *E. curvula* hay was firstly presented with tulp hexane extracts (equivalent to 10 g tulp DM) in forage meal (500 g) on Days 0 and 1. The mixtures were consumed in both cases, showing that they were palatable and not in themselves aversive. The steer was then dosed with epoxyscillirosidin (0.125 mg kg<sup>-1</sup> BW) plus a tulp hexane extract (equivalent to 5 g tulp DM) on Day 5 and tested for intake of a 1.5% tulp-forage meal mixture (500 g) the next day. The tulp mixture was totally refused, indicating aversion to tulp. The results show that artificial induction of aversion to tulp was possible if epoxyscillirosidin was dosed together with a tulp hexane extract, representing the identification factor(s) of tulp.

### Induction of aversion to tulp on a tulp-kikuyu grazing

Applicability of this aversion inducing method was tested with cattle exposed to tulp on a small tulp-kikuyu grazing (c. 10 x 10 m). The experimental details are given in Table 80.3. Trials were performed during the pre-bloom, mid-bloom, and post-bloom growth-stages of tulp. Yearling steers, raised on tulp-free field, received *E. curvula* hay *ad libitum* in a pen at the laboratory for a week before aversion treatment was instituted. At each growth stage of the tulp, three steers were averted by dosing epoxyscillirosidin (*per os*) together with a tulp hexane extract. The epoxyscillirosidin and evaporated hexane extracts, suspended in 50 ml propylene glycol, were administered orally 17–21 hours prior to exposure to the tulp-kikuyu grazing. Feed and water were withheld from the animals during this time. An equal number of control animals were treated similarly, except that they were not dosed with the aversive mixture. The animals were exposed to the tulp-kikuyu grazing for 48 hours and observed for clinical signs of tulp poisoning. Severely poisoned animals were removed from the tulp-kikuyu grazing and treated with activated charcoal (2 g kg<sup>-1</sup> BW).

Degree of poisoning was classified according to the criteria given in Table 80.4.

The occurrence of one or more of the following clinical signs, namely posterior paresis with the animal unable to stand, rumen stasis, and a consistent occurrence of heart blocks, was classified as severe poisoning. Posterior paresis

**Table 80.3.** Experimental detail for aversion treatment of cattle at three growth stages of tulip.

Treatment	Growth stage		
	Pre-bloom	Mid-bloom	Post-bloom
Epoxydiscillirosidin (mg kg <sup>-1</sup> BW)	0.07	0.06	0.07
Tulip-hexane extract (tulip equivalent per animal):			
• fresh tulip (g)	15	15	25
• dry tulip (g)	10	10	10
Time period (hours) between aversion treatment and exposure to tulip (withheld from food and water)	18	21	17
Time period exposed to the pasture (hours)	48	48	48

**Table 80.4.** Criteria for classifying the degree of tulip poisoning.

Clinical signs	Degree of poisoning (one or more signs)	
	Mild	Severe
Posterior paresis	Remains standing	Unable to stand
Heart blocks	Sporadically	Consistently
Inhibition of rumen movements	1–2 per 5 min	Rumen stasis

with the animal being able to remain standing, inhibition of rumen movements to 1–2 per 5 min, or a sporadic occurrence of heart blocks was classified as mild poisoning.

Results of the trial on the tulip-kikuyu grazing are shown in Table 80.5.

A total number of six averted and seven control animals were poisoned over the three growth stages. Fewer of the averted animals were severely poisoned, namely three, compared to six of the controls, of which two died. The occurrence of severe poisoning among averted animals, however, contradicted observations that averted animals tried to avoid tulip plants on the grazing. This anomaly can only be explained by a possible toxic contribution by the aversive agent.

#### Contribution of the aversive agent to poisoning

In order to determine the toxic contribution by the aversive agent (epoxydiscillirosidin), each of six cattle was dosed (*per os*) with a different amount of epoxydiscillirosidin and examined for poisoning as indicated in Table 80.6.

**Table 80.5.** Number of averted and control cattle poisoned on a small tulp-kikuyu grazing at various growth stages of the tulp.

Degree of poisoning	Averted animals				Control animals			
	Pre-bloom	Mid-bloom	Post-bloom	Total	Pre-bloom	Mid-bloom	Post-bloom	Total
Unaffected	0	2	1	3	0	1	1	2
Mildly	2	0	1	3	0	0	1	1
Severely	1	1	1	3	1	2	1	4
Deadly	0	0	0	0	2	0	0	2

The results indicate that the lowest dose that caused clinical signs of poisoning was 0.100 mg kg<sup>-1</sup> BW. The aversion doses used (0.060–0.070 mg kg<sup>-1</sup> BW) in the former trial were 60–70% of this dose, suggesting that the aversive dose must have made an important contribution towards poisoning of averted animals.

**Aversion with epoxyscillirosidin plus lithium chloride as aversive agents**

The problem was addressed by partial replacement (*c.* 70%) of epoxyscillirosidin with lithium chloride (LiCl). Lithium chloride is a suitable aversive agent to replace epoxyscillirosidin as it rapidly induces gastrointestinal distress, is not lethal, and is available commercially (Ralphs and Olsen, 1990). Lithium chloride has no effect on the heart and therefore would not complement the toxic action of epoxyscillirosidin. The efficiency of this aversive mixture was investigated in cattle grazing on a small tulp-kikuyu grazing (10 x 10 m) at the laboratory of the institute. The tulp on the grazings to which the cattle were exposed were respectively 70%, 80% and 90% in bloom. The cattle were made accustomed to an adjacent kikuyu grazing by daily exposures prior to the trial. The detail of the treatments is shown in Table 80.7. An equal number of control animals were treated similarly except that they were not dosed with the aversive mixture. The results of this trial are shown in Table 80.8.

**Table 80.6.** Effect of varying doses (*per os*) of epoxyscillirosidin on poisoning of cattle.

Dose (mg kg <sup>-1</sup> BW)	Clinical signs of poisoning		
	Posterior paresis	Heart blocks	Rumen stasis
0.075	No	No	No
0.100	No	Mild	No
0.112	Severe	Severe	No
0.125	Mild	No	No
0.137	Severe	Severe	Severe
0.150	Severe	Severe	No

**Table 80.7.** Experimental detail of treatments for aversion to tulip.

Treatment	Growth stage		
	70% bloom	80% bloom	90% bloom
Epoxydiscillirosidin (mg kg <sup>-1</sup> BW)	0.02	0.02	0.02
Lithium chloride (mg kg <sup>-1</sup> BW)	80	80	120
Tulp-hexane extract (equivalent fresh tulip [g] per animal)	20	20	20
Time period (hours):			
• Between aversion treatment and exposure to tulip	24	24	24
• Withheld from food and water before exposure to tulip	17	16	0
• Exposed to the tulip-kikuyu grazing	48	48	48

In total over the three growth stages, five averted animals were poisoned compared to eight of the controls. None of the averted animals was severely poisoned or died, compared to four of the control animals, of which one died. The results indicate that aversion treatment with a combination of epoxydiscillirosidin and LiCl, with animals being made accustomed to an adjacent kikuyu pasture, reduced the number and severity of tulip poisoning.

## Conclusion

The properties involved in natural aversion of cattle to tulip were identified and exploited for controlled artificial aversion of naïve cattle to tulip. The risk of tulip poisoning on a small tulip-kikuyu grazing at the laboratory was notably reduced, suggesting that this technique might be useful to limit tulip poisoning on tulip-infested grazings.

**Table 80.8.** Number of averted and control cattle poisoned on a small tulip-kikuyu grazing at various growth (bloom) stages of the tulip.

Degree of poisoning	Averted animals				Control animals			
	Tulp bloom stage				Tulp bloom stage			
	70%	80%	90%	Total	70%	80%	90%	Total
Unaffected	0	1	3	4	0	0	1	1
Slightly	3	2	0	5	2	1	1	4
Severely	0	0	0	0	1	1	1	3
Deadly	0	0	0	0	0	1	0	1

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## Chapter 81

# Plant-associated Diseases, Either New or New to the State, Encountered Over the Last Decade (1991–2001) in Western Australia

J.G. Allen<sup>1</sup>, J.H. Creeper<sup>1</sup>, D. Forshaw<sup>2</sup>, M.J. Kabay<sup>1</sup>, D.C. Main<sup>1</sup> and R.B. Richards<sup>2</sup>

<sup>1</sup>Department of Agriculture, 3 Baron-Hay Court, South Perth, WA 6151, Australia; <sup>2</sup>Department of Agriculture, 444 Albany Highway, Albany, WA 6330, Australia

The past decade has been a remarkable period in Western Australia for the identification of completely new plant-associated diseases, and for the first time occurrence within the state of plant-associated diseases reported elsewhere in Australia or the world. Newly identified plant-associated diseases have included *Cucumis melo* ssp. *agrestis* intoxication in cattle (Jubb *et al.*, 1995), *Stemodia kingii* intoxication in sheep (Allen and Mitchell, 1998), lupin-associated rumenitis in sheep and cattle (Allen *et al.*, 1998), black soil blindness in sheep and cattle caused by the fungus *Corallocytophthora ornico-preoides* on Mitchell grass (*Astrebula* spp.) (Jubb *et al.*, 1998), and *Heliotropium ovalifolium* intoxication in horses (Creeper *et al.*, 1999). We describe here numerous other plant-associated diseases in livestock that have either never been reported before, or have not previously occurred in Western Australia.

### New Plant-associated Diseases

#### Diseases associated with tagasaste (*Chamaecytisus proliferus*)

Tagasaste is a perennial leguminous fodder shrub grown throughout the south-west of Western Australia. The plant is known to contain flavone phenolics (Edwards, 2000) and there have been three disease syndromes associated with the grazing of it by livestock.

#### *Nephropathy and hepatopathy syndrome*

Two cases in mature cows (5/88 and a single animal dead) and a single case in 2-year-old ewes (25/770 dead) have been reported. The cases occurred in autumn,

winter and spring. The microscopic pathology in each included acute to subacute necrosis of tubular epithelium in the kidney cortex, and widespread necrosis of individual hepatocytes in the liver.

#### *Tagasaste staggers*

This is a regularly occurring condition in cattle grazing tagasaste. It occurs throughout the year and is usually only noticed when the cattle are moved. It has been reported in yearling to mature stock of both sexes, and it generally does not result in death. Most cases involve one to a few animals, but up to 60/400 have been affected. The clinical signs include tremors over the whole body, but particularly the face, an ataxia and stumbling affecting all limbs (staggering), and falling and temporary collapse if the animals are forced to keep moving. If affected animals are left alone they recover. Only two affected animals have been necropsied, and examination of an extensive range of tissues, including the brain and spinal cord, revealed no lesions. Clinical chemistry involving a wide range of tests has been conducted on a large number of affected animals, and the only consistent findings have been increased concentrations of plasma ammonia, urea, glucose and L-lactate, compared to unaffected animals in the same herds. The increased ammonia and urea concentrations are believed to indicate that these animals are eating more of the tagasaste than other animals.

#### *Leucodystrophy in neonatal calves – 'dancer calves'*

This condition has occurred on at least five properties where cows have grazed tagasaste during the last month of their gestation. It has occurred in more than 1 year on some properties, and has occurred in 3 consecutive years on one. It is thought more likely to occur if the tagasaste is stressed by lack of water, cold or waterlogging, which results in the leaves of the plant developing a yellowish colour. Affected calves seem to be born in a cluster within the calving period, and up to six may be born within a group of 30-40 cows. Affected calves are either stillborn, born alive but so weak that they cannot stand, or born alive and able to stand but showing various degrees of dysmetria. The consistent sudden and wild swinging and twirling of the tail ('dancing' tail, hence the name 'dancer calves'), as well as the awkward gait of calves that can stand, provide evidence of the latter. If calves are able to feed, or are nursed, property owners report that in some the clinical signs gradually disappear over about 3 months.

The characteristic microscopic lesion is the presence of plaques and spaces, up to 100 µm in diameter, within the white matter of the central nervous system and optic nerve, but predominantly in the hindbrain and the entire spinal cord. In the spinal cord all tracts are affected, but changes are most severe in the ventral and lateral tracts. The spaces are thought to result from plaques falling out during processing. In addition, within affected areas there is gliosis, oligodendrocytes with vacuolar cytoplasm and pycnotic nuclei, and macrophages within the plaques or spaces, to varying degrees. The axons usually appear unaffected and

can be seen to traverse the plaques and spaces, but in severely affected calves some axonal spheroids are present.

Electron microscopy has revealed that the plaques are intramyelinic proliferations of vesicular membranous profiles, together with myelin bodies and a granular matrix that may be remnants of microtubules. The nearby glial cells and their processes also contain these vesicular membranous profiles, so the condition may primarily result from toxic damage to the oligodendrocytes.

### **Hairy toadflax (*Kickxia* sp.) in sheep**

Gardner and Bennetts (1956) reported that hairy toadflax (*Kickxia* sp., formerly *Linaria elatine* L.) in Western Australia was suspected to be toxic but presented no evidence. Toadflax (*Linaria vulgaris*) is reported to be toxic in Europe where it has caused reduced appetite, dyspnoea, diarrhoea and gastroenteritis in sheep, cattle and horses (Cooper and Johnson, 1984).

Following the death of several sheep seen to be eating hairy toadflax on a property approximately 200 km north of Perth, the plant was collected, dried, milled, and drenched in an aqueous slurry to a Merino wether hogget. The sheep received a total of 1.95 kg (55.5 g kg<sup>-1</sup> bodyweight) of the plant in seven doses over 3 days. It had a reduced appetite at 24 hours and had stopped eating completely by 48 hours, at which time it had developed diarrhoea. By 72 hours it had profuse watery diarrhoea, but was not depressed. However, within hours it became depressed and was euthanized at 3.5 days.

There was patchy focal necrosis and sloughing of superficial epithelial cells in the abomasum and caecum. In the ileum there was complete loss of villi and severe atrophy of surviving villi, with oedema of the lamina propria. In the heart there was multifocal acute necrosis of myocytes throughout the myocardium of both ventricles. These pathological changes are similar to those caused by cardiac glycosides, which are the toxins suspected to be in *L. vulgaris*.

### **Stressed *Acacia saligna* in goats**

*Acacia saligna* is occasionally grown as a fodder shrub in southern Australia. It is not considered to produce cyanogenic glycosides (Conn *et al.*, 1985). In the case investigated a group of mature female goats had been provided daily with cuttings from a plantation of *A. saligna*, when three died suddenly with marked pulmonary oedema. The investigating veterinarian diagnosed acute cyanide intoxication and submitted fresh plant for analysis. It contained no cyanide. Some weeks later the owner recalled that at the time of the deaths the cuttings had been taken from shrubs with very large malformations of the stems and leaves. He inspected the area from which they were collected and found similarly malformed stems, but not to the same extent as they had been earlier. He submitted these, together with normal stems from the same plants, for analysis. The malformed stems were found to contain 20 mg cyanide (as HCN) kg<sup>-1</sup> wet weight, while the normal stems contained no cyanide. Plant pathologists reported that the malformations were typical of those induced by a heavy thrips infestation, where the thrips

carried a pathogenic virus. It is considered possible that this insect/virus damage induced affected stems to produce cyanogenic glycosides, and that the malformed stems originally fed contained higher concentrations of cyanide than those stems eventually analysed.

## **Plant-associated Diseases New to Western Australia**

### **Chinkerinchee (*Ornithogalum thyrsoides*) in sheep**

Chinkerinchee (or Star of Bethlehem) is well recognized as a poisonous plant in South Africa (Kellerman *et al.*, 1988), and this first case of poisoning in Australia resulted following the escape of this bulbous perennial herb from garden cultivation into nearby farmland.

Four days after 220 Merino ewe hoggets were introduced into a paddock heavily infested with the chinkerinchee, five sheep were found dead, another two had a staggering gait, another two had subcutaneous oedema of the face and ears, and 33% of the sheep had a severe, foetid, watery diarrhoea. There had been similar occurrences in previous years when sheep were put into this paddock soon after the first winter rains, which caused the chinkerinchee to start growing.

Two sheep were necropsied and found to have severe villus atrophy in the duodenum and ileum, with some proteinaceous fluid in the lamina propria of the ileum, and patchy necrosis and sloughing of the superficial epithelium in the colon. In the heart there was acute to subacute multifocal myocardial necrosis involving both ventricles and the interventricular septum.

The clinical signs and pathology in the gastrointestinal tract were similar to the descriptions of chinkerinchee poisoning in South Africa (Kellerman *et al.*, 1988). However, the cardiac lesions have not previously been reported. Myocardial necrosis has been seen in *O. toxicarum* intoxication in sheep, and cardenolides have been isolated from *O. magnum* and *O. umbellatum*, and prasinolides from *O. prassinum* and *O. thyrsoides* (C.J. Botha, Onderstepoort, 1999, personal communication).

### **Lesser loosestrife (*Lythrum hyssopifolium*) in sheep**

Deaths occurred in two separate flocks of weaner sheep introduced into canola stubbles in early summer. At that time the only green feed in the paddocks was lesser loosestrife. Deaths commenced 7 and 15 days after introduction into the paddocks, and continued for 6 weeks after removal, which was done on the first day that deaths occurred. Clinical signs included sudden death initially, then in the rest of the flock loss of condition, depression, weakness, recumbency, and occasionally photosensitization. The mortality rates were 35/300 in mixed sex sheep, and 125/300 in rams.

Sheep necropsied at the start of the outbreaks had severe, acute, lobular or midzonal necrosis in the liver, with or without haemorrhage. Many of these sheep

also had severe, acute necrosis of tubular epithelium in the renal cortex, with casts staining like haemoglobin casts in many dilated tubules. Sheep necropsied 20 days into the outbreaks had widespread interstitial and periglomerular fibrosis in the kidneys, with dilation of many of the remaining tubules. Proteinaceous casts were present in some tubules and tubular epithelium frequently contained large amounts of haemosiderin.

Similar cases of lesser loosestrife poisoning have been reported in the eastern states of Australia following the introduction of sheep into the stubbles of canola, wheat and oat crops (Glastonbury *et al.*, 1991; Nimmo Wilkie and Lancaster, 1993). A case has also occurred associated with canola stubbles in South Africa, where the condition was reproduced by immediately feeding the fresh green plant (T. Gous, Stellenbosch, 2000, personal communication).

### **Green canola (*Brassica napus*) in sheep**

Canola is the registered name for cultivars of *Brassica napus* that have reduced concentrations of alkenyl glucosinolates and erucic acid in their seed (Cheeke, 1998). We have encountered two syndromes associated with the unusual circumstance of grazing green canola.

The first involved a flock of weaner sheep introduced into a canola stubble early in summer. Late seasonal rain had caused a lot of the harvested canola to reshoot, and the sheep were attracted to this green material. Within 3 to 4 days some sheep were found dead with a blood stained froth oozing from their nostrils, and other sheep exhibited respiratory distress and had froth around their mouths. The sheep were removed from the paddock and recovered with no more deaths. This case was similar to some reported in New South Wales where sheep grazed failed canola crops and developed interstitial pneumonia and pulmonary oedema (Marshall, 1995). The cause was suspected to be indole glucosinolates in the canola. These compounds are hydrolysed in the alimentary tract to 3-hydroxymethylindole, which has caused interstitial pneumonia in cattle (Seawright, 1989).

The second syndrome occurred on two separate properties when flocks of weaner sheep were introduced to canola stubbles in mid-summer. There had been unseasonable heavy rainfall and canola seed still on the ground had germinated, providing a substantial sward of young green canola for the sheep. Within days 90% of the sheep in each flock had developed a putrid scour and were removed immediately. Similar cases have been seen in New South Wales where sheep that have been necropsied have had a rumenitis and a gastroenteritis (C.A. Bourke, Orange, 2000, personal communication). It is presumed the condition is caused by extremely irritant isothiocyanates, produced by hydrolysis of glucosinolates in the canola, which have produced a similar condition in cattle (Seawright, 1989).

### **Sorghum (*Sorghum spp.*) in sheep and cattle**

Following heavy summer rains, a flock of 800 weaner sheep was introduced into a paddock of *Sorghum* that was growing and was about 45 cm high. Within a

week eight sheep were seen to be depressed, staggering and showing hyperextension of their forelimbs. They also had muscle tremors affecting the head and neck, and nystagmus. Microscopic examination revealed axonal spheroids close to nuclei throughout the brain, and particularly adjacent to the cerebellar roof nuclei. In the spinal cord the spheroids were within the grey matter of the ventral horns near to the motor neurones, in the white matter immediately adjacent to the ventral horns, and in the ventral and lateral white matter tracts. There was also focal Wallerian degeneration within the white matter of the cerebellum and spinal cord. Similar clinical conditions and pathology have been reported in young sheep grazing *Sorghum* spp. (Bradley *et al.*, 1995; Glastonbury *et al.*, 1997).

A herd of 70 Hereford x Angus cows grazed *Sorghum* from 6 to 8 months of their gestation. Three cows aborted at about 8 months, and between then and the estimated start of calving, four calves were born unable to stand, two were born alive with arthrogryposis, two were born dead with arthrogryposis, there was a late-term abortion and one normal calf was born. The calves that could not stand developed tonic spasms on stimulation. Microscopic examination of the brains from calves that could not stand revealed extensive Wallerian degeneration of white matter tracts within the brain stem and cerebellar peduncles, and occasional axonal spheroids in the cerebellar white matter (spinal cord not submitted). Similar clinical conditions and microscopic pathology have been reported in neonatal calves from dams that had grazed *Sorghum* spp. during pregnancy (Seaman *et al.*, 1981; Moloney and Treloar, 1997).

The cause of these *Sorghum* neurotoxicities is not known, but some believe it is a form of lathyrism (Bradley *et al.*, 1995). It has also been stated that the pathology in sheep and cattle is quite different, with sheep developing an axonopathy and cattle a leucomyelopathy (Bourke, 1995; Glastonbury *et al.*, 1997). In the sheep case reported here Wallerian degeneration was a feature of the pathology, and in the case in calves reported by Moloney and Treloar (1997) there was an axonopathy.

## **Other Plant-associated Diseases Encountered that were New to Western Australia**

Nitrate poisoning in cattle following the feeding of *Sorghum* hay and the grazing of a standing *Sorghum*/millet crop that had been accidentally fertilized with a liquid nitrate and borax fertilizer meant for cauliflowers growing adjacent; *Malva parviflora* (marshmallow) toxicity in sheep (skeletal and cardiac myopathy); increased thyroid size in pigs fed canola meal (Mullan *et al.*, 2000); big head (a secondary hyperparathyroidism due to chronic oxalate toxicity) in horses grazing *Cenchrus ciliaris* (buffel grass) and *C. setiger* (birdwood grass); tryptophan-induced acute bovine pulmonary emphysema; photosensitization in sheep grazing *Panicum miliaceum* (French millet) and in sheep grazing *Echinochloa frumentacea* (Japanese millet); green oat poisoning in cattle (sudden onset of severe diarrhoea with accompanying drop in milk production); *Cynodon dactylon*

(Bermuda or couch grass) staggers in cattle; redgut (a haemorrhagic enteritis associated with torsion) in sheep grazing *Medicago sativa* (lucerne); and the occurrence of toxic blooms of *Cylindrospermopsis raciborskii*.

## Acknowledgements

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## Chapter 82

# Steroidal Sapogenins and Saponins in *Narthecium ossifragum* from Scotland

A.L. Wilkins<sup>1</sup>, A. Flåøyen<sup>2,3</sup> and J.I. Loader<sup>1</sup>

<sup>1</sup>Chemistry Department, School of Science and Technology, The University of Waikato, Private Bag 3105, Hamilton, New Zealand; <sup>2</sup>National Veterinary Institute, PO Box 8156 Dep., 0033 Oslo, Norway; <sup>3</sup>Department of Large Animal Clinical Sciences, Norwegian School of Veterinary Science, PO Box 8146 Dep., 0033 Oslo, Norway

### Introduction

*Narthecium ossifragum* (Eng.: Bog asphodel) is a saponin containing plant implicated in the hepatogenous photosensitization of sheep (Flåøyen, 1999) in Northern Europe. Most reported outbreaks of *N. ossifragum*-associated photosensitization of sheep originate from Norway (Ender, 1955; Ceh and Hauge, 1981; Abdelkader *et al.*, 1984; Flåøyen, 1999), however there are also reports of *N. ossifragum*-associated photosensitization of sheep grazing *N. ossifragum* containing pastures in the Faroe Islands, Scotland (Flåøyen *et al.*, 1995) and the North of England (Ford, 1964).

While there is some knowledge of the levels of saponins (steroidal sapogenin glycosides) in Norwegian collections of *N. ossifragum* (Flåøyen *et al.*, Chapter 11 this volume), there is no information concerning the levels of steroidal sapogenins in Scottish collections of *N. ossifragum*.

In this chapter we report an evaluation of the levels of free and conjugated sapogenins (saponins) found in *N. ossifragum* plants collected from Auchtertyre Farm, Strathfillian, West Perthshire, Scotland. At the time of sampling two photosensitized lambs had recently been removed from a pasture that included an appreciable quantity of *N. ossifragum*.

### Experimental

Plant material (leaf, stems, flower heads and roots) were collected on 8 August 2001 from two sites on Auchtertyre Farm, Strathfillian, West Perthshire, Scotland (56° 26' North, 4° 39' West), adjacent to the access road leading to the West Highland Railway Line bridge, c. 100 and 150 m above the farm workshop area (sites 1 and 2, young, recently grazed plants) and from a hillside slope c. 500 m above the railway bridge (site 3, mature plants that had not been grazed). Plant

samples were frozen within 6 h of collection, and maintained at freezer temperature, other than for the period (10 h) when they were transported to the National Veterinary Institute, Oslo, where they were refrozen and freeze dried.

Accurately weighed portions of the freeze dried plant materials (*c.* 0.2–0.3 g) were placed in cellulose extraction thimbles and sequentially extracted for 3 h with dichloromethane, and (after overnight drying of the extraction thimble) with methanol, using a SoxTech extractor. After the evaporation of solvent under a stream of warm nitrogen, conjugated extracts were hydrolysed for 90 min at *c.* 90–95°C using 0.5 M HCl (7 ml), and extracted with dichloromethane (3 x 4 ml). All extracts were acetylated with pyridine/acetic anhydride, spiked with sarsasapogenin propionate as internal standard, and analysed by selected ion mode (SIM) GC/MS as described previously (Wilkins *et al.*, 1994), other than that a 25 m x 0.22 mm id HP-5 capillary column (Hewlett Packard, USA), a Karlo-Erba 8000 GC and a VG Trio 1000 mass spectrometer were used. SIM-GC/MS analyses were temperature programmed from 200°C (1 min hold) to 265°C at 40°C min<sup>-1</sup> and then to 290°C at 5°C min<sup>-1</sup> (16 min hold). Quantification was performed relative to sarsasapogenin propionate. The *m/z* 139 ion response factor of sarsasapogenin acetate relative to that of sarsasapogenin propionate was 1.15.

## Results and Discussion

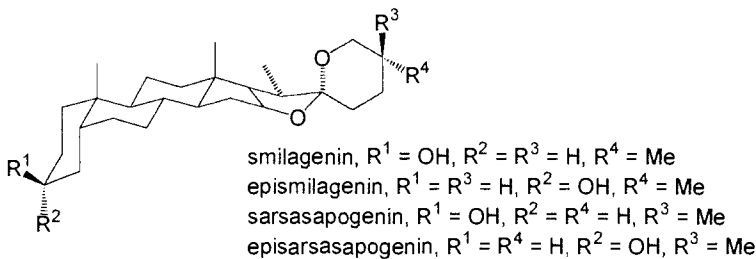
The dry matter (DM) content and levels of free and conjugated sapogenins determined for the freeze dried plant materials are presented in Table 82.1. Confirmation of the presence of smilagenin and sarsasapogenin acetates (25*R* and 25*S*-epimers respectively of the same sapogenin; see Fig. 82.1) in the acetylated extracts was established by comparison of total ion chromatogram mode mass spectra, *m/z* 255/*m/z* 315, *m/z* 269/*m/z* 329 and *m/z* 284/*m/z* 344 ion ratio (Wilkins *et al.*, 1994) and retention time data for the acetylated sapogenins and for authentic specimens of smilagenin and sarsasapogenin acetates. The total levels of conjugated (glycosylated) sapogenins found in leaf samples, with the exception of the basal region of the mature plants from site 3, (2040–4618 mg kg<sup>-1</sup> DM) are comparable to those present in summer collections of Norwegian *N. ossifragum* (Flåøyen *et al.*, Chapter 11 this volume).

Since plant material was collected from only two sites on a single Scottish farm, caution must therefore be exercised when comparing the characteristics of Norwegian and Scottish collections of *N. ossifragum*. The *c.* 45–65% contribution of smilagenin to total sapogenins determined for the Scottish collections of *N. ossifragum* (Table 82.1) can be compared to the *c.* 10–15% contribution of smilagenin to total sapogenins in Norwegian collections. Miles *et al.* (1991) have suggested that following the ovine metabolism of sapogenins to episapogenins, epismilagenin (25*R*-epimer) may be more lithogenic (crystal-forming) than episarsasapogenin (25*S*-epimer) (Fig. 82.1).

**Table 82.1.** Percentage dry matter (DM) and free and conjugated sapogenin levels (mg kg<sup>-1</sup> DM) determined for *N. ossifragum* plant samples.

Site/sample	Extract	% DM	Smilagenin	Sarsasapogenin	Total <sup>c</sup>
Site 1 <sup>a</sup>					
Leaf (0–15 cm)	Free	34.3	tr	tr	tr
	Conjugated		1,047	993	2,040
Flower heads	Free	75.4	3	55	58
	Conjugated		8,139	10,245	18,384
Stems	Free	52.7	tr	tr	0
	Conjugated		26	28	54
Roots	Free	54.1	145	687	832
	Conjugated		44	167	211
Site 2 <sup>a</sup>					
Leaf (0–15 cm)	Free	44.6	tr	tr	tr
	Conjugated		2,788	1,830	4,618
Site 3 <sup>b</sup>					
Leaf (0–10 cm)	Free	30.4	tr	tr	tr
	Conjugated		75	36	111
Leaf (10–20 cm)	Free	30.8	tr	tr	tr
	Conjugated		2,119	1,454	3,573
Leaf (20–30 cm)	Free	40.2	tr	tr	tr
	Conjugated		1,870	1,408	3,278
Leaf (bulk)	Free	32.3	6	12	18
	Conjugated		1,262	1,029	2,291
Stems	Free	36.0	12	20	32
	Conjugated		23	24	47
Flower heads and stems	Free	57.5	24	32	56
	Conjugated		42	30	72
Roots	Free	62.0	341	841	1,182
	Conjugated		14	54	68

<sup>a</sup> Young plants; <sup>b</sup> mature plants; <sup>c</sup> total sapogenins; tr = trace.

**Fig. 82.1.** Chemical structures of smilagenin, epismilagenin, sarsasapogenin and episarsasapogenin.

Intriguing aspects of the results were the detection of a substantial level of conjugated sapogenins (saponins) in the flower heads of young plants from site 1 (c. 18 g kg<sup>-1</sup> DM) and the presence of significant levels of free sapogenins in the roots of plants from sites 1 and 3 (832 and 1184 mg kg<sup>-1</sup> DM respectively). It is tempting to speculate that sapogenins are initially synthesized in the roots, and following glycosylation, they are transported to leaf tips and flower heads, where they may possibly act as antifungal agents (Flåøyen *et al.*, Chapter 11 this volume). More detailed studies are however required to validate these hypotheses.

## Conclusions

The presence in Scottish *N. ossifragum* samples, from a pasture on which sheep were photosensitized, of elevated levels of conjugated steroidal sapogenins has been established. The percentage contribution of smilagenin (a 25R-genin) to the Scottish samples was typically two to four times greater than was the case for Norwegian collections of *N. ossifragum*.

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## Chapter 83

# **Transfer of Cyanide and its Main Metabolite Thiocyanate in Milk: Study of Cyanogenic Plants Ingestion during Lactation in Goats**

B. Soto-Blanco and S.L. Górniak

*Research Centre for Veterinary Toxicology (CEPTOX), Department of Pathology, School of Veterinary Medicine, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, 05508-900, Brazil*

### **Introduction**

Having an important impact on animal production and food safety, several plant toxins and/or their products of biotransformation are known to be transferred through milk. Examples include the indolizidine (James *et al.*, 1990), piperidine (López *et al.*, 1999), pyrrolizidine (Eastman *et al.*, 1982) and quinolizidine alkaloids (Ortega and Lazerson, 1987), the glucosinolates (White and Cheeke, 1983; Garg and Gupta, 1987; Subuh *et al.*, 1995), the sesquiterpene lactones (Ivie *et al.*, 1975) and ptaquiloside (Evans *et al.*, 1972). Although excretion of plant toxins through mammary glands reduces the toxicity to lactating animals, it is an important source of such toxins to the consumers. Moreover, neonates are usually more sensitive to the toxins than adults (Panter and James, 1990). The toxicological hazard of this route of elimination is most significant in the cases of repetitive exposures rather than single.

A number of plants utilized in animal feeding are cyanogenic, such as cassava, sorghum, and *Cynodon* grasses. Long-term cyanide ingestion, which occurs when cyanogenic plants are part of the diet, has been associated to pancreatic diabetes, hypothyroidism, and several neuropathies in both humans and animals (Poulton, 1983). Nonetheless, no work was found in the literature aiming to understand the effects of exposure to cyanogenic plants or to cyanide on lactation in ruminants.

Thus, the objective of the present work was to evaluate the effects on both the dams and litter of the maternal exposure to potassium cyanide (KCN) during the lactation period in goats. An experimental model for study of the transfer of plant toxins through milk in ruminants is proposed.

## Materials and Methods

Twenty-eight mixed-breed female goats, 1–3 years old, were bred to one Alpine buck. At the day of birth, the dams were divided into four equal groups and were dosed with 0, 1.0, 2.0, or 3.0 mg KCN (Merck Co, Darmstadt, Germany)  $\text{kg}^{-1}$  bodyweight  $\text{day}^{-1}$  administered orally with tap water for 3 months. The experimental animals received KCN twice a day, between 7:30 and 8:00 and between 16:30 and 17:00.

From the jugular vein of each goat, blood samples were collected from both dams and kids. Whole blood cyanide and plasma thiocyanate levels were measured at the 30th, 60th, and 90th days of experiment, and plasma thyroxine (T4) and triiodo-thyronine (T3) concentrations were measured at the 90th day. Plasma samples were held at  $-10\text{ }^{\circ}\text{C}$  until analysis, whereas whole blood samples for cyanide determination were used immediately after collection. Plasma thiocyanate concentrations were determined spectrophotometrically by the method of Pettigrew and Fell (1972). Blood cyanide concentrations were measured by the procedure of Holzbecher and Ellenberger (1985), which we modified. In this methodology, glass headspace vials (30 mm x 49 mm o.d., outer chamber) with special tubes inside (14 mm x 30 mm o.d., inner chamber) were used. One ml of whole blood plus one drop of 10% EDTA followed by 1 ml of 50%  $\text{H}_2\text{SO}_4$  was put into the external portion of the microdiffusion chamber. The internal chamber contained 2 ml of 0.1 M NaOH. After 2 hours at room temperature, 0.5 ml of the solution in the internal portion of the chamber was transferred to a tube containing 1.5 ml of 0.1 M  $\text{NaH}_2\text{PO}_4$  and then was added 0.5 ml 0.1% of Chloramin T. After 2 minutes, 1.5 ml of pyridine-barbituric acid solution (21 ml deionized water, 10 ml pyridine, 2 ml 12 M HCl and 2 g barbituric acid) was mixed. After 2 minutes, spectrophotometric measurement of the colour complex was done at 584 nm.

At the end of the experimental period, one doe from each group and all the male goats from every litter were euthanized for histopathological analyses. Data is reported as mean  $\pm$  SEM and was analysed statistically by two-way and one-way analysis of variance (ANOVA), followed by Dunnett's test. The level of significance was set at  $P < 0.05$ .

## Results

One dam from the 3.0 mg KCN  $\text{kg}^{-1}$   $\text{day}^{-1}$  group was excluded because its kid couldn't suck maternal milk. No clinical signs of toxicity were seen in any goat from any group. However, a dam that received 3.0 mg KCN  $\text{kg}^{-1}$   $\text{day}^{-1}$  died at the 55th day of lactation. There were no differences between the bodyweights of the goats receiving cyanide and the controls. The bodyweights from the dams had no interaction between KCN doses and period of lactation and were not affected by KCN doses, but were significantly affected by the period of lactation. The

comparison between male and female kids showed that males were heavier than females, but this parameter was not affected by the treatment.

The whole blood cyanide and plasma thiocyanate concentrations in the dams and kids are presented in Figs 83.1 and 83.2, respectively. Both thiocyanate and cyanide levels were increased in all experimental mothers. In the kids, thiocyanate levels were increased at all evaluated times with a peak at the 30th day. The concentrations of cyanide in the treated offspring were increased at days 30 and 60, being higher at the 30th day, and undetectable at the 90th day. No control animal presented detectable levels of cyanide in blood.

Plasma levels of T3 and T4 were not affected by cyanide treatment (Table 83.1). Observed histological changes included increased number of reabsorption vacuoles on the colloid of thyroidal follicles and mild hepatocellular vacuolation and degeneration in both dams and kids from experimental groups. No lesions were found in the pancreas from any animal, or in any tissue from controls.

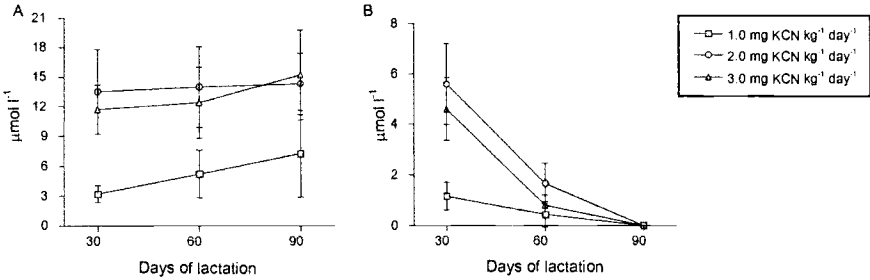


Fig. 83.1. Blood cyanide ( $\mu\text{mol l}^{-1}$ ) levels from dams treated with KCN for the lactation (A) and their kids (B).

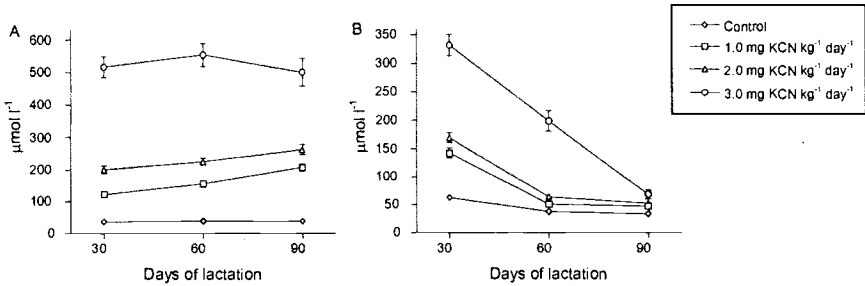


Fig. 83.2. Plasma thiocyanate ( $\mu\text{mol l}^{-1}$ ) concentrations from dams treated with KCN for the lactation (A) and their kids (B).

**Table 83.1.** Plasma triiodo-thyronine (T3, ng dl<sup>-1</sup>) and thyroxine (T4, µg dl<sup>-1</sup>) levels from dams treated with KCN for the lactation and in their kids.

Plasma hormone	Control	KCN (mg kg <sup>-1</sup> day <sup>-1</sup> )		
		1.0	2.0	3.0
<b>Dams</b>				
T3	85.6 ± 3.07	71.4 ± 5.79	105.0 ± 15.1	122.0 ± 17.9
T4	2.93 ± 0.11	2.62 ± 0.29	3.16 ± 0.36	3.54 ± 0.13
<b>Kids</b>				
T3	25.0 ± 14.4	97.1 ± 17.6	94.1 ± 12.3	137.0 ± 9.29
T4	3.00 ± 0.20	2.80 ± 0.14	3.02 ± 0.26	3.40 ± 0.11

## Discussion

Increased blood levels of thiocyanate and cyanide in the sucking kids from the experimental groups in the present study demonstrate that both these substances were transferred from the maternal bloodstream through milk. In fact, it is well known that thiocyanate is a ubiquitous substance in milk, and its concentration is influenced by diet (Garg and Gupta, 1987; Subuh *et al.*, 1995). Furthermore, cyanide was detected in milk from cows (Chikamoto *et al.*, 1983), but it is possible that it could be generated from thiocyanate. The amount of this substance in the blood of the kids suggests the transfer of cyanide occurs at low levels, whereas thiocyanate was transmitted to the offspring at higher levels by this method. The decrease in levels of both cyanide and thiocyanate in the offspring as the number of days of lactation increase is probably due to a reduction in milk consumption concomitant with an increase in consumption of other foods (grass and ration).

Glucosinolates are toxins that produce goitre and are present in plants of the Cruciferae, Capparaceae, Limnathaceae, and Resedaceae families. The hydrolysis of glucosinolates releases thiocyanate, isothiocyanate, organic nitriles, and goitrin (5-vinyl-oxazolidine-2-thione). Thyroid disturbances were found in goats and rabbits fed milk from goats treated with glucosinolate-containing plants (White and Cheeke, 1983). Subuh *et al.* (1995) found increased thiocyanate levels in the milk of cows fed meals with different glucosinolate concentrations; however, isothiocyanate, goitrin and 1-cyano-2-hydroxy-3-butene were not detected. Thus, it is feasible to suppose thiocyanate is responsible for the impaired thyroid function present in milk. Furthermore, thiocyanate, but not cyanide itself, was probably the most responsible for the effects observed in the sucking goats.

Long-term cyanide exposure has been associated with increased number of resorption vacuoles in the thyroidal follicles (Sousa, 2000; Soto-Blanco *et al.*, 2001a) and mild degenerative lesions in liver (Okolie and Osagie, 1999; Sousa,



2000). These findings were also present in both dams and kids in the present work, showing milk from treated dams affected the sucking offspring.

Tropical pancreatic diabetes has been associated with chronic cyanide exposure through consumption of cassava in man (McMillan and Geevarghese, 1979). As verified earlier in male goats (Soto-Blanco *et al.*, 2001b), no significant alterations were found in the morphology of the pancreas from any animal. Results from the present study reinforce our previous supposition that cyanide itself does not induce a diabetogenic effect (Soto-Blanco *et al.*, 2001b).

Previous work performed in our laboratory administrating KCN for a prolonged period to goats revealed degenerative lesions on the central nervous system (Soto-Blanco and Górnjak, unpublished data). However, in the present study no lesion was found in this system from any animal. It can be supposed the excretion of thiocyanate and probably cyanide in the milk could protect the mothers, whereas the levels of these substances were not sufficient to promote lesions in the kids.

Summarizing, goats that suckle from mothers exposed to cyanogenic plants can be affected as thiocyanate and probably cyanide can be transferred from the maternal bloodstream to the offspring through milk. It is feasible to suppose that in this way kids can be indirectly intoxicated by cyanogenic plants.

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## Chapter 84

# The Effect of Feeding Broilers Tall Fescue Grass (*Festuca arundinacea*) Seeds Containing the Endophyte Toxin Ergovaline on Ascites Syndrome Morbidity

A. Shlosberg<sup>1,3</sup>, J.C. Hermes<sup>2</sup>, L. SmithWood<sup>1</sup> and A.M. Craig<sup>1</sup>

<sup>1</sup>College of Veterinary Medicine, Oregon State University, Corvallis OR 97331, USA; <sup>2</sup>Faculty of Animal Science, Oregon State University, Corvallis OR 97331, USA; <sup>3</sup>Present address, Kimron Veterinary Institute, PO Box 12, Bet Dagan, Israel

### Introduction

Tall fescue grass (*Festuca arundinacea*) plants grown in the USA and other countries for cattle feed and for seed production are often contaminated by the growth of an endophyte fungus (*Neotyphodium coenophialum*) that has been shown to produce alkaloids toxic to animals (Oliver, 1997). Billion dollar losses to the cattle industry have been recorded in the USA from this grassland toxicosis (Oliver, 1997). Tall fescue grass straw is a by-product of the Oregon grass-seed industry that has been proposed as a source of bedding for poultry as a less costly alternative to sawdust (Hermes *et al.*, 1999).

The ascites syndrome (AS) is a major cause of losses in modern broilers (Shlosberg *et al.*, 1992) and AS incidence is likely to increase as intensive selection based mainly on faster bodyweight gain continues, giving the grower a bird that is inherently susceptible to factors that may induce hypoxemia and consequently death with ascites (Wideman and Bottje, 1993). The precipitators of AS in a flock are multi-faceted (Julian, 1993), with cold exposure probably being the most important (Shlosberg *et al.*, 1998b). Cold exposure may lead to pulmonary vasoconstriction and increased pulmonary vascular resistance and thence to pulmonary hypertension, which precipitates a chain of physiological, biochemical and pathological changes ultimately leading to manifestations of AS and death by asphyxia (Julian, 1993; Wideman and Bottje, 1993). It is known that ergot alkaloids such as ergovaline (EV) cause vasoconstriction, probably mediated by endothelial damage (Oliver, 1997), and constriction of pulmonary blood vessels may intensify a state of incipient hypoxaemia present in rapidly growing broilers, and so could exacerbate and precipitate the development of AS.

This trial was conducted to investigate whether *N. coenophialum*-infested tall fescue grass seeds with high levels of EV therein may indeed deleteriously affect the development of AS in cold-stressed broilers.

## Materials and Methods

### Broiler husbandry and treatments

This study was approved and conducted according to the guidelines of the Oregon State University Animal Welfare Committee. Mixed sex commercial broiler chicks were wing-banded and grown under normal commercial conditions to produce maximum initial growth; a standard broiler starter mash feed was given *ad lib*. At 20 days of age, 300 chicks were weighed and randomly allocated into six groups of 50 birds, each group comprising two replicates of 25 birds. Each subgroup was housed in a separate floor pen, bedded on sawdust; heating was by infrared lights. Feed was formulated with endophyte-contaminated tall fescue grass seed to produce theoretical total EV concentrations in the mash feed of 0, 500, 1000 and 1500  $\mu\text{g kg}^{-1}$ . The 0 (control) feed was constituted as for the toxic feeds, using tall fescue grass seed with minimal EV contamination. As the maximal level of endophyte found in seeds was about 2500  $\mu\text{g g}^{-1}$ , this necessitated making the seed a large proportion of the feed. Repeated analyses of the theoretical 0, 500, 1000 and 1500  $\mu\text{g kg}^{-1}$  diets showed that they contained 55, 450, 650 and 1325  $\mu\text{g kg}^{-1}$  EV, respectively. Analysis of the feeds showed that the protein levels were 18.8, 18.2, 17.7 and 16.8 for the no EV, low EV, medium EV and high EV groups, respectively; energy levels were 3908, 3927, 3906 and 3883  $\text{kcal kg}^{-1}$ , respectively. Four groups of birds given these four dietary levels (and termed no EV, low EV, medium EV and high EV) were kept under normal (warm) conditions of husbandry, whereas two other groups, no EV and high EV, were cold-exposed. This was performed by ceasing heating at 21 days, and opening ventilation vents to allow exposure to cold air in early winter in Corvallis, Oregon.

### Examinations

Hourly temperatures in the warm and cold pens were recorded using Optic Stowaway temperature recorders (Onset Computer Corporation, Pocasset, MA, USA) placed at the other end of the pens from the heaters. Birds were weighed at Days 20, 28, 35 and 42. Ten birds from each pen weighing between 600–700 g on Day 20 were randomly chosen and were colour marked. On Days 28, 35 and 42, various measurements were made on these 120 birds. Comb temperature (CT) was determined with an electronic infrared thermometer (Vet-Temp, model VT-100, Advanced Monitors Corporation, San Diego, CA) on Days 28, 35 and 42. Body temperature (BT) was measured by inserting the probe of an electronic thermometer into the cloaca of birds until a stable reading was recorded, at Days

28 and 35. Oximetry was performed on Days 28, 35 and 42 using a veterinary oximeter (8600 V pulse oximeter with transreflectance sensor, Nonin Medical Inc., Plymouth, MN) with the sensor placed over the wing cephalic vein; oxygen saturation of haemoglobin in the arterial blood (PaO<sub>2</sub>) and heart rate (HR) were so determined. Any birds dying after Day 21 were necropsied and examined for gross pathological lesions. The trial was terminated on Day 49, when the birds were killed, necropsied and sex was determined. Statistical analysis of all data was carried out using the JMP software (SAS Institute, 1994) using appropriate mixed-effect linear models.

## Results

The mean ambient minimum and maximum temperatures in the cold pens for Weeks 4, 5, 6 and 7, were 5.3 and 18.1, 8.0 and 17.0, 7.9 and 13.0 and 6.9 and 11.4°C, respectively. This was considered a severe cold exposure, as for instance in Week 4, the mean temperature was < 10°C for 8 h day<sup>-1</sup> and < 16°C for 18 h day<sup>-1</sup>. The mean ambient minimum and maximum temperatures in the warm pens for Weeks 4, 5, 6 and 7, were 11.6 and 21.0, 14.1 and 20.4, 13.6 and 18.0 and 10.4 and 17.5°C, respectively; these temperatures are lower than recommended in normal broiler husbandry. There was no significant effect of EV additive or temperature on BW, except for a mild reduction at 42 days in cold-exposed birds (data not shown). The BW of the no-EV group in the warm environment was less than normally recorded in commercial flocks (data not shown). Table 84.1 shows the PaO<sub>2</sub> data. At Day 28 the EV treatment caused a mildly significant elevation in PaO<sub>2</sub>, whereas the cold treatment induced a markedly significant reduction. At Day 35 a mildly significant reduction was found in the cold; at Day 42 no changes were seen. The PaO<sub>2</sub> was very high in all groups at all measurements. There was no significant effect of EV or temperature on HR (data not shown). There was no significant ( $P < 0.05$ ) correlation at any age between PaO<sub>2</sub> and BW, or HR. Correlations were found between PaO<sub>2</sub> on Day 21 and PaO<sub>2</sub> at Day 28 ( $P < 0.05$ ), between PaO<sub>2</sub> on Day 28 and PaO<sub>2</sub> on Day 35 ( $P < 0.01$ ) and Day 42 ( $P < 0.01$ ) and HR on Day 28 ( $P < 0.0001$ ). Correlations were also found between PaO<sub>2</sub> at Day 21 and BT ( $P < 0.0001$ ) and CT ( $P < 0.05$ ) at Day 28, and between PaO<sub>2</sub> at Day 28 and CT at Days 28 and 35 ( $P < 0.05$ ) and BT at Day 28 ( $P < 0.001$ ).

One bird died during the 4 weeks, but no signs of AS were present. Necropsy of the remaining birds at Day 49 revealed none of the pathological lesions associated with AS (Julian, 1993). At Day 42 the high EV group showed a significant ( $P < 0.005$ ) elevation in CT (data not shown). The effect of temperature was not marked, except at Day 42, when a mild elevation ( $P < 0.05$ ) was noted in the cold groups. No significant ( $P < 0.05$ ) correlations between CT and BT, BW, PaO<sub>2</sub>, or HR were found. A correlation was found between CT at Day 42 and CT at Days 21, 28 and 35 ( $P < 0.005$ ). Cold exposure caused a highly significant ( $P < 0.005$ ) elevation in BT (data not shown) on Day 28, with an EV x

**Table 84.1.** Main effects of endophyte additives (ergovaline, EV) on oxygen saturation of arterial blood of broilers in warm and cold environments.

Main effect	Treatment	% Oxygen saturation of arterial blood at age:		
		28 days <sup>a</sup>	35 days <sup>a</sup>	42 days <sup>a</sup>
Additive	None	92.8	96.0	95.9
	Low EV	95.4	96.5	96.5
	Medium EV	97.2 <sup>b</sup>	96.9	96.6
	High EV	95.0	96.2	96.7
Temperature	Warm	95.6	96.6	96.5
	Cold	92.2	95.1	96.2
Source of variation		P(F)		
Additive		0.0358	0.8930	0.2716
Temperature		0.0013	0.0246	0.7357
Additive x temperature		0.0500	0.6290	0.6485

<sup>a</sup>Birds divided equally between warm and cold environments on Day 21.

<sup>b</sup>Value significantly different from no-additive group at  $P < 0.05$ .

temperature interaction, but there was no main effect of EV on either measurement. No significant ( $P < 0.05$ ) correlations were found between sex of the birds and PaO<sub>2</sub> or HR. Expected correlations ( $P < 0.0001$ ) were found between sex of the birds and all BW measurements.

## Discussion

The temperatures measured in the cold-exposed groups in this trial compared with other AS-induction protocols (Scheele *et al.*, 1991; Lubritz and McPherson, 1994) indicated that the birds were exposed to severe cold. It was therefore surprising that no AS was recorded. However, temperatures in the warm pens were considerably below normal recommended levels, so a true comparison between 'warm' and 'cold' is not totally valid. If the cold treatment is compared with the warm treatment, the few significant effects of the cold in the course of the trial were a higher body temperature at Day 28, and a reduction in PaO<sub>2</sub> on Days 28 and 35. A lowered PaO<sub>2</sub> is typical for cold exposure (Shlosberg *et al.*, 1998a), with levels  $< 85\%$  being considered abnormal.

The comb temperature was lower in the higher levels of EV at Day 28, as was expected if EV induces vasoconstriction, but was higher at Day 42. The latter finding would indicate peripheral vasodilation, perhaps induced by some adaptive mechanism to continuous feeding of the EV; if this was the case, it might indicate a similar effect on pulmonary blood vessels. Although there was a highly significant rise in body temperature in cold exposed birds at Day 28, this was not

recorded a week later. The endophyte toxins are said to have a hyperthermic effect in some mammals (Oliver, 1997), but this was shown to be secondary to vasoconstrictive over-heating and thus could not ameliorate the effect of cold exposure as a trigger of ascites development. In this work no effect of EV was seen on BT, although CT was elevated at Day 42 in the high EV group.

The levels of EV in the feed were much lower than those that caused feed aversion or even a mild toxicosis in fowl (Bragg *et al.*, 1970; Bakau and Bryden, 1988; Rotter *et al.*, 1985), so it is not surprising that overt manifestations were not seen. However, despite the severe exposure to cold, it was surprising there were no consistent indications of development of AS (reduced PaO<sub>2</sub>, reduced HR, cyanosis, mortality, pathological findings) (Julian, 1993; Wideman and Bottje, 1993; Olkowski and Classen, 1998; Shlosberg *et al.*, 1998a). This may be due to the reduced BW of the birds compared with commercial flocks, that in itself would preclude development of AS (Shlosberg *et al.*, 1998a). The main easily measured physiological manifestation of developing AS is a hypoxaemia, whereas in this trial, PaO<sub>2</sub> was extremely high on all measurements, and in all groups (Table 84.1). This could also be a manifestation of slower growth, but if these data are compared with a previous work (Shlosberg *et al.*, 1998a), it may be surmised that other factors are expressed. Then, broilers exposed to a similar cold regimen, and weighing 1729 g at Day 42 (compared to 1757 g in this trial) had a mean PaO<sub>2</sub> of only 72% (compared to 96% in this trial). It is possible that the uniquely high PaO<sub>2</sub> recorded in this trial could have been connected with some compound(s) in the fescue seeds that prevented the typical hypoxaemia and the consequent development of the AS. As there was no correlation between PaO<sub>2</sub> and the presence or absence of EV, it seems unlikely that EV has any role in the very high blood oxygenation recorded in all groups. Apart from such ergopeptine alkaloids, infected fescue seed contains many other bioactive compounds (Lorenz, 1979; Porter, 1995; Oliver, 1997). The separate or combined effects of these compounds are largely unknown in ruminant species fed such grasses, and completely unexplored in poultry. The putative effect may be on the diameter of the pulmonary blood vessels or the bronchial tree, or cardiac function, or by a change in oxygen binding to haemoglobin. If this augmented oxygenation of blood can be proven to be valid, in a better-controlled replicate work, and considering the fact that no toxic effects were seen in this trial, then the addition of fescue seed might be a valid prophylactic measure against the development of AS.

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## Chapter 85

# Bovine Enzootic Haematuria on São Miguel Island – Azores

C. Pinto<sup>1</sup>, T. Januário<sup>1</sup>, M. Gerales<sup>1</sup>, J. Machado<sup>2</sup>, D.R. Lauren<sup>3</sup>, B.L. Smith<sup>4</sup> and R.C. Robinson<sup>5</sup>

<sup>1</sup>*Serviço de Desenvolvimento Agrário de São Miguel, 9504-541 Ponta Delgada, Azores, Portugal;* <sup>2</sup>*Universidade dos Açores, Angra do Heroísmo, Portugal;* <sup>3</sup>*Food and Biological Chemistry Group, Hort Research, Ruakura Research Centre, Hamilton, New Zealand;* <sup>4</sup>*Toxinology and Food Safety Research Group, AgResearch, Ruakura Research Centre, Hamilton, New Zealand;* <sup>5</sup>*International Application Technology Group, Aventis CropScience, North Newbald, York, UK*

### Bovine Enzootic Haematuria – Current Situation

#### Nature of the disease

Bovine enzootic haematuria (BEH) is a severe chronic, non-infectious disease caused by prolonged ingestion of bracken fern (*Pteridium* spp.), which is toxic in all its parts. It is characterized by the development of inflammatory and neoplastic lesions of the inner wall of the urinary bladder and clinically by intermittent haematuria and death due to anaemia (Radostits *et al.*, 1994). Cattle over 1 year of age can be affected but the disease occurs most commonly in animals over 4 years old.

Urinary bladder tumours (UBT) are the main lesional feature of BEH. These neoplastic structures are identified during routine post-mortem meat inspection at the sole São Miguel Island slaughterhouse. Since October 1996, all urinary bladders from slaughtered cattle have been opened to look for lesions present on the inner surface of the bladder.

Histopathological examination of bladders from 319 animals with suspected UBT revealed the presence of neoplasia in 89% (284) of the cases. Malignant lesions were identified in 71.8% (204) of all neoplastic cases. Transitional cell carcinoma was the main histological type, present in 49.3% of the cases where UBT were diagnosed (Pinto *et al.*, 1999).

#### The Azores archipelago

The Azores archipelago is composed of nine islands of volcanic origin, with a total land surface area of 2333 km<sup>2</sup>; São Miguel Island (SMI), the largest, is 760 km<sup>2</sup>. The climate is typically oceanic, rainy (annual rainfall is 900–3000 mm)

with mild temperatures ( $17^{\circ}\text{C} \pm 9^{\circ}\text{C}$ ) and only small differences between summer and winter, with no frosts below 600 m altitude. The lowlands (< 200 m) have dry summers with drought periods. More than 50% of the land surface is mountainous. The soils are of the Andasol type, acid (pH 5.5–6.5), with nitrogen, phosphorus and cobalt deficiency (Oliveira, 1989; SREA, 1998).

### Dairying on São Miguel Island

The Azores economy is agriculture based, dairy production being the most important sector. Agriculture employs 17.6% of the total active population (SREA, 1998). About 83% of the usable agricultural area (41,076 ha) on SMI is permanent pasture (34,124 ha), mostly down to perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). There are about 108,000 cattle on SMI of which 55,000 are dairy cows (RGA, 1999), almost all being Holstein-Friesians. There are 2026 herds with an average of 26.4 cows per farm and 19.7 ha of land area per herd.

Farm grazing land is usually fragmented and split-up into several small paddocks or fields. The most common grazing management system for dairy cows is strip grazing controlled by electric fencing. Cattle are moved regularly and frequently between the different areas and are milked in the fields using mobile milking bails. At milking time all production cows are fed concentrated supplements.

### Incidence of the disease and its economic relevance

During 2000, about 28% of SMI dairy herds sent to slaughter contained at least one cow with UBT. There has been a dramatic increase of both UBT cases and UBT affected herds during the last 6 years. It may be noted that the increase in UBT cases has been occurring despite small decreases in the numbers of herds (Table 85.1).

**Table 85.1.** Annual incidence of urinary bladder tumours (UBT) in dairy herds of São Miguel Island.

Year	Dairy cows	Dairy herds	UBT case herds	UBT	% UBT case herds
1995	47,922	2,093	190	232	9.07
1996	49,790	2,102	155	182	7.37
1997	50,108	2,101	130	160	6.18
1998	52,119	2,132	326	418	15.29
1999	53,037	2,089	439	628	21.01
2000	53,476	2,026	573	1,028	28.28

Source: Meat Inspection Service of Ponta Delgada slaughterhouse; SIARA and POSEIMA database for dairy cows.

**Fig. 85.1.** Incidence of urinary bladder tumours in slaughtered cows at the Ponta Delgada abattoir from July 1989 to December 2000.

Slaughterhouse records show that the D Group (all cows after calving and over 2 years old) is most affected by BEH. This group is represented in almost all cases by dairy cows at the end of their productive life. During 2000, 8096 cows were slaughtered at the SMI abattoir of which 14% (1134 carcasses) were rejected due to the presence of UBT. Whole carcass condemnations due to UBT have shown a dramatic increase since 1997 (Fig. 85.1). UBT are the main cause of whole carcass condemnations in cattle at the Ponta Delgada abattoir. This type of neoplasia was present in 1149 (73.5%) of the 1563 cattle carcasses condemned. The proportion of UBT relative to other cattle neoplasias is increasing gradually: In 2000, UBT represented 87.6% of the all observed neoplasias.

Probable reasons for the pronounced increase in numbers of neoplasms are:

1. The regional compensation programme for cows with suspected tumours in 1995 (grant available all that year) and since 1998 to 2000 (grant paid in bimonthly periods only);
2. The systematic opening of all urinary bladders from slaughtered cattle during routine post-mortem meat inspection (since October 1996);
3. Increasing cattle stocking rates without concomitant increase in pasture land;
4. Greater use of marginal land infested with weeds (e.g. *Pteridium aquilinum*, *Lantana camara*, *Phytollaca americana*, *Mentha suaveolens* and *Solanum mauritanus*) for rearing yearlings and heifers.

The cost of BEH to the regional government of the Azores is currently over 120,000 contos per annum (€600,000 approx.) paid in compensation to farmers for the slaughter of cows with suspected tumours. However, there are further

costs to the farmers since many cows die before reaching the slaughterhouse, and there are losses in milk production, costs of weed control and other necessary husbandry procedures. Consequently, BEH is now a significant constraint on the SMI dairy industry.

## Relationship between Bovine Enzootic Haematuria and Bracken Fern

Bracken (*Pteridium* genus) is noted as the principal cause of BEH by different researchers in several regions of the world (Dobereiner *et al.*, 1967; Price and Pamucku, 1968; Bringuier and Jean-Blain, 1987; Villalobos-Salazar *et al.*, 1989; Xu, 1992; Smith, 1997). Recent studies on SMI (Pinto *et al.*, 2000) confirmed the association between consumption of bracken and BEH/UBT in the Azores. Bracken in the Azores archipelago belongs to the *Pteridium aquilinum* species only (Page, 1976; Thompson, 2000), where it is called *feto* or *feito* (Fernandes and Fernandes, 1983).

### Bracken and its implications for animal health

There are five well-defined syndromes associated with the ingestion of bracken by domestic livestock (Smith, 1990):

1. *Thiamine deficiency* induced by a thiaminase type I. This disease occurs in horses and pigs, which eat either the croziers or rhizomes of bracken (Evans, 1976);
2. *Acute bracken poisoning* of ruminants, with gut epithelium necrosis and bone marrow aplasia, usually with fatal consequences (Tokarnia *et al.*, 1967; Evans *et al.*, 1982). The megakaryocytes cease to bud off platelets resulting in a severe thrombocytopenia. Other precursors of granulocytes are also affected, leading to severe leucopenia and consequent increase in susceptibility to infectious diseases. This syndrome was identified in calves grazing on bracken-infested pastures on SMI (Pinto *et al.*, 1998);
3. *Bright blindness of sheep*, by progressive retinal atrophy;
4. *Upper alimentary tract carcinoma*; tumours of the nasopharynx have been reported, as well as tumours of the oesophagus and the fore-stomachs of cattle when grazing areas infested with *Pteridium* spp. in Brazil, Kenya and the UK (Dobereiner *et al.*, 1967; Jarrett *et al.*, 1978). These kinds of tumours have been diagnosed in the Azores although they are not common there (Pinto *et al.*, 1996b). It has been suggested that bovine papilloma virus type four (BPV-4) cooperates with bracken toxins to produce upper alimentary squamous cell carcinoma in cattle (Campo, 1997);
5. *Enzootic haematuria of sheep and cattle* due to the occurrence of multiple tumours in the urinary bladder after prolonged exposure to bracken, both in natural and in experimental conditions (Price and Pamucku, 1968; McCrea and Head, 1981; Bringuier and Jean-Blain, 1987; Campo *et al.*, 1992; Xu, 1992).

## Ptaquiloside

Several metabolites found in bracken constitute a true arsenal of defensive molecules (Cooper-Driver, 1976; Cooper-Driver and Swain, 1976). The most important of these, due to its effects on animals, is ptaquiloside, a water-soluble norsesquiterpenoid glycoside with mutagenic and carcinogenic properties (Van der Hoeven *et al.*, 1983; Hirono *et al.*, 1984; Evans, 1986). Ptaquiloside (PT) is the major carcinogen isolated from bracken. It has been shown to cause cancer in laboratory animals and has revealed mutagenic and clastogenic properties (Van der Hoeven *et al.*, 1983; Smith *et al.*, 1988; Smith, 1990; Smith and Seawright, 1995).

The biological effects of ptaquiloside are similar to those of classical alkylating agents which induce cancer by reaction with DNA (Povey *et al.*, 1994). Under alkaline conditions PT converts to dienone, which, by electrophilic reaction, can form adducts with the DNA bases (especially adenosine in specific base sequences) or to form inactive pterosina B (Smith *et al.*, 1994). Alkylation of DNA is believed to be the first step in the initiation of chemically induced carcinogenesis (Miller and Miller, 1986; Shahin *et al.*, 1999). The location of neoplasia in the upper alimentary tract of cattle, the urinary bladder of herbivores, and the ileum of rodents, may be due to alkaline conditions in these organs (Van der Hoeven, 1983; Smith and Seawright, 1995).

Work in New Zealand shows that the incidence of BEH appears to be greatest in areas where bracken PT levels have been shown to be high, especially in the young growing parts of the plant in late spring and early summer (Smith *et al.*, 1993, 1994). High levels of PT (average = 3084  $\mu\text{g g}^{-1}$ ) have also been found in bracken samples (green and soft parts of the plant) from pastures located in different parts of SMI (Table 85.2). Levels of PT over 500  $\mu\text{g g}^{-1}$  are considered to be significant (Smith *et al.*, 1994). High levels of PT have also been found on farms from SMI where acute bracken poisoning outbreaks were diagnosed (Pinto *et al.*, 1998).

## Geographical distribution of bracken

Bracken is abundant on all the islands in the Azores archipelago (Ward, 1970; Wilmanns and Rasbach, 1973). Bracken is especially common in grazing lands

**Table 85.2.** Ptaquiloside levels in bracken samples collected from different districts of São Miguel Island.

Sample type	No. of samples	Ptaquiloside levels ( $\mu\text{g g}^{-1}$ )		
		Average	Maximum	Minimum
Croziers	3	4924	6526	3795
Croziers and fronds (soft parts)	9	2471	5928	560
Dry fronds	2	24	33	15

on SMI. Climatic conditions, relief, soil and farm management practices create an environment favourable to the luxuriant growth of this weed. Bracken is abundant at the base of hedgerows and stone walls separating fields and paddocks, and wherever the ground is very steep or uncultivated (Wilmanns and Rasbach, 1973). However, higher altitude pastures (> 400 m), exposed, windy, with raised soil moisture (water-logging) and cooler temperatures, do not favour bracken.

### **Factors that favour bracken herbivory**

All parts of bracken are toxic (Tokarnia *et al.*, 1967), including rhizomes and spores (Villalobos-Salazar *et al.*, 1994), when ingested or inhaled by animals. Hunger associated with periods of forage scarcity seems to be a determinant factor in bracken consumption (Hannam, 1986; Lawson, 1989; Pinto *et al.*, 1996a). During the drought periods, which occur in late summer on SMI, bracken is the sole plant that remains green on lowland pastures, especially near the boundary walls and hedgerows. Consequently, during this period bracken consumption tends to be greatest. A similar effect occurs also at other times of year when animals are allowed to graze areas cut for silage.

Grazing animals prefer the soft parts of the plant (Bringuier and Jean-Blain, 1987) that are the portions richest in ptaquiloside (Smith *et al.*, 1993) (Table 85.2). In consequence, the cutting of bracken after grazing, practised by the majority of farmers on SMI, stimulates the growth of more palatable croziers and fronds with higher concentrations of toxins.

Further, the widespread use of fertilizers rich in nitrogen and poor in potassium and phosphorus may be promoting soil acidification, conditions which are detrimental to ryegrass and clovers but favour bracken and its encroachment.

The nature of volcanic soils, poor in phosphorus, copper, selenium and cobalt (Oliveira, 1989), may also cause deficiencies in grazing animals that induce consumption of particular weeds and their associated toxins. It is thought that the same deficiencies may even exacerbate the effects of certain toxins.

### **Geographical Distribution of Urinary Bladder Tumours**

The geographical distribution by district and by herd of 2648 UBT identified at the Ponta Delgada slaughterhouse between Jan. 1995 and Dec. 2000, led to the conclusion that all but one district (Ribeira Quente where no herds existed) had UBT cases. This survey also made it possible to identify the areas most affected, as well as their evolution during the last 6 years. It is evident that there has been a dramatic increase in UBT cases during the last few years. Currently, the districts located on the north and south slopes of the central volcanic cone of Lagoa do Fogo have more than 60% of the herds with UBT cases.

### **Relationship with the altitude and relief**

Districts located at low altitude (< 200m) and with more irregular relief seem to have higher numbers of herds with UBT cases. In these regions bracken is present all year round due to higher temperatures, less exposure and less water logging compared with higher altitude pastures. By favouring drainage and making ploughing more difficult, steeper slopes create favourable conditions for bracken and other weeds. It has also been noted that districts with pastures located on the margins of small rivers seem to have more UBT cases, possibly due to the steeper slopes and uncultivated land at these locations.

### **Relationship with dairy cow numbers**

A comparison between the map of dairy cow density and UBT distribution seems to indicate a greater number of UBT affected herds in areas with high stock densities (RGA, 1999). This relationship is most evident on the western half of the island.

## **Preventive Measures against Bovine Enzootic Haematuria**

### **Immediate preventive measures**

Until comprehensive, long-term measures are in place to control the incidence of BEH, the local animal health authorities have been advised to issue general stock-keeping guidelines to farmers to keep dairy cattle from eating bracken (Robinson, 2000a):

- Better use of electric fencing to keep the cows from reaching any bracken fronds;
- Cutting of bracken and removing it from the field before an area is grazed (not afterwards);
- Ensuring that bracken is not accidentally included in any silage, hay or other feed supplement;
- Not putting cattle to graze fields where silage or hay has been cut and where bracken is still growing;
- Not using mobile milking stalls where bracken is present nearby;
- Not putting cows to graze fields where bracken is growing during the dry summer months.

### **Longer-term preventive measures (husbandry and agronomy)**

Certain longer-term stock husbandry measures are also expected to reduce levels of BEH:

- Reduction in grazing pressure;
- Use of more balanced fertilizers (N, P, K, not just N);
- Regular liming to improve forage quality;
- Tree-planting on land located on steep slopes and/or with poor soil that is difficult to manage as pasture;
- Use of the herbicide 'Asulox' (Bayer CropScience UK Ltd, Hauxton, Cambridge CB2 5HU) to eradicate bracken from affected pastures.

'Asulox', a selective, systemic herbicide (40% asulam), is used to control bracken where a degree of selectivity is required to protect other desirable vegetation that may be associated with the bracken ('Asulox' may also be used to control *Rumex* spp. in pastures). Considerable experience has been gained with asulam in other countries to achieve permanent bracken clearance, which traditional cutting methods fail to achieve (Robinson, 1999, 2000b). Most bracken in the Azores can be treated by ground-based methods (not aerial) and where the bulk of applications will comprise spot-treatments using a minimum of chemical. Because stock-farmers on SMI are not routinely familiar with application of pesticides, provision must be made to advise farmers on the correct use of the chemical and equipment. In some cases, it may be more effective for specialist contractors to be appointed for such work. Bracken clearance is never an instant process while the management of associated vegetation recovery also needs to be considered over the long term. These matters mean that a fund of expert advice on bracken control needs to be established locally in the Azores.

#### **Practical measures to be developed by the local animal health authorities and Agronomy Department**

- Farmer advisory meetings to impart information on stock husbandry and bracken control methods to eliminate BEH from the dairy herds;
- Distribution of leaflets with advice to farmers on BEH control measures;
- Re-registration of 'Asulox' in the Azores for control of bracken and *Rumex* spp;
- On-farm applications of 'Asulox' to demonstrate to stock-farmers its efficacy, safety and correct use;
- Distribution arrangements to supply farmers in the Azores with suitable herbicide application equipment for bracken control;
- The appointment of one or more contractors to carry out herbicide treatments of bracken on São Miguel;
- The appointment of a specialist to coordinate bracken control in the Azores and the practical methods required.

#### **Future Perspectives in the Veterinary Field**

Recent studies indicate a relationship between BEH and bovine papilloma virus (BPV) infections in experimental and natural conditions (Campo *et al.*, 1992;



Campo, 1997; Stocco dos Santos, 1998). In addition to current studies, further research will be initiated on SMI in the near future to:

1. Investigate the role of BPV infections in BEH cases seen in the field;
2. Identify and characterize the early pathological effects of grazing in cattle at risk to BEH by using haematological procedures;
3. Identify and study the UBT oncogenes of pathological relevance;
4. Establish a model for investigating ptaquiloside carcinogenesis in cattle.

It may also prove important to investigate other detrimental health factors such as bovine viral diarrhoea, copper, selenium and cobalt deficiencies and their relationship with the development of BEH.

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## Chapter 86

# Toxicophenology and Grazing Risk Models of Tall Larkspur

M.H. Ralphs, D.R. Gardner and J.A. Pfister

USDA/ARS Poisonous Plant Lab, 1150 E. 1400 N., Logan UT 84341, USA

### Introduction

Tall larkspurs (*Delphinium barbeyi*, *D. occidentale*, *D. glaucum*) are the most important poisonous plant problem on mountain rangelands in the western USA. They are palatable to livestock and acutely toxic to cattle. Cattle deaths average 2 to 5% on mountain rangelands, but may exceed 15% in areas where larkspurs are abundant (Pfister *et al.*, 1999). In addition to cattle deaths, significant amounts of forage are wasted and management costs increase as producers defer or avoid grazing larkspur-infested areas. Total cost to the livestock industry exceeds \$20 million annually.

Larkspur plants contain many norditerpenoid alkaloids. Alkaloids containing the N-(methylsuccinimido)-anthranilic ester group (MSAL) are most toxic, with methyllycaconitine (MLA) being the most prominent (Manners *et al.*, 1995) (Fig. 86.1). MLA is a potent neuromuscular blocker, causing paralysis and rapid death from respiratory failure (Dobelis *et al.*, 1999).

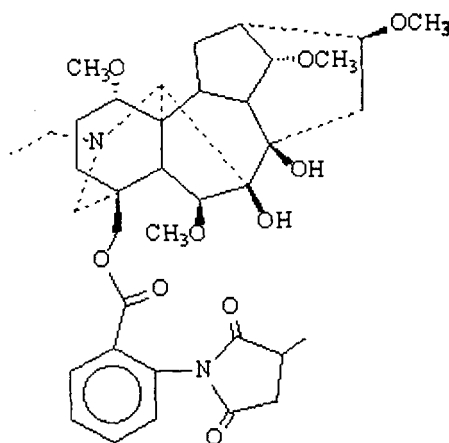


Fig. 86.1. Methyllycaconitine (MLA), the principal toxic alkaloid in larkspur.

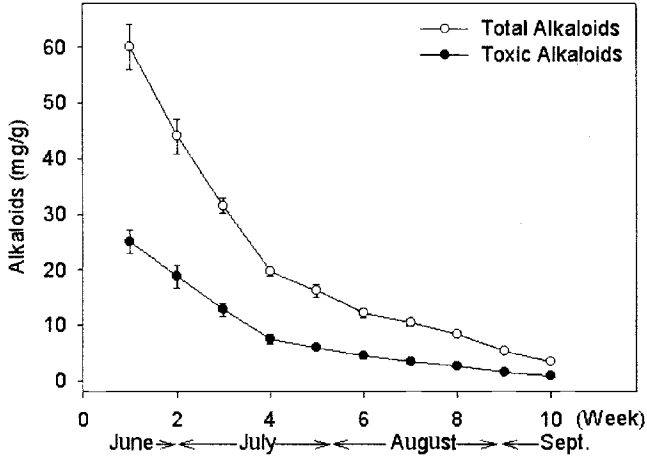


Fig. 86.2. Total and toxic alkaloid concentration in tall larkspur over the growing season.

## Chemical Phenology

Early research (Marsh and Clawson, 1916) reported that tall larkspur was most toxic in its early growth and toxicity declined as it matured. Williams and Cronin (1966) measured total alkaloids using colorimetric techniques in tall and Duncceap larkspur and reported a nearly linear decline in total alkaloid concentration as the season progressed. Olsen (1983) confirmed that toxicity followed these trends. Manners *et al.* (1993) reported the concentration of the dominant toxic alkaloid (MLA) declined in plant parts as the plant matured. Gardner *et al.* (1997) developed a Fourier-transformed infrared spectroscopy (FTIR) method to quantify MSAL and total alkaloids. Concentration of toxic and total alkaloids decline in a concave curvilinear manner as the plants mature through the phenological growth stages over the growing season (Fig. 86.2) (Pfister *et al.*, 1994; Ralphs *et al.*, 1997).

## Environmental Stress

Environmental stresses do not alter toxic alkaloid levels. Short-term shade and photosynthesis inhibition decreased the dry weight of the plants, thus increasing the concentration of alkaloids in the plant, but the total amount of alkaloids remained constant (Ralphs *et al.*, 1998a). Varying both light and temperature in a

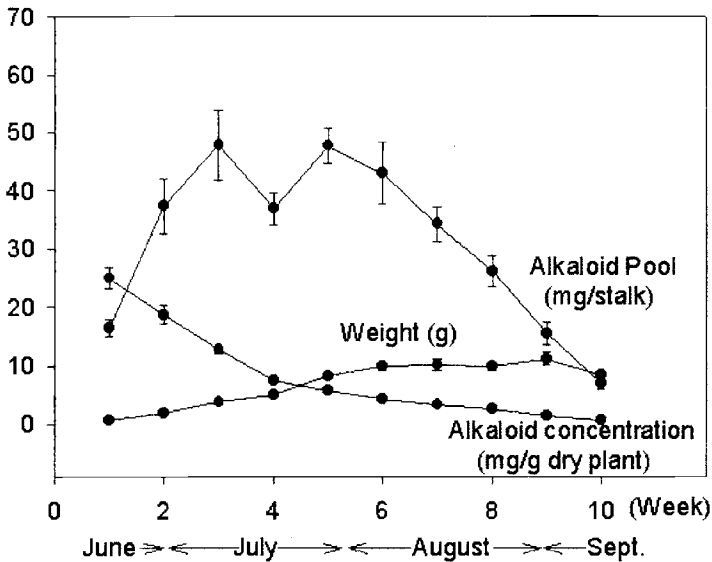


Fig. 86.3. Alkaloid pools in larkspur stalks, calculated from the alkaloid concentration ( $\text{mg g}^{-1}$  dry plant) multiplied by the mass or weight (g) of the stalk.

climate box study did not significantly affect alkaloid concentrations (Ralphs, unpublished data). The herbicide glyphosate (Roundup) rapidly desiccates larkspur plants, thus increasing alkaloid concentration as the plant desiccated, but the amount of alkaloids in the stalks was similar to untreated controls (Ralphs *et al.*, 1998b). Treatment with metsulfuron (Escort) increased both concentration and absolute amount of alkaloids in tall larkspur. This has been the only treatment that has substantially increased the amount of alkaloids in larkspur. Contrary to the plant defence theory, insect damage from the larkspur mirid (*Hoplomachus affiguratus*), which is host specific to tall larkspur, reduced toxic alkaloid concentration (Ralphs *et al.*, 1998c). Also clipping tall larkspur greatly reduced both vigour and alkaloid pools the following year (Ralphs and Gardner, 2000a).

## Alkaloid Pools

A functional explanation of alkaloid levels in tall larkspur was developed by measuring alkaloid pools in larkspur plant parts (Ralphs *et al.*, 2000b). Evidence suggests that alkaloid synthesis occurs during the first 3–4 weeks of growth in the spring. Alkaloid pools in the above ground stalks increased for 3 weeks, levelled off for 4–5 weeks, then declined precipitously following flowering (Fig. 86.3). The linear decline in concentration of alkaloids described in earlier research can

be explained by the dilution of a fixed amount of alkaloid within the increasing biomass as the plants grow in size and mass.

The alkaloid pools declined rapidly following flowering even though the biomass of the plants remains constant until senescence several weeks later. Alkaloids are either being translocated or catabolized (Ralphs and Gardner, unpublished data). Translocation of toxic alkaloids to the floral sinks (6.8 mg per flower and pods, 4.2 mg per seed) was much less than the decline from leaves and stems (54.1 mg per stalk). The slight increase in alkaloid concentration in the crown is unlikely to account from the remainder of translocated alkaloids. Therefore, we speculate that the alkaloids are being catabolized.

### Predicting Toxic Alkaloid Levels

The characteristic decline in alkaloid concentration in tall larkspur allowed us to predict toxic alkaloid concentration throughout the growing season. Various plant (height and weight) and weather (days since snow melt, precipitation, temperature, and growing degree days, which integrates time and temperature suitable for growing since the plants emerged from under snow) parameters were all considered in a multiple regression predictive equation using Mallows Cp model selection procedure. This procedure selects for the smallest total squared error. It selected plant height and growing degree days (gdd) since snow melt as the best predictors for toxic alkaloid concentration in the following equation.

$$y = 30.71 - 0.35ht + 0.00165 ht^2 - 0.0153 gdd \quad (R^2 = 0.73)$$

where  $y$  = concentration of toxic alkaloids mg g<sup>-1</sup> plant dry wt.  
 $ht$  = height of the plant in cm, and the height term squared.  
 $gdd$  = growing degree days since snow melt.

The equation was developed from a data set encompassing two locations, 4 years, and periodic sampling over the growing season. The equation was verified internally on the original data set and on an external data set of four new populations.

### Predicting Risk of Poisoning

Knowledge of the characteristic decline in alkaloid concentration and grazing behaviour of cattle led to conceptual models for predicting risk of poisoning (Pfister *et al.*, 1997a). Alkaloid concentration is high as the plants begin growth and decline as the plants mature. Livestock will not consume larkspur in its early growth, but begin eating it as it begins to flower, and will consume large amounts

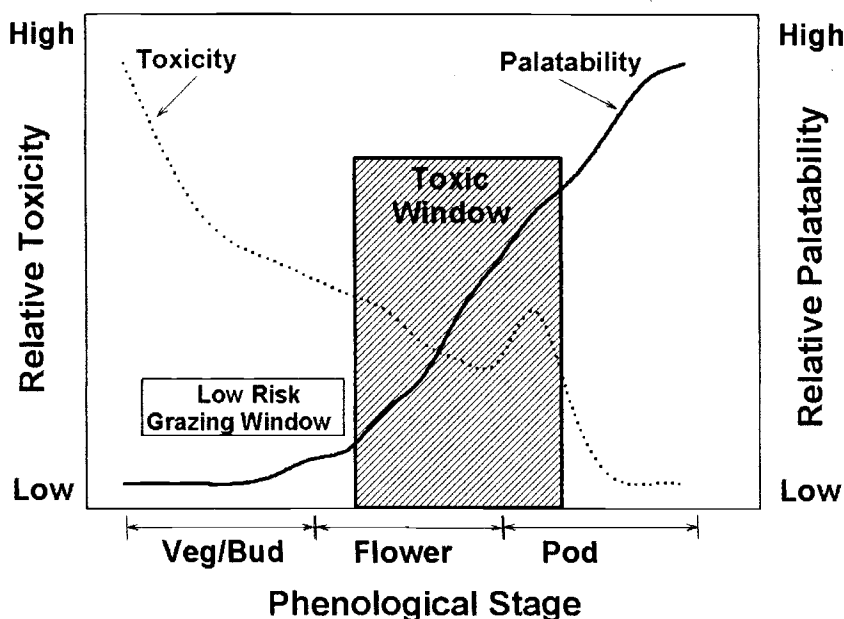


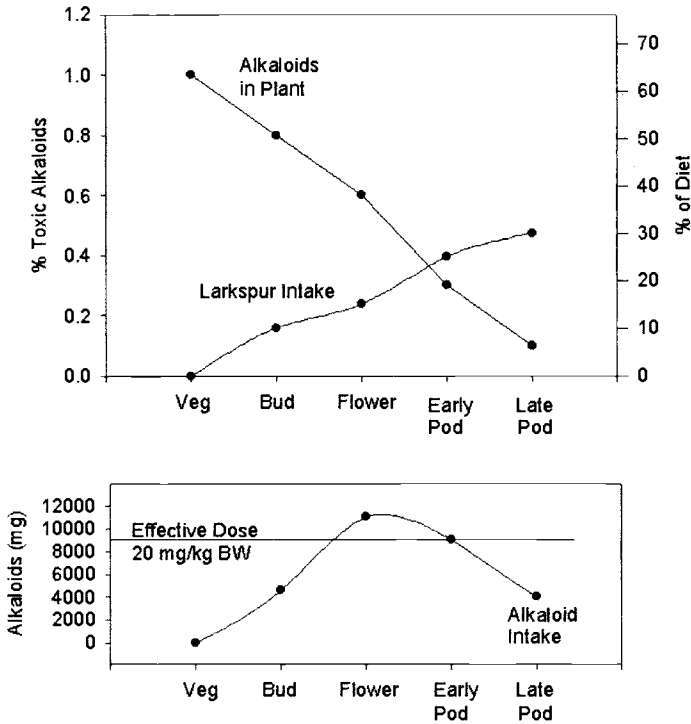
Fig. 86.4. Toxic window of poisoning based on increasing palatability of larkspur to the animal and decreasing alkaloid concentration in the plant.

during the pod stage when alkaloid levels are below the toxic threshold. Combining grazing behaviour and alkaloid levels defines a critical period of risk from flowering to early pod stage when alkaloid concentration is sufficiently high and cows consume enough larkspur to receive a lethal dose (Fig. 86.4).

Another model compared the predicted intake of toxic alkaloids (average consumption patterns over the grazing season, and toxic alkaloid concentration in the plants) with the threshold for intoxication (Fig. 86.5). The threshold is based on an effective dose of MLA ( $21 \text{ mg kg}^{-1} \text{ BW}$ ) that will cause collapse, but not death (Pfister *et al.*, 1997b). The short period during flowering and early pod poses the greatest risk of poisoning.

From these models, the management recommendation was to graze cattle in larkspur areas early in the growing season when larkspur is unpalatable, remove them during the flower to early pod stage of growth when the risk of poisoning is highest, then allow them to graze larkspur following pod drop when the alkaloid levels are not high enough to poison them (Pfister *et al.*, 1997a).





**Fig. 86.5.** Risk of poisoning expressed as the intake of toxic alkaloids exceeds the effective dose. The intake of alkaloids is calculated from increasing levels of larkspur consumption by the cow but declining alkaloid concentration in the plant.

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