NITROGEN FIXATION IN TROPICAL CROPPING SYSTEMS
2nd Edition

For Irene, Onno and Tessa
And many more adventures to come!

Nitrogen Fixation in Tropical Cropping Systems 2nd Edition

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Preface to Second Edition

When approached to write a second edition of this book, Kate Wilson and I readily agreed that developments in the field of N_2-fixation in tropical agriculture warranted a complete revision. We initially undertook this as a joint venture and it was a great disappointment when Kate realized that commitments to motherhood and to her new field of marine biology would preclude her co-authoring this edition. I only hope that the absence of her keen and critical mind and flowing style is not too apparent!

In the last decade we have witnessed a number of exciting developments in the field of N_2-fixation: a completely novel mechanism for N_2-fixation; refinements in our understanding of nod factors; the burgeoning of new rhizobial species – to name but a few. In aspects more directly related to N_2-fixation in agriculture, the emphasis on inoculation and the preoccupation with measuring N_2-fixation which was apparent in the 1980s has matured into a much fuller understanding of how N_2-fixation fits within the N cycle in production systems. Yet despite these significant developments in our understanding of N_2-fixation at all scales it is still fairly difficult to find clear examples where this knowledge has been made full use of in practice. Although governments must take considerable responsibility for this, there is still a strong tendency among those of us directly involved in research for agricultural development to underestimate the problems which smallholder farmers face in deciding to alter their methods of crop production; decisions which have a major impact on the immediate and future well-being of their families.

I have maintained the format of the first edition, that is of three sections which describe in turn: the organisms and process of N_2-fixation; the ways in which N_2-fixation can contribute to the wide variety of tropical production systems; and how inputs from N_2-fixation have been harnessed in tropical agriculture and may be increased in future. Two new chapters replace an earlier chapter in the first edition on

'Legumes in Multiple Cropping', namely Chapter 5 which discusses the common principles by which N can be contributed from N_2-fixation in different types of cropping systems, and Chapter 9 which is devoted to green manure legumes in annual crop production. A further major change is an increased emphasis in the final section on the problems of developing appealing and appropriate technologies that are likely to gain widespread adoption among smallholder farmers in the tropics. As with the first edition my aim is to provide a critical analysis that may assist in focusing future research in this field.

A primary target audience for whom the book is written is students and researchers in developing countries who do not have ready access to the abundant literature on N_2-fixation. I hope that this book will also be read by scientists working in research on N_2-fixation and related topics in the developed world, and that it may serve to dispel some of the naivety often expressed that manipulation of a particular gene or process in the laboratory is likely to lead spontaneously to changes in agricultural productivity in the immediate future. Writing at a time of political and economic turmoil in Zimbabwe has given me even greater sympathy with those who continue to work with commitment to agricultural development in the tropics under difficult conditions. It has been salutary to witness how rapidly circumstances may change and undermine groundwork that has taken years to develop.

As one who has repeatedly witnessed the phenomenal productivity of legumes wholly dependent on N_2-fixation in depleted soils of the tropics, I remain confident that N_2-fixation has a major role to play in future agricultural development. If this book can assist in a small way in spurring success in utilization of N_2-fixation to improve the livelihoods of smallholder farmers in the tropics, then the effort involved in its preparation will have been worthwhile!

K.E. Giller

Preface to First Edition

In the world of biological N_2-fixation research we have been witness to some rapid and fascinating developments, particularly in the understanding of symbiotic relationships, which have relevance to many areas of biology. For instance the report that a single small molecule at very low concentration can elicit the development of a fully differentiated nodule on a legume would a few years ago have seemed impossible. But what lessons have we learnt for use in agriculture? When first approached with the general enquiry 'What gaps do you see in your field for academic texts?' we realized that there was no detailed synthesis of the research on N_2-fixation relevant to agriculture in the topics. In discussing research with scientists in the tropics it was clear that their lack of access to the literature, as well as to the informal network of researchers who often share results and information long before publication, hampers their research. It was also apparent that scientists in other disciplines, whether agroforesters, breeders or agronomists with an interest in N_2-fixation – or even people researching the more fundamental aspects of the molecular biology or chemistry of N_2-fixation – needed access to a synthesis of the knowledge already available if the benefits of N_2-fixation in agriculture are to be realized.

We therefore set out to fill this gap, and to do so in a way that would be accessible to as wide an audience as possible. Our own collaboration was very helpful in this regard, as one of us (Ken Giller) has experience in tropical agriculture, while the other (Kate Wilson) has specialized in research into the genetics and molecular biology of tropical rhizobial strains. In working together in the laboratory and in writing this book, we were often confronted with the problem of a concept or fact being self-evident to one of us, and completely obscure to the other. Discussions of this nature have helped to make the book clearer and also more critical in content.

We hope this book is timely in many ways. On the one hand, the scientific literature is super-saturated with books and articles about biological N_2-fixation and

there has been a definite wane in funding of this research, at least in many developed countries. On the other hand, the field is becoming ever more fascinating as we begin to really understand the underlying biology and chemistry of many processes in biological N_2-fixation, and it will perhaps be one of the first subjects in which it will truly be possible for scientists from very different disciplines to work together.

Another reason why this book is timely is the changing emphasis in agricultural research and practice around the world. In both developing and developed countries it is increasingly being realized that we must strive for a sustainable agriculture, one which can feed the population of the world but not at the environmental cost exacted by present-day intensive farming practices. A sustainable agriculture clearly requires that all nutrients removed in the crops or lost from the cropping system must be replenished and in the long-term there is no option but to restore the supplies of phosphorus and potassium by using fertilizers. But this is not the case for nitrogen, one of the nutrients required in the largest quantities for plant growth, and one which is commonly limiting for agricultural production. Nitrogen can be directly captured – 'fixed' – from the atmosphere.

Biologically fixed nitrogen can contribute directly to the needs of a growing crop or can be added to the soil so contributing to its fertility. For long term sustainability we can make a choice between reliance on the use of inorganic nitrogen fertilizers or a greater reliance on the biological fixation of nitrogen in tropical agriculture. Technologies based on the use of nitrogen-fixing plants are more likely to be accessible for use by farmers in the tropics who in many regions do not have the financial resources to take advantage of fertilizers even when they are available. In the long term therefore it seems clear that biological N_2-fixation, with its ability to capture an inexhaustible nutrient resource, must play a fundamental role in agriculture.

Our aim in this book has been to look at the actual and potential role of biological N_2-fixation in tropical agriculture. We begin by describing the process itself and the organisms which are capable of fixing N_2 in cropping systems of the tropics. Subsequently we consider the cropping systems in terms of the main crop components and examine how N_2-fixation can contribute to agricultural production. The interactions between crops in mixtures, in rotation and in agroforestry are also considered. In the concluding section we assess how biological N_2-fixation technology and research has been used to improve the inputs from N_2-fixation for agriculture, and examine possible directions for the future.

We both agreed from the earliest stages that we preferred to be provocative in places, and if we stimulate disagreement and discussion with any viewpoints presented in the book, we shall be pleased – and look forward to hearing about them. Overall we hope that we present a cautiously optimistic view of the role of biological N_2-fixation in the diversity of tropical cropping systems and of the possibilities for the future.

K.E. Giller
K.J. Wilson

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Abbreviations

ACIAR	Australian Centre for International Agricultural Research
CGIAR	Consultative Group for International Agricultural Research
CIAT	Centro Internacional de Agricultura Tropical
FAO	Food and Agriculture Organisation
GTZ	Deutsche Gesellschaft für Technische Zusammenarbeit
IAEA	International Atomic Energy Agency
ICARDA	International Centre for Agricultural Research in Dry Areas
ICRAF	International Centre for Research in Agroforestry
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IDRC	International Development Research Centre
IIED	International Institute for Environment and Development
IITA	International Institute for Tropical Agriculture
ILRI	International Livestock Research Institute
IRRI	International Rice Research Institute
IT	Intermediate Technology
SADCC	Southern African Development Coordination Committee
TSBF	Tropical Soil Biology and Fertility Programme
UNDP	United Nations Development Programme
USAID	United States Agency for International Development
USDA	United States Department of Agriculture

Part I

Introduction

Chapter 1

Tropical Environments: Climates, Soils and Cropping Systems

The tropics have the potential to be the most productive cropping environments in the world. Plants need heat, light and moisture to grow and all of these are available in abundance in the tropics. Where rainfall is sufficient, crops can be grown year-round, rather than only in the warm seasons as in temperate regions. And yet, despite these natural advantages, yields in tropical cropping systems are often pitifully small. The unpredictability of the climate – in particular the timing of the rains – and the lack of nutrients for plant growth in many soils, combine to limit crop production in the tropics. Whilst we can do little to modify the climate we can use various approaches to solve the problems of soil fertility. The most obvious solution is to import nutrients in the form of mineral fertilizers, but for a variety of social, economic and political reasons this is generally difficult, especially in Africa. The alternative is to increase the biological inputs of nutrients and it is here that biological fixation of atmospheric nitrogen (N_2) has a crucial role to play in increasing the sustainability of yields with minimal external inputs. The actual and possible contribution of biological N_2-fixation in tropical cropping systems is the subject of this book.

The tropics are precisely defined as the region between the Tropic of Cancer (23.5°N) and the Tropic of Capricorn (23.5°S). However, in this book we will follow the lead of many other writers and use the term 'tropics' loosely to encompass the true tropics and also the subtropics, namely latitudes between 30° north or south of the equator.

So what are the characteristics of tropical environments? At the coast on the equator the mean temperature varies from 26 to 27°C by only 2–3°C between months and diurnal variation is no more than 6–10°C. And yet, close to the equator we can find the snow-capped peaks of Kilimanjaro in northern Tanzania, or the

Ruwenzori Mountains on the border between Uganda and Zaire. The seasonality of climates and the opportunities for crop production depend largely on the rainfall and the tropics encompass climates ranging from arid deserts to those with the highest rainfall in the world. Tropical soils are notoriously highly leached and acid but, as we shall see, some tropical soils are highly fertile. The environment in which crops are produced is determined by the climate, the soil and local modifications of these resulting from the cropping system, in which the crop itself plays an important role. We shall consider each of these in turn.

Tropical Climates

The major factors that give rise to the diversity of climates in the tropics are the topography, the rainfall and the winds. Total incident radiation varies seasonally with latitude, but never by more than 15% within the tropics. Thick cloud cover can reduce penetration of radiation to a much greater extent and therefore the potential for production is much higher in the dry seasons without cloud as long as sufficient irrigation water is available. However, daylength can vary with latitude in the tropics by up to 1.5 h from the constant 12 h found on the equator and this can have an effect. Some plants in the tropics are so sensitive to small changes in daylength that flowering is only triggered at certain times of the year.

Topography rather than latitude is the major factor determining temperature. The temperature falls by 0.65°C with every 100 m increase in altitude, and thus temperatures in the highlands are substantially below those near sea level. Topography can also influence cloud cover, and thereby both temperature, through its effect on penetration of radiation, and rainfall. Temperature regulates the rate of plant growth and so is a primary determinant of the time crops take to reach maturity in different zones. However, it is rainfall which is usually the most important factor in determining the potential productivity and the major climatic zones in the tropics are distinguished primarily by the amount and distribution of the rainfall throughout the year. Here we will follow the classification of climates given by Norman *et al.* (1995).

The major climatic zones

The wet tropics

The 'humid' or 'wet' tropics have a mean monthly air temperature greater than 18°C and rainfall above 1800 mm $year^{-1}$. For at least 10 months of the year rainfall exceeds evaporation from the soil and vegetation and so crops can be grown virtually the whole year round. This zone includes the majority of the great river basins of the Amazon and the Congo and much of lowland Southeast Asia.

The wet and dry tropics

The 'wet and dry' regions also have mean monthly air temperatures above 18°C but have strongly seasonal rainfall patterns with a dry period of at least 2 months

when crops cannot be grown without irrigation. The rainfall can have a bimodal distribution with two main rainy seasons or there may be a single rainy season with total rainfall between 300 and 1800 mm year^{-1}. This large category includes what are often referred to as the 'sub-humid' and the 'semiarid' tropics and covers most parts of Asia dominated by monsoon season(s) and the large savannah areas of South America and Africa. In West Africa there is a marked gradation of climates, with rainfall decreasing further inland from the humid, tropical coast to the semiarid climate of the Sahel. In East Africa rainfall is bimodal, with a short and a long rainy season; in southern Africa there is only a single rainy season. The length of the cropping season is determined not simply by the rainfall. It also depends critically on the capacity of the soil to retain moisture and on the additional water that can be collected by runoff from the surrounding land. Thus in some areas in this category cropping is possible throughout the year but in others crop growth can only be sustained for less than 3 months. The great problem of agricultural production in such areas is the unpredictability of both the onset of the rainy season and the distribution of rainfall during crop growth. Where the dry season is long, the farming system must allow for provision of food for both humans and animals during the period when crops cannot be grown.

The dry tropics

The 'dry' tropics are regions with rainfall of less than 300 mm year^{-1} in which crop production is possible only with irrigation. Such areas include most of tropical Africa north of 15°N and Australia south of 15°S. In the absence of irrigation, the only agricultural production feasible in these regions is extensive grazing.

The cool tropics

The final category, that of the 'cool' tropics, encompasses areas where the mean monthly temperature falls below 18°C but stays above −3°C. It is made up of regions at higher altitudes and these have a marked variability in rainfall. Crops are produced at altitudes up to 3000 m above sea level on the equator in the Andes, although we would more readily associate many of the crop species grown there with temperate climates.

Tropical Soils

Soils of the tropics are extremely diverse. The single common characteristic of all tropical soils is the constancy of soil temperatures throughout the year. The widely held misconception that all tropical soils are highly leached and infertile originated from the early writings of rather ill-travelled scientists from northern, temperate regions (Sanchez, 1976). The term 'tropical soil' became synonymous with 'lateritic soil', used for those soils with layers rich in iron oxides that harden irreversibly on exposure to air. In reality, soils of the tropics vary from young volcanic or alluvial soils to some of the oldest, most highly weathered and leached soils in the world. Two classification systems that divide soils into groups on the basis of their physical and

chemical structure are now widely used: the World Reference Base for Soil Resources (ISSS/ISRIC/FAO, 1998), which replaces the FAO/UNESCO Legend (FAO/UNESCO, 1974); and the USDA Soil Taxonomy (Soil Survey Staff, 1999). The major groups of each classification, and how the two can be related to each other, are summarized in Table 1.1. The USDA Soil Taxonomy will be used here in further discussion.

Not all of the groups of soils can be readily interrelated between the two systems as some of the criteria used to separate groups differ, and many generalizations are made at this broad 'order' scale of description. Both systems further subdivide the major orders of soils into many subgroupings, which give somewhat more detailed information as to their moisture, temperature and nutrient status. A detailed discussion of these subdivisions is beyond the scope of this book.

An analysis of the relative frequency of different soil groupings indicates that highly weathered, leached soils (Oxisols, Ultisols and the less-leached Alfisols) cover more than half of the land area of the tropics (von Uexküll and Mutert, 1995). Desert soils (Aridisols) occupy some 16% of the tropics, leaving only 30% of the land area covered by younger soil formations (Table 1.2). Soil classification is not necessarily a good guide to soil fertility, as classification depends more on the characteristics of the subsoil, whilst the ability of soils to support crop growth, at least in the short term, is dependent on the surface soil horizons. However, classifications do provide a useful framework within which to discuss the distribution and uses of soils and generalizations can be made concerning the advantages and problems for agriculture in the different soil types.

Table 1.1. The major soil orders of the USDA Soil Taxonomy and their approximate equivalent in the World Reference Base for Soil Resources. (Updated from Norman *et al.*, 1995.)

USDA Soil Taxonomy	World Reference Base[a]
Oxisols	*Ferralsols*, Gleysols
Ultisols	*Acrisols*, Nitisols
Entisols	*Fluvisols*, *Plinthosols*, *Durosols*, *Regosols*, Arenosols, Gleysols
Alfisols	*Luvisols*, *Alisols*, Planosols, Albeluvisols, Solonetz
Inceptisols	*Cambisols*, *Gleysols*
Vertisols	*Vertisols*
Aridisols	*Yermosols*, *Xerosols*, *Cambisols*, Solonetz, Solonchaks, Gleysols, Rendzinas, Albeluvisols, Phaeozems, Nitisols
Mollisols	*Chernozems*, *Phaeozems*, *Kastanozems*, *Umbrisols*, *Rendzinas*
Andisols	*Andosols*
Histosols	*Histosols*
Spodosols	*Podzols*

[a]Italics indicate the predominant corresponding group.

Soils are formed by the chemical and physical weathering (or breakdown) of parent materials and the high temperatures and rainfall of many parts of the tropics ensure that weathering can be very rapid. High rates of leaching (that is, the removal of nutrients in water percolating through the soil) go hand-in-hand with rapid weathering. This, coupled with the fact that large areas of soils are developed from rocks such as granites which contain small amounts of weatherable bases, means that many inherently infertile soils occur in the tropics. The rapid weathering of exposed rocks also means that some deposits of volcanic origin, which are often young in geological terms, can also be highly leached. Likewise, alluvial soils will not necessarily be fertile if they are formed by deposition of particles originating from erosion of old, weathered surfaces. When considering the processes that brought about the formation of a particular type of soil it is important to remember that the climate prevalent at the time when the soil was formed may have differed markedly from the present climate.

Younger, more fertile soil formations are characterized by the presence of unweathered minerals, in which the fertility of the soil is maintained by the release of nutrients by weathering. The more fertile soils therefore tend to occur in areas where there has been relatively recent (in geological terms) addition of volcanic ash or alluvium containing weatherable minerals, or in climates where a long dry season slows down the rate of weathering and leaching. In areas subject to tectonic activity, repeated landslides can restrict soil development so that the soils remain shallow and unweathered minerals remain within the reach of plant roots.

Table 1.2. Area and distribution of soils in the tropics. (Based on Sanchez and Salinas, 1981; Norman *et al.*, 1995 with modifications from Soil Survey Staff, 1999.)

Major soil associations	Tropical America (Mha)	Tropical Africa (Mha)	Tropical Asia (Mha)	Tropical Australia (Mha)	Total (Mha)	% of the tropics
Oxisols	452	495	14	0	961	25
Ultisols	325	137	291	8	761	20
Entisols	126	305	76	95	602	16
Alfisols	96	289	65	29	478	12
Inceptisols	232	178	193	3	606	16
Vertisols	18	42	60	28	149	4
Aridisols	7	166	5	8	186	5
Mollisols	32	0	4	0	36	1
Andisols	32	1	12	0	45	1
Histosols	4	4	24	0	32	1
Spodosols	3	1	2	0	6	< 1
Total	1328	1618	745	171	3862	100

Soils with low activity clays

Highly weathered soils, which used to be called latosols, are now classified as the Oxisols, Ultisols and Alfisols with low activity clays. These are deep, well-drained soils with low activity clays, characteristic of old landscapes with very high rainfall. They also occur in parts of West Africa and Australia that are now dry but used to be much wetter. Oxisols are soils with weathered horizons of kaolinite, iron oxides and sand with a small capacity for cation exchange (< 16 mmol 100 g^{-1} of clay). They are usually very deep, well-drained, red or yellow soils with poor fertility but excellent physical structure. Oxisols cover a huge area of the Amazon basin, the South American savannahs and Central Africa. There are smaller areas of Oxisols in Southeast Asia. Ultisols can be distinguished from Oxisols on the basis of a distinct horizon enriched in clay with less than 35% base saturation (see below). They generally contain more weatherable minerals than Oxisols but have a poorer structure and are infertile. Most of the uplands of Southeast Asia are dominated by Ultisols and these soils also cover large areas of South America and Africa. The low activity clay Alfisols are soils with a distinct horizon enriched in clay with a base saturation greater than 35%. They are similar to Ultisols but generally less acid and more fertile.

Soils with high activity clays

Many Alfisols have high activity 2 : 1 clays, are fertile and have no major management problems. Some Alfisols with a subsoil rich in laterite can become uncultivatable if the topsoil is eroded, and these are found mainly in West Africa, India and Sri Lanka.

Most Alfisols, Entisols and Inceptisols are young soils with little differentiation. Inceptisols have a moderately weathered subsoil (cambic horizon) but no other diagnostic horizons. An important group of Inceptisols, the Sulfaquepts or 'acid-sulphate soils', occur in coastal plains and river deltas where iron-rich soils have been inundated with sea water. Sea water contains a lot of sulphate, which is reduced to H_2S and then forms pyrite (FeS_2). On exposure to air the pyrite is oxidized to ferric sulphate and sulphuric acid, giving soil pH values as low as pH 2 in some cases.

Vertisols or 'black cotton soils' are deep soils with a high proportion of 2 : 1 clays (see below) which swell on wetting and crack on drying, such that a self-mulching effect occurs. They cover large areas of India, Java, Ethiopia and the Sudan, and lower topographic positions throughout 'wet-and-dry' climates in Africa, but a small part of the tropics as a whole. Spodosols are usually developed on sandy materials and characteristically have an 'iron pan' formed below a bleached horizon with a surface organic layer, usually as a result of a fluctuating high water table. Histosols are soils generally developed in wet conditions in which more than half of the top 80 cm is organic matter. Neither of these last two groups covers large areas of the tropics but both are of local importance.

Andisols are usually black soils developed from volcanic deposits with a high organic matter content; they are usually found in mountainous regions. Although

Andisols are often relatively young soils, with a large amount of weatherable minerals, they are not all fertile as they can become rapidly leached and can have a very high capacity to fix phosphorus. They do tend to have good physical properties and although they only cover some 2% of the land surface of the earth they support roughly 10% of the world's population – indicating their capacity for agriculture.

Mollisols are soils with a soft surface horizon rich in organic matter, and base saturation > 50%; they are found in northern India, Mexico and Paraguay. Aridisols are soils of dry regions which have little importance for agriculture, unless irrigated, but cover a large area of the African tropics (Table 1.2).

Chemical characteristics of leached soils

Leaching removes large amounts of nutrients from the soil. The cation exchange capacity (CEC) is a measure of the net negative charge of a soil, and this determines the soil's ability to retain positively charged ions or cations. The CEC results from negative charges on the surface of clays and on the soil organic matter. In most of the highly leached tropical soils, iron and aluminium oxides and hydroxides are abundant and the dominant clay fraction is kaolinite. Kaolinite has a structure of 1 : 1 silica : alumina layers and carries an inherently small negative charge compared with the 2 : 1 clay minerals (such as smectites, illites or vermiculites) that are predominant in soils of temperate regions. Further, whilst the 2 : 1 clays carry a permanent charge, the negative charge on kaolinite and on organic matter varies depending on the pH and the ionic strength of the soil solution. At low soil pH the CEC is small compared with that at high pH, rendering the capacity to protect cations from leaching even less. In some tropical soils a net positive charge can develop so that the rate of movement of anions (such as nitrate) through the soil can be retarded, but this is fairly rare (Wong *et al.*, 1990). This process may assist in enabling deep-rooting species, especially trees, to capture and recycle nitrate from the subsoils of low activity clay soils (Buresh and Tian, 1997).

In soils where the parent material contains much aluminium it can become the predominant cation when the soils have been leached of other cations. Thus the proportion of the CEC occupied by aluminium ions (the % aluminium saturation) can be as high as 80–90% and the base saturation (i.e. the proportion of the CEC occupied by the cations that predominate in most soils: Ca^{2+}, Mg^{2+} and K^{+}) is low. Acidity *per se* is not harmful to plants, except in extreme cases, and the problems of plant growth on acid soils are largely due to the large amounts of aluminium, and in some soils iron and manganese, that come into solution under acid conditions and are highly toxic.

Warm, wet conditions in soil are ideal for the rapid decomposition of organic matter added to soil. This can be an advantage as nutrients are released rapidly but it also means that little organic matter generally accumulates. Organic matter provides an important component of the CEC where the contribution of the clay fraction is small and it also contributes to the physical properties of the soil by helping to hold soil particles together in large aggregates, which are important for aeration of the soil

and infiltration of water. There is often a marked seasonality of organic matter decomposition in the wet and dry tropics, due to a flush of decomposition associated with the rewetting of very dry soils – known as the 'Birch effect' (Birch, 1964). This can lead to a pronounced flush of nitrate in the soil at the onset of the rainy season that is susceptible to leaching in cultivated soils, as the high concentrations of nitrate occur before the roots of crops are sufficiently well developed to absorb.

Deficiencies of many essential nutrients are common in leached soils, and soils that have developed over parent materials that contain small amounts of particular elements will be especially prone to problems. In the highly acid Oxisols and Ultisols it is not uncommon to have an inadequate supply of nitrogen, phosphorus, sulphur, calcium, magnesium, zinc, boron and copper. Phosphorus tends to be chemically bound or 'fixed' in a form not available for plant uptake in acid soils rich in iron and aluminium hydroxides. Molybdenum, which occurs in soils as the molybdate ion (MoO_4^{2-}), is held in the same way so that deficiencies in plants may be acute even in soils that are not inherently depleted of phosphorus or molybdenum. These deficiencies, coupled with the toxicity problems described above, may make one marvel that agriculture can be practised on such soils at all, but with proper management continuous cultivation is possible (Sanchez and Salinas, 1981; von Uexküll and Mutert, 1995).

Tropical Cropping Systems

In parts of the forests of Southeast Asia and the Amazon, indigenous tribes can still obtain a large amount of their food by hunting and gathering, but the area of forest that can support this is rapidly diminishing. All other societies derive the major part of their food from the cultivation of crops or from animal production in grazing systems (Ruthenberg, 1980).

Shifting cultivation

The oldest form of crop production known is that of shifting cultivation or 'swidden' agriculture. Crops are produced on land from which the native vegetation (most often forest) has been cleared, and usually burned, and after the cropping period the land is abandoned and the vegetation allowed to regenerate. The lengths of the different phases of the cycle of shifting cultivation – clearance and burning, cropping, and finally abandonment and regeneration – vary enormously between different regions. The cropping phase is usually short, only 1 or 2 years, and when land is plentiful is followed by a regeneration or fallow period of 15 years or more. Shifting cultivation can be a sustainable form of agriculture provided that sufficient time is allowed for the store of nutrients in the soil and the vegetation to be fully replenished, but only a limited population can be supported. If the land is brought back into cultivation too soon for the land to regenerate fully then the balance of the cycle will be upset and the

organic matter of the soils, the key to soil fertility, will be gradually depleted (Nye and Greenland, 1960).

Fallow systems

An intensified form of truly 'shifting' cultivation, in which settlements are moved slowly as new areas are brought into production, occurs through various rotation systems (Ruthenberg, 1980). Fallow systems are those in which crops are grown with short, intervening fallow periods during which the land is left to revegetate, but they tend to give way to continuous cultivation as population pressure increases. As with most artificial classification schemes, the boundaries between different forms of cropping system are often not clear, but fallow systems can be identified as systems with one-third to two-thirds of the land under cultivation at any one time, whereas continuous or 'permanent' cultivation systems are those where more than two-thirds of the land is cultivated at any one time. Various types of fallow systems can be identified: bush-fallow systems, in which the 'bush' – usually grasses, shrubs and trees – regrows during the fallow; savannah-fallow systems, in which the fallow comprises grasses; or ley systems, in which the land is also dominated by grasses during the fallow and is used for grazing.

As the fallow periods are not long enough to restore the fertility of the soil fully, productivity decreases with the intensity of soil use on all but the most fertile soils, unless nutrients are imported to replace those leached or removed in the crops. In most of the tropics the supply of organic manures is sufficient to sustain only a moderate output, and mineral fertilizers are often beyond the means of smallholders. Production thus continues by mining the reserves of the soil.

Permanent farming

Classifications of permanent agricultural production systems are generally based on one of three criteria: the nature of the crop rotation; whether crops or animals are the major outputs; or on the water supply, whether rainfed or irrigated. In the tropics there are few farmers who do not have any animals for milk or meat production and few pastoralists who grow no crops, particularly when we consider small-scale production. This means that no classifications can account properly for the diversity of crop rotations and combinations likely to be encountered. The common features of permanent farming that tend to distinguish them from fallow systems are: a permanent division of land between that used for arable crops and that used for grazing; clearly defined fields; and a predominance of annual and biennial food crops (Ruthenberg, 1980).

A feature common to agriculture in most regions of the tropics is the widespread use of multiple cropping in which several crops are grown in the same field, either in rotation within a year, or in combination in various forms of intercropping. Most of these cropping systems contain some component of perennial crops, but a separate

form of system can be recognized where perennial crops are planted as the main source of income in plantations.

Conclusions

The major determinants of agricultural productivity in the tropics are climate and soil fertility. A number of climatic zones can be identified and these change more with topography than with latitude. The fertility of soils in the tropics varies widely. Soils derived from geologically recent deposits, such as unweathered volcanic deposits or alluvial material, are the most fertile. Many tropical soils are derived from ancient parent rock poor in bases and are highly weathered and leached, with consequent problems of nutrient deficiencies, or of acidity and associated toxicities. A number of different cropping systems are practised in the tropics, ranging from shifting cultivation through fallow systems to permanent agriculture. With increasing population pressures there is a tendency towards permanent agriculture and a serious danger of a steady depletion of soil fertility. This book addresses the present and possible future contribution of N_2-fixation in the maintenance of soil fertility in tropical cropping systems.

Chapter 2

N_2-fixing Organisms in the Tropics

All organisms that are able to fix N_2 share two properties in common. One, of course, is the ability to carry out this important and difficult reaction. The second is that all such organisms are prokaryotes. No higher organism (eukaryote) capable of N_2-fixation on its own has ever been discovered. Many eukaryotes do derive direct benefit from intimate associations with N_2-fixing bacteria, and these are the subject of detailed discussion in later chapters. This chapter concentrates on the biology of the N_2-fixing organisms themselves and of the symbioses they form that are of importance in tropical cropping systems.

The variety of N_2-fixing organisms is so great that it is impossible to describe them all in detail. A good overview of the diversity of prokaryotes capable of N_2-fixation is given by Postgate (1998) and their probable evolutionary relationships to each other are described by Young (1992). One of the main conclusions resulting from this survey is that prokaryotes capable of N_2-fixation are scattered liberally throughout many 'divisions' of bacteria, where divisions are defined as distinct phylogenetic lineages of bacteria (Hugenholtz *et al.*, 1998). Indeed, quite remarkably N_2-fixing species are found among both the Bacteria and the Archaea[1], which are now recognized as two entirely separate 'domains' of prokaryotes (Woese, 1987). When one considers that there is only one further domain making up all of life on earth – the Eukarya or eukaryotes – this phylogenetic distribution of diazotrophy appears all the more remarkable.

[1] The Bacteria (sometimes referred to as the Eubacteria) and the Archaea are the formal names for the two domains of prokaryotes. In this book, species belonging to these groups are referred to as eubacteria or archaebacteria, respectively, and the word bacteria (with a lower case b) refers collectively to both, i.e. it is equivalent to the word prokaryotes.

The explosion of new discoveries in bacterial phylogenetics since the 1980s is due to the advent of DNA sequencing, which has allowed phylogenetic analysis in groups where shared morphological characters are few (Woese, 1987). This has revealed that earlier methods of grouping bacteria according to morphological, physiological or ecological properties often did not reflect true evolutionary relationships. For example, the automatic classification of all bacteria that form N_2-fixing associations with legumes as members of a single genus, *Rhizobium*, actually obscured for many decades the fact that there are several different groups of bacteria that form such associations with legumes, as will be discussed later in this chapter.

Thus, while the subject of bacterial systematics may seem to be primarily of academic interest, it is actually of practical importance. For example, conditions for the culture or genetic manipulation of one group of bacteria are likely to be applicable to closely related bacteria, but not necessarily to groups of bacteria that may share certain properties in common – such as the ability to induce N_2-fixing nodules on the roots of legume plants – but are in fact only distantly related and exhibit very different physiologies. To give one practical example, many *Bradyrhizobium* strains are unable to use sucrose as a carbon source (Martinez de Drets *et al.*, 1973). Thus molasses, which can be a convenient, cheap and locally available material for use as a carbon source in the growth of bacteria in tropical countries, often cannot be used directly for the production of *Bradyrhizobium* inoculants. Instead it must first be converted to monosaccharides using a process such as fermentation with yeast strains (T.A. Lie, personal communication, 1990). By contrast, most *Rhizobium* species will grow very well on sucrose.

This chapter, therefore, begins by giving an overview of present-day bacterial classification and the implications this has for the study of N_2-fixing organisms. The biology of some of these N_2-fixing organisms is then described. Rather than try to give a comprehensive survey, it concentrates specifically on groups of N_2-fixing bacteria that are found in the tropics and that are most likely to make significant contributions to the N balance of tropical cropping systems. Several other reviews are available that give more general coverage of the biology of all N_2-fixing organisms (e.g. Gallon and Chaplin, 1987; Sprent and Sprent, 1990; Postgate, 1998).

Prokaryote Classification

The terms prokaryote and eukaryote actually refer to the basic structure of the cell (*karyos* = Greek for kernel or nucleus). Prokaryotes (*pro* = primitive) have a cell structure that appears simple: a single heterogeneous compartment containing nucleic acids, sites of protein synthesis, enzymes for metabolic reactions and myriad other cell components. By contrast, eukaryotic (*eu* = true) cells appear complex, containing a nucleus and many other separate membrane-bound compartments or organelles in which specialized conditions for specific sets of cellular reactions are created. This structural specialization of eukaryotic cells creates a very clear demarcation separating eukaryotes – whether an amoeba, a rice plant or a camel – from prokaryotes, which,

although they may carry out similar sets of metabolic reactions, show no obvious intracellular structural sophistication.

The taxonomic classification of eukaryotes continues to be based primarily on morphological differences, though molecular analyses are also increasingly applied in combination with morphological criteria. For prokaryotes, this is not really possible or useful. At school we may have been taught that bacteria were either rods, cocci or spirilla – an attempt to classify bacteria according to their shape – but this classification is almost meaningless. For example, application of antibiotics or simple mutations can cause bacteria of several species, which normally exist as cocci, to form rods instead (e.g. Lleo *et al.*, 1990). Prokaryote classification has also been based on other criteria such as the ability to carry out photosynthesis or to fix N_2 but, as will be repeatedly emphasized, such classification based on a limited set of phenotypic properties can in fact obscure significant similarities or differences.

The classification of prokaryotes remains very difficult, but the application of molecular biology has made possible a prokaryote classification that is systematic, in other words that does indicate evolutionary relationships between groups. This change is reflected in the renaming of the key book used for the identification of bacteria, 'Bergey's Manual'. Formerly called *Bergey's Manual of Determinative Bacteriology*, indicating only that the identity of a particular bacterial isolate could be determined, the 8th edition was renamed *Bergey's Manual of Systematic Bacteriology*, indicating that the classifications actually try to reflect true evolutionary relationships (Krieg and Holt, 1984). The history of prokaryote classification is reviewed in an article defending the concept of the Bacteria and Archaea as two separate domains of prokaryotes (Woese, 1998).

The molecular method that has had the greatest impact on bacterial classification is the use of nucleic acid sequence homologies (Woese, 1987). There are many advantages to the use of nucleic acid sequences as a tool for taxonomic analysis. First, unlike characters such as metabolic capabilities, protein profiles, or even morphology, the structure of DNA of any organism remains constant throughout its life cycle (with very few exceptions). Thus, whether *Myxobacteria* are present in the soil as a loose colony of single cells or aggregated together forming a multicellular fruiting body, the sequence of their DNA, and the information that can be derived from it, remains unaltered (Shimkets and Woese, 1992).

Secondly, the DNA sequence does in fact change with time through the process of random mutations, but, with certain exceptions, this is time measured in billions of generations and is tempered by the process of natural selection. Thus, while changes may occur at equal rates in all regions of the DNA, organisms that bear deleterious changes, such as those that lead to loss of an essential enzyme, will not survive. The result is that perceived rates of change of nucleic acid sequence – changes that can be seen in surviving organisms – vary in different regions of the genome. This is very fortunate for molecular evolutionists because it means that, to them, the DNA sequence can be viewed as a series of molecular clocks all ticking at different rates.

Thus, in one region of the genome there might be sufficient differences to discriminate between two very closely related bacteria, whereas in another the rate of accumulation of nucleotide changes may be so slow that comparisons can be

made between bacteria separated by hundreds of millions of years of evolution. For example, sequence differences in the genes encoding the respiratory-chain protein cytochrome *c* have been useful in the reordering of one bacterial subdivision, the α-purple bacteria, but the changes are too great to allow cytochrome *c* genes to be useful in determining phylogenetic relationships between more widely dispersed groups of bacteria. For these purposes, the sequences of ribosomal RNA genes (5S, 16S or 23S) have proved most useful (Woese, 1987) (Fig. 2.1). This pervasive application of molecular tools to bacterial systematics is reflected in a spurt of handbooks to molecular prokaryotic taxonomy published in the mid 1990s (Goodfellow and O'Donnell, 1993; Priest and Austin, 1993; Towner and Cockayne, 1993; Logan, 1994) and, above all, in the massive rise in sequence information available. At the end of 1999 there were more than 12,000 16S rRNA gene sequences from eubacterial species in the public nucleic acid databases and a further 600–700 from Archaea.

To return specifically to the subject of N_2-fixing organisms, these developments in molecular phylogeny of prokaryotes have underscored the variety of prokaryotic taxa that contain N_2-fixing species, including both Bacteria and Archaea. This begs the questions of whether all N_2-fixing organisms are derived from a common ancestor (one that pre-dates the divergence of archaebacteria and eubacteria), whether the capacity for N_2-fixation has evolved independently several times, or whether genes encoding functions needed for N_2-fixation have spread between organisms more recently by lateral gene transfer.

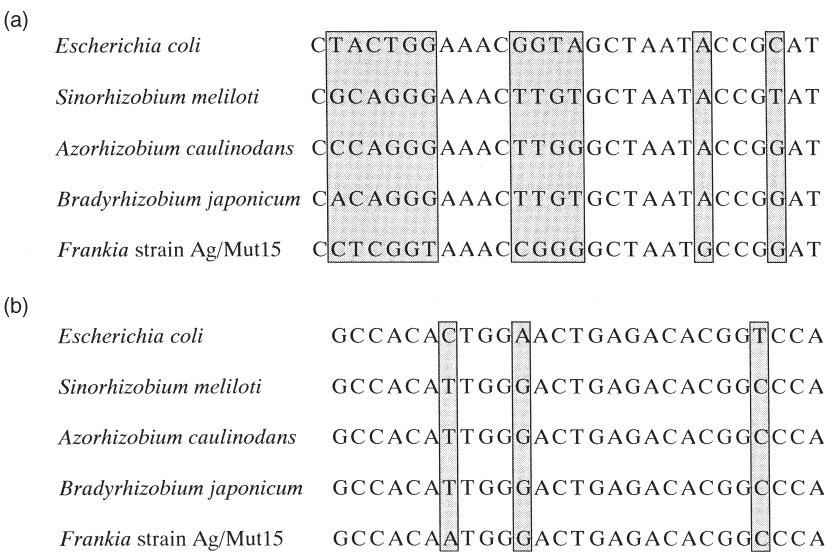

Fig. 2.1. Sequence divergence between (a) variable and (b) conserved regions of genes encoding 16S rRNA from several bacteria (differences between sequences are shaded).

Nitrogenase genes found among diazotrophic archaebacteria show strong similarity to genes found among eubacteria, and thus it is generally accepted that this system of N_2-fixation (there is now one other system known – see section on other actinomycetes in this chapter) has evolved only once. A corollary of this is that N_2-fixation capability must have been lost many times in descendants of this 'common ancestor' to account for its current sporadic phylogenetic distribution (Young, 2000).

When the genes encoding the two components of dinitrogenase, *nifD* and *nifK*, and their homologues are considered, the genes cluster according to the metal requirements of the nitrogenase (Mo, Vn, Fe; see Chapter 3), suggesting that the presumed gene duplication that led to subsequent evolution of the alternative nitrogenases occurred only once (Kessler *et al*., 1997). However, when Component 1 nitrogenase genes are considered (*nifH*, *vnfH*, *anfH*), they fall into four groups and there is no clear separation according to metal requirements; *anfH* genes cluster with group II *nifH* genes, and *vnfH* genes cluster with group I *nifH* genes (Kessler *et al*., 1997). Furthermore, a *nifH* sequence-based phylogeny shows only partial concordance with accepted 16S rRNA-based phylogenies, suggesting that there has been either some gene duplication or lateral gene transfer in the course of the evolution of nitrogenase genes (Young, 1996, 2000).

N_2-fixing Species in Tropical Cropping Systems

There are relatively few N_2-fixing species shown to make a real contribution to tropical cropping systems. The predominant group are the rhizobia – bacteria that form symbiotic associations with legume plants. As we shall see, these bacteria contribute the greatest amounts of biologically fixed N in agriculture and are therefore the focus of this book.

The second most economically important group is the cyanobacteria (blue-green algae), which are found both as free-living species and in associations with a variety of plants, most notably the aquatic fern *Azolla*. This symbiotic association is deliberately introduced into rice paddy fields in China and Vietnam and contributes significant amounts of fixed N to rice crops (Chapter 7). While cyanobacteria form associations with many other organisms, ranging from fungi (constituting lichens) to the Angiosperm genus *Gunnera*, no other cyanobacterial symbioses are of such direct importance in tropical cropping systems.

A third group of important N_2-fixers are the actinomycete *Frankia* species, which form symbiotic associations with flowering plants from a number of different families. Almost all of their host plants are woody perennials (trees and shrubs) and their importance in agroforestry is increasingly being realized. While most of the known host plants for *Frankia* are temperate species, *Frankia* do form active N_2-fixing symbioses with some tropical plants. The fast-growing tree genus *Casuarina* is the most prominent example, and is widely used in agroforestry in the tropics.

A fourth group of N_2-fixers is more loosely associated with plants. This group includes *Azospirillum* species, which colonize the root epidermis of host species

including wheat, maize and rice (Vande Broek *et al*., 1993). *Azoarcus* species were isolated from the roots of Kallar grass, a salt-tolerant species of no economic importance, but it has also been shown capable of invading the roots of rice and promoting growth, albeit not through N_2-fixation (Hurek *et al*., 1994). Species of *Herbaspirillum* and *Acetobacter*, *H. seropedicae* and *A. diazotrophicus*, have been found endophytically within the roots, shoots and stems of a variety of graminaceous plants, including sugarcane, wheat, maize and rice (James and Olivares, 1998). N_2-fixing members of these two genera were first identified in sugarcane plants grown in Brazil (Chapter 6) although it remains formally unproven whether they contribute fixed N to the cane plants (James and Olivares, 1998).

The last group of N_2-fixing organisms that contribute to the N balance in tropical cropping systems is the free-living N_2-fixers. Organisms such as *Klebsiella* and *Azotobacter* live in the soil and fix N_2 when other forms of N are unavailable.

An important point to make is that knowledge of prokaryotic microorganisms is heavily biased towards those that can be cultured or at least readily detected by human beings. Over the past 10 years nucleic acid analysis techniques have been applied to detect and analyse bacteria without the necessity for any culturing step. It was already known that the numbers of bacteria cultured from any environmental sample represented only 1% or less of the bacteria visible through a microscope. Molecular techniques have revealed that indeed prokaryotic diversity far exceeds what was known based only on cultured species, and has confirmed the belief that a vast number of microorganisms on earth have never been detected (Hugenholtz *et al*., 1998). It is not a wild overstatement to say that these newly identified groups will certainly include many N_2-fixing species.

Bacteria that Fix N_2 with Legume Host Plants

This group of bacteria can collectively be referred to as 'rhizobia', referring to bacteria of several genera that induce and infect nodules on the roots and/or stems of plants of the family *Leguminosae*. According to this definition, bacteria may be considered as rhizobia irrespective of whether they actually fix N_2. However, this definition excludes bacteria that may be very closely related to nodule-forming bacteria either in other phenotypic properties or according to data derived from nucleic acid sequences, but which do not form nodules on any legume plant. As we shall see, this grouping of bacteria based on occupation of a common ecological niche does in fact encompass an enormous amount of genetic and phenotypic diversity.

Common elements of the symbiosis

Before discussing the variations in bacterial identity, host range, pathway of infection, nodule structure, efficiency of N_2-fixation and so on, we shall begin by describing the common core elements of the legume symbiosis. Here a note of

semantic caution must be sounded: the word 'symbiosis' is being used loosely on occasion, as our definition of rhizobia includes bacteria able to induce and infect nodules without actually fixing N_2, and therefore in effect behaving as parasites.

Perhaps the most important general principle is that the symbioses established between rhizobia and their host plants are specific. That is, only certain strains of rhizobia can form a symbiosis with a given legume – and these may be termed 'compatible' rhizobia. Incompatible rhizobia, which by definition (as rhizobia) do form a symbiosis with some other legume species, cannot form a symbiosis with the host plant in question. This observation indicates that there must be recognition between the plant and bacteria.

Thus the first step in the establishment of a rhizobial–legume symbiosis is an interaction between a legume species that is susceptible to nodulation and compatible rhizobia. For the time being the discussion will be restricted to root nodules, and so it can further be specified that this is an interaction between the roots of the plant and rhizobia present in the soil. These rhizobia may have been introduced deliberately into the soil by inoculation, or they may have been already present in the soil as free-living bacteria, in which case they are termed 'indigenous' rhizobia. As will be discussed in Chapter 14, most soils contain some indigenous rhizobia capable of nodulating most legume species that are planted in them. While this is a testament either to the diversity of the bacterial population in any given soil, or to the promiscuity of certain rhizobia, it is also a major handicap to the improvement of legume yield through inoculation technology.

A great deal is now understood about the molecular events underlying the recognition between bacterial and plant partners (for reviews see van Rhijn and Vanderleyden, 1995; Denarié *et al.*, 1996; Heidstra and Bisseling, 1996; Long, 1996; Cohn *et al.*, 1998). A major component of this initial interaction consists of stimulation of biochemical activity in the rhizobial strains by flavonoid and isoflavonoid molecules in the plant root exudate. These compounds stimulate the activity of *nod* (nodulation) genes – that is, genes whose products are required to enable nodulation of the cognate legume host. There is some specificity in this interaction as different flavonoid and isoflavonoid compounds from different legumes have been shown to activate the *nod* genes of their compatible rhizobia preferentially, and the *nod* genes of the broad-host-range *Rhizobium* strain NGR234 are activated by a correspondingly broad range of phenolic compounds. However, this stimulation is by no means completely specific, as exudates from incompatible legume species can often activate the *nod* genes of a given *Rhizobium* strain to some degree, and in some cases exudates even from non-legumes may cause this stimulation.

These flavonoids or isoflavonoids enter the bacterial cell, where they bind to a protein termed NodD. The effect of this binding is to convert NodD into a transcriptional activator – that is, a factor that stimulates expression of specific genes. NodD then activates the remaining nodulation genes, protein products of which cooperate to synthesize a 'Nod factor', a signal molecule that is secreted into the plant rhizosphere. These Nod factors share a common ground plan, but it is the chemical details that confer specificity of recognition. The basic structure is a β-1,4-linked *N*-acetyl-D-glucosamine backbone with three to six sugar units which is acylated on

the non-reducing terminal sugar residue. The degree of saturation of this acyl group and the presence of other groups varies between rhizobial species and appears to determine host specificity (Fig. 2.2). An example would be the first Nod factor characterized, NodRm-1 from *Sinorhizobium meliloti*. The recognition by the host plant is so exquisite that absence of a critical sulphate group switches the specificity of this molecule from recognition by *Medicago* to recognition by *Vicia* (Denarié *et al.*, 1996; Schultze and Kondorosi, 1998).

The role of the Nod factors appears to be to trigger responses in the plant that lead to rhizobial infection and development of a root nodule. Nod factors trigger a number of responses in the plant epidermis: membrane depolarization of cells in the zone of emerging root hairs; deformation of root hairs; and induction of expression of nodule specific genes ('nodulins'). In addition, Nod factors contribute to induction of infection thread formation. Nod factors are also able to mitotically reactivate quiescent cells in the root cortex that become the nodule meristem and in some cases purified Nod factors are sufficient to induce development of a complete nodule structure. Finally, Nod factors also induce expression of nodulins in the pericycle (Heidstra and Bisseling, 1996). A major focus of current research is the search for the plant proteins that recognize and initiate the response to the Nod factors – the Nod factor receptors. Factors that bind or otherwise interact with chemically synthesized Nod factors have been identified from lucerne and *Dolichus biflorus*, but further data are required to demonstrate whether these candidates are the true receptors (Etzler *et al.*, 1999; Gressent *et al.*, 1999; Roberts *et al.*, 1999; Stacey, 2000). Moreover,

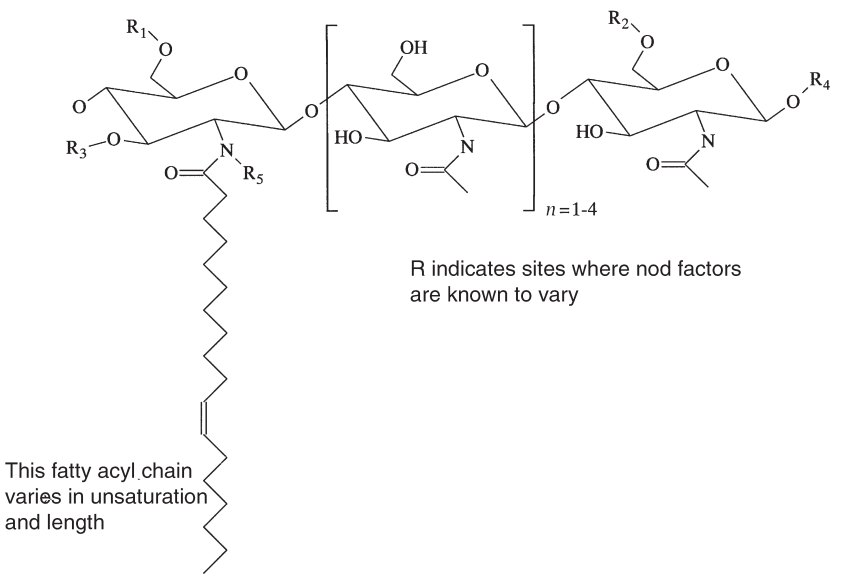

Fig. 2.2. A generic structure for rhizobial nodulation factors (Pacios Bras *et al.*, 2000). These molecules can elicit formation of nodules on legume hosts when present at very small concentrations and with a high degree of host specificity.

genetic studies suggest that legumes may actually have two Nod factor receptors – one that is responsible for allowing entry of the bacteria into the plant and one that transmits the signal to the nodule cortex to initiate cell division (Ardourel *et al.*, 1994; Minami *et al.*, 1996; Geurts *et al.*, 1997; Bono *et al.*, 2000).

The net effect of the chemical signalling between plant and bacterium is that compatible rhizobia that are closely attached to the root surface gain entry to the root, and the two processes of plant cell division and concurrent rhizobial invasion continue until the mature, N_2-fixing nodule is formed. There are many variations on the pathway of development and in the final structure of the nodule. All that can be said to be in common is that the bacteria eventually, at some location within the mature nodule, undergo biochemical and morphological differentiation and begin to fix N_2. It is at this point that they are referred to as bacteroids (Oke and Long, 1999b).

Variations on the theme

Infection mechanism

Three different pathways of legume root infection have been described: root hair infection; crack entry; and direct penetration between epidermal cells. The first is clearly different from the second two, but the evidence to distinguish the latter two mechanisms is more circumstantial.

Root hair infection

Root hairs are outgrowths of root epidermal cells, the primary function of which is to increase the volume of soil that may be exploited by the root for nutrients and for water. In many legumes, these outgrowths serve as the conduit by which rhizobia first gain entry to the plant root. The Nod factors produced by the rhizobia cause changes in the growth patterns of the root hairs, producing many deformations, including the characteristic 'shepherd's crooks'. At the centre of the crook, disruption of the plant cell wall occurs, enabling the rhizobia to enter the root hair.

As they do so, a new structure, the infection thread, forms within the plant cell and encloses the rhizobia. Thus the rhizobia remain topologically outside the plant cell cytoplasm. The wall of the infection thread is composed of plant cell wall material, and the bacteria inside the thread are embedded in a glycoprotein matrix that is probably of both plant and bacterial origin (VandenBosch *et al.*, 1989). At the same time, cells within the root cortex undergo cytological rearrangements to create a series of radially aligned cytoplasmic bridges termed 'pre-infection threads', which are traversed by the actual infection thread as it passes through the root (van Brussel *et al.*, 1992). The infection thread grows by continual deposition of plant cell wall material at its tip, and ramifies within the root tissue, so delivering the rhizobia to numerous plant cells within the emerging root nodule. The rhizobia meanwhile divide within the infection thread, and eventually are 'released' from unwalled segments of the infection thread, thus gaining entry to the plant cell cytoplasm. This release process is essentially endocytosis. The rhizobia become surrounded by fragments of the

host-cell plasma membrane that formerly separated the host cytoplasm from the infection thread wall and this forms the peribacteroid membrane (Newcomb, 1981). The bacteria generally undergo significant morphological change, which appears to be host determined (see Fig. 2.4), and are now termed bacteroids. These visible changes are accompanied by biochemical changes: nitrogenase is synthesized, the bacterial enzymes of ammonia assimilation are repressed, and N_2-fixation begins.

Crack entry and epidermal penetration

In these two mechanisms of infection, penetration does not occur via root hairs, but directly at the root surface. Key evidence for this pathway is provided by nodulation in legume species that have no or very few root hairs. This is particularly common among woody legume species and epidermal infection has been directly demonstrated for the Brazilian tree species *Mimosa scabrella* (de Faria *et al.*, 1988). In this example rhizobia were seen to penetrate the primary cell wall at the junction of two epidermal cells, and no infection threads were ever observed in the rare root hairs that did occur.

'Crack entry' refers to a similar pathway of infection where the rhizobia also gain entry between two epidermal cells rather than via a root hair (Boogerd and van Rossum, 1997). The key difference is the purported necessity for a 'wound' at the root surface before penetration of rhizobia can occur. This wounding is believed to be caused by the emergence of lateral roots, as nodules are found only at the junctions between lateral and main roots in the three species for which crack entry has been described: *Arachis hypogaea* (Chandler, 1978), and *Stylosanthes capitata* and *S. hamata* (Chandler *et al.*, 1982). The presence of axillary root hairs appears to be involved in the infection process, as these are absent in non-nodulating genotypes of *Arachis* (Nambiar *et al.*, 1983b). Given the difficulty in obtaining direct evidence for wounding, the fact that it is clearly not considered essential for all forms of direct epidermal penetration, and the fact that non-nodulating mutants of *A. hypogaea* still develop lateral roots and presumably the associated 'wounds' (Nigam *et al.*, 1980), this hypothesis may be treated with caution. It is of interest to note that both host plant genera known to exhibit crack entry are members of the tribe *Aeschynomeneae*.

In either case, as with root hair infection, the rhizobia within the developing nodule are initially extracellular. In *A. hypogaea* files of bacteria can be seen filling the spaces between the cells in the young nodule. Eventually, presumably by some form of endocytotic process at areas where the host cell wall is weakened, these rhizobia gain entry into the host cell cytoplasm, again surrounded by peribacteroid membranes derived from the plant cytoplasmic membrane. N_2-fixation is not believed to begin until this stage. In *Stylosanthes*, infection appears to occur by a system of progressive infection and then collapse of cortical cells, enabling inward spread of the rhizobia. In *Mimosa*, bacteria were seen to progress in the cortical region actually through the cell walls, rather than by separating cells at the middle lamella. Where intracellular infection occurred, the bacteria remained surrounded by cell wall material (de Faria *et al.*, 1988).

Persistent infection threads

The idea that N_2-fixation by rhizobia is always carried out by bacteroids present in the host cell cytoplasm was first challenged when the structure of nodules induced on the non-legume tree *Parasponia andersonii* was examined (Becking, 1992). It was found that infection threads persisted, and N_2-fixation by the bacteria took place within these structures without bacterial release (Trinick, 1979). The structure of the threads was seen (in the transmission electron microscope, following staining with osmium tetraoxide) to change from a tightly packed thread with a darkly staining wall to a more loosely packed thread with a lightly stained wall (Trinick, 1979; Price *et al*., 1984). The latter threads were assumed to be the sites of active N_2-fixation and were therefore termed fixation threads (Price *et al*., 1984).

Persistent infection threads have subsequently been found in legume species, including many nodulated legumes from the subfamily *Caesalpinioideae* (de Faria *et al*., 1987). Infection threads therefore represent an alternative pathway of nodule development rather than an isolated exception.

Nodule structure

The variation in rhizobial infection and nodule development already described begs the question: what is a legume root nodule? Sprent (1989) suggested that the only property held in common by all legume nodules is the stem-like character of the peripheral vascular system, which contrasts with the central vascular system observed in roots, and in actinorhizal and *Parasponia* nodules. Nevertheless, a basic nodule structure can be described. In all cases there is an outer, uninfected cortical region containing the peripheral vasculature. This is separated from the inner, infected zone by a nodule endodermis. The central zone contains the infected cells where active N_2-fixation takes place. These are often interspersed with uninfected cells. In plants that assimilate fixed N in the form of ureides (Chapter 4) these are the sites of ureide synthesis, but the specific role, if any, in plants that assimilate fixed N as amides is unknown. In nodules of some legumes, such as *A. hypogaea*, all cells in the central zone are infected.

Two primary types of nodule structure can be discerned: the determinate type and indeterminate type (Fig. 2.3). These terms refer to differences in the nodule meristem, which is persistent in the latter but not in the former case. Determinate nodules grow for a fixed period, all parts of the nodule differentiating at the same time; thus senescence also occurs at one time and they have a finite life span. In contrast, indeterminate nodules have an apical meristem which continues to be active throughout the lifetime of the nodule, producing new zones of infection, and so giving rise to a gradient of differentiation progressing back towards the root – the apical meristem being most distal, followed by the invasion zone where infection thread growth continues (now reversed in direction to follow the apical meristem) with concomitant bacterial release. Progressing towards the root are then found the early, mature and late symbiotic zones, respectively. Perennial nodules are always of the indeterminate type, the persistent meristem being able to resume activity in each new growing season.

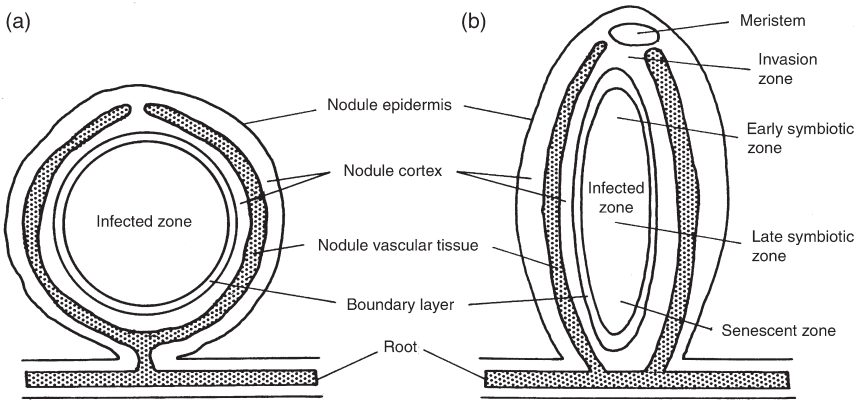

Fig. 2.3. Structure of (a) determinate and (b) indeterminate legume nodules.

Other obvious key differences are in nodule shape, which is always spherical for determinate nodules but can vary from cylindrical to coralloid for indeterminate nodules, and the location of the root cells that initially form the nodule meristem: in determinate nodules these are cells of the outer root cortex, whereas in indeterminate nodules the initial nodule is formed of cells of the inner root cortex. There are a number of detailed reviews of the biology and molecular biology of the infection process and subsequent nodule development (Nap and Bisseling, 1990; Hirsch, 1992; Brewin, 1998; Cohn *et al.*, 1998).

Many features of the symbiosis are host controlled

Most, if not all, of these differences in nodule development and structure are host controlled. This is clear because a single rhizobial strain is often capable of infecting different host plants by different means, and giving rise to nodules of different structure. It may be released into the host cell in one species and remain in fixation threads in another (e.g. Price *et al.*, 1984). In fact, the symbiosis appears to be so closely controlled by the plant that even bacteroid shape is determined by the plant host. Figure 2.4 illustrates such differences, showing light and scanning electron micrographs of nodule structure and bacteroids in a groundnut plant and a siratro plant, both induced by the same strain, *Bradyrhizobium* sp. (*Arachis*) strain NC92.

Stem nodulation

Stem nodulation occurs in three genera of legumes that have the capacity to grow in waterlogged conditions: *Aeschynomene* (several species), *Discolobium* (at least two species) and *Sesbania* (*S. rostrata* only). Nodulation can be induced along the whole length of the stem (Fig. 2.5), which in *S. rostrata* can mean that nodules sometimes form up to 3 m above the ground. Each of these plants also has the ability to form root nodules.

Infection and development of stem nodules are quite different from those of root nodules, even though both may be induced by the same rhizobial strain. In all

Fig. 2.4. Structure of nodules induced on (a, c) siratro and (b, d) groundnut by *Bradyrhizobium* sp. (*Arachis*) strain NC92. Light (a, b; bar 100 μm) and transmission electron (c, d; bar 1 μm) micrographs. Note the absence of infected cells in the infected zone of groundnut nodules. B, bacteroid; CW, plant cell wall; I, infected cell; N, nucleus; S, starch grains; U, uninfected cell; V, vacuole; VB, vascular bundle.

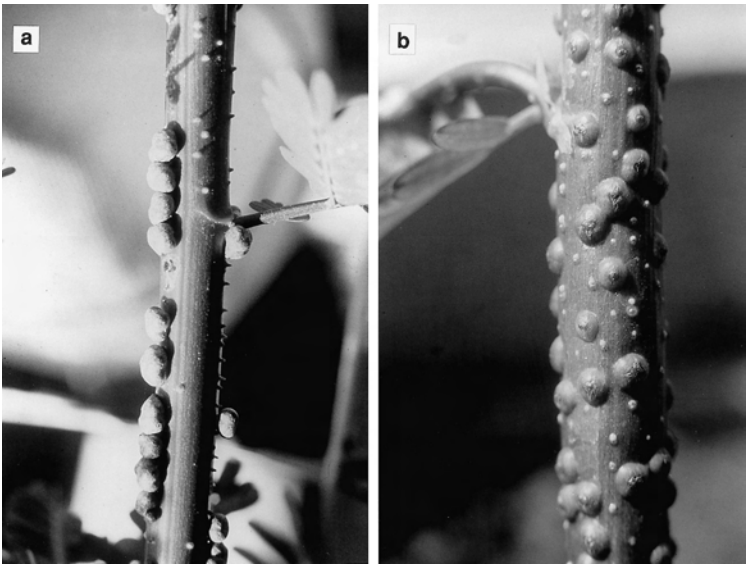

Fig. 2.5. Stem nodulation on (a) *Sesbania rostrata* and (b) *Aeschynomene afraspera*. (Photographs: M. Becker.)

species examined, stem nodules form at regular intervals along the stem at preformed incipient but dormant primordia. In different species these primordia may be completely hidden within the stem or they may be slightly protruding, the latter showing much greater susceptibility to infection. These dormant primordia have a typical root structure, and in waterlogged conditions, if nodules do not form, adventitious roots may develop from them (Dreyfus *et al.*, 1984).

S. rostrata shows the most profuse stem nodulation. The root primordia protrude up to 3 mm in three or four vertical rows up the stem. These nodulation sites, in contrast to sites for root nodule formation, remain susceptible to infection throughout the life of the plant. Thus infection can be induced simply by spraying the plants with an aerial suspension of compatible rhizobia. In the field, infection is probably achieved by epiphytic rhizobia – in one study as many as 5×10^5 stem-nodulating rhizobia were found on each square centimetre of leaf (Adebayo *et al.*, 1989) – but is often sporadic. Thus, there is a good likelihood of increasing stem nodulation of *S. rostrata* by spray inoculation, particularly as nodule development is favoured by increasing humidity (Parsons *et al.*, 1993a). Epiphytic rhizobia occur in greater numbers on leaves of *S. rostrata* than other plants found in the same habitats, suggesting that their association with *Sesbania* is also beneficial to the rhizobia (Robertson *et al.*, 1995).

Infection actually occurs in three steps. In the first, rhizobia colonize the intercellular spaces of the root primordia and form infection pockets. At the same time meristematic activity is induced in some of the primordial cells (Ndoye *et al.*, 1994). An infection thread then forms within the root primordium and this penetrates host cells and enables eventual intracellular release of the rhizobia. Once

released, the bacteria are enclosed in host-derived membranes just as in root nodules. Thus stem nodulation in *S. rostrata* combines aspects of direct intercellular infection and infection via infection threads (Boivin *et al.*, 1997).

Infection is generally less profuse in the genus *Aeschynomene*. In one species, *A. afraspera*, the root primordia are as prominent as in *S. rostrata*, and the species is correspondingly highly susceptible to infection (Alazard and Duhoux, 1990). Other species have less prominent primordia and are therefore less susceptible to infection. This group includes the species *A. scabra*, *A. indica*, *A. paniculata* and *A. sensitiva*. A third group, including *A. crassiculus* and *A. elaphroxylon*, only forms nodules on adventitious roots that arise from the primordia on the stem in waterlogged conditions, and thus does not form true stem nodules (Dreyfus *et al.*, 1984; Boivin *et al.*, 1997). This is also a feature of the aquatic legume *Neptunia plena*, which was earlier thought to form stem nodules (James *et al.*, 1992), whereas in the third genus in which stem nodulation was described, *Discolobium*, true stem nodules are formed that have vascular connections directly with the stem (Loureiro *et al.*, 1994). The genus *Discolobium* is a further member of the legume tribe *Aeschynomeneae* that occurs in swamps and rivers in South America (Loureiro *et al.*, 1998). The two species examined, *D. pulchellum* and *D. psoraleaefolium*, are both able to form stem nodules.

Nodulation in the *Leguminosae*

By no means all legume genera examined are able to form rhizobial symbioses. This is perhaps not surprising considering the vastness and diversity of the family, currently estimated to contain 16,000–19,000 species in about 750 different genera (Allen and Allen, 1981). The family *Leguminosae* is divided into three subfamilies: the *Papilionoideae* (pea-like flowers), the *Mimosoideae* (compound inflorescences with reduced petals) and the *Caesalpinioideae* (flowers usually with five petals, apparently radially symmetrical) (Polhill and Raven, 1981).

The *Mimosoideae* and the *Caesalpinioideae* are almost completely restricted to the tropics. The *Papilionoideae* contains the majority of the most important grain legumes. The 42 tribes of the *Leguminosae* along with examples of genera in each are listed in Table 2.1. Nodulation capacity has been surveyed in several thousand species, representing about 20% of leguminous species and including members of about 60% of legume genera (Corby, 1988; de Faria *et al.*, 1989; Sprent, 2001). Of these, 97% of examined papilionoid species form nodules, as do more than 90% of mimosoid species. In contrast, only 23% of examined caesalpinioid species nodulate. These species all fall within eight genera – seven in the tribe *Caesalpinieae* and one sole genus, *Chamaecrista*, within the tribe *Cassieae* (Sprent and Raven, 1992; Sprent, 1995). The survey by Corby (1988) recognized three types of indeterminate nodule: caesalpinioid, crotalarioid and lupin type; and two types of determinate nodule: aeschynomenoid and desmodoid. The taxonomic distribution of these nodule types was also described.

Table 2.1. Taxonomy of the family *Leguminosae*.

Subfamily	*Caesalpinioideae*	
Tribe	1. *Caesalpinieae*	*Caesalpinia, Peltophorum, Parkinsonia*
	2. *Cassieae*	*Cassia, Ceratonia, Senna, Chamaecrista*
	3. *Cercideae*	*Bauhinia*
	4. *Detarieae*	*Afzelia, Intsia, Peltogyne*
	5. *Amherstieae*	*Brachystegia, Julbernardia, Tamarindus*
Subfamily	*Mimosoideae*	
Tribe	1. *Parkieae*	*Parkia*
	2. *Mimozygantheae*	*Mimozyganthus*
	3. *Mimoseae*	*Desmanthus, Leucaena, Mimosa, Prosopis*
	4. *Acacieae*	*Acacia, Faidherbia*
	5. *Ingeae*	*Albizia, Calliandra, Inga, Pithecellobium*
Subfamily	*Papilionoideae*	
Tribe	1. *Swartzieae*	*Swartzia*
	2. *Sophoreae*	*Castanospermum*
	3. *Dipteryxeae*	*Pterodon*
	4. *Dalbergieae*	*Andira, Dalbergia, Pterocarpus*
	5. *Abreae*	*Abrus*
	6. *Tephrosieae*	*Tephrosia, Derris, Lonchocarpus, Wisteria*
	7. *Robinieae*[a]	*Gliricidia, Sesbania*
	8. *Indigoferae*	*Cyamopsis, Indigofera*
	9. *Desmodieae*	*Cordariocalyx, Desmodium*
	10. *Phaseoleae*	*Flemingia, Glycine, Pueraria, Phaseolus, Vigna*
	11. *Psoraleae*	*Psoralea*
	12. *Amorpheae*	*Dalea*
	14. *Aeschynomeneae*	*Aeschynomene, Arachis, Stylosanthes, Zornia*
	15. *Adesmieae*	*Adesmia*
	16. *Galegeae*	*Astragalus, Galega, Glycyrrhiza, Oxytropis*
	17. *Carmichaelieae*	*Carmichaelia*
	18. *Hedysareae*	*Hedysarum, Onobrychis*
	19. *Loteae*	*Anthyllis, Lotus*
	20. *Coronilleae*	*Coronilla, Ornithopus*
	21. *Vicieae*	*Vicia, Lathyrus, Pisum*
	22. *Cicereae*	*Cicer*
	23. *Trifolieae*	*Medicago, Melilotus, Trifolium, Trigonella*
	24. *Brongniartieae*	*Brongniartia*
	25. *Mirbelieae*	*Brachysema, Mirbelia*
	26. *Bossiaeae*	*Bossiaea*
	27. *Podalyrieae*	*Podalyria*
	28. *Liparieae*	*Liparia*
	29. *Crotalarieae*	*Crotalaria, Lotononis*
	30. *Euchresteae*	*Euchresta*
	31. *Thermopsideae*	*Piptanthus, Thermopsis*
	32. *Genisteae*	*Genista, Lupinus*

[a]Tribe 13. *Sesbanieae* is now included in tribe 7, *Robinieae*.

Revisions of legume taxonomy, particularly within the tribe *Cassieae* of the *Caesalpinioideae* (Irwin and Barneby, 1981) (Chapter 12) have converged with the distribution of the ability of legumes to nodulate to give a better understanding of evolutionary relationships. The *Caesalpinioideae* is accepted as the most primitive group and the *Papilionoideae* and *Mimosoideae* are likely to have evolved from a common, nodulated caesalpinioid ancestor (Sprent and Raven, 1992). Members of the *Papilionoideae* with persistent infection threads are presumed to represent an evolutionary link with the *Caesalpinioideae*. The caesalpinioid genus *Chamaecrista* is of particular note in relation to the evolution of nodule structure. Almost all of the species in this genus that have been examined form nodules, but the nodules differ markedly in morphology (Naisbitt *et al*., 1992) (Fig. 2.6). The central nodule cells in *C. ensiformis* contain persistent infection threads, whereas in *C. nictans* the bacteria are free in the nodule cells and resemble bacteroids in *C. nictitans.* Two further species, *C. flexuosa* and *C. desvauxii,* have infection structures that appear to be interediate, as if the history of nodule evolution can be revealed within this one legume genus.

An intriguing theory has been proposed to explain the occurrence of nodulation in the *Leguminosae* (McKey, 1994). A feature common to all legumes, which tends to differentiate them from most other families of plants, is the high concentration of N in their leaves. Although it has generally been assumed that these N-rich leaves are a consequence of N_2-fixation, this is a feature shared by all caesalpinioid legumes, including those that do not nodulate. McKey (1994) suggested that the ability to fix N_2 in association with bacteria actually evolved in legumes *in response* to the demands of maintaining high leaf N concentrations. A corollary to this theory is that legumes developed N-rich leaves to begin with because the efficiency of photosynthesis increases with tissue N concentrations.

Despite some extensive surveys of nodulation ability (e.g. de Faria *et al*., 1984, 1999; Athar and Mahmood, 1990; Moreira *et al*., 1992; Athar, 1997), there remains an enormous number of legumes (particularly in the tropics) whose capacity to nodulate has not been confirmed. A word of caution must be uttered about the occurrence of 'false nodulation' in which hypertrophies that may resemble nodules to the inexperienced eye are seen on roots (Allen and Allen, 1981); methods for distinguishing these from true root nodules are straightforward (Truchet *et al*., 1989). Clearly, knowledge of nodulation ability is of considerable importance in the choice of legumes for use in agriculture, particularly in the selection of trees for agroforestry. It certainly cannot be assumed that a plant must be able to nodulate and fix N_2 just because it is a legume.

Classification of rhizobia

The recognition that exploitation of N_2 by legumes is due to the presence of bacteria in the root nodules occurred over a century ago (Hellriegel and Wilfarth, 1888). In the same year, root nodule bacteria were isolated from nodules of a number of legume plants (Beijerinck, 1888), and pure cultures of the bacteria were shown to induce nodules when reinoculated on to the same host plants. These

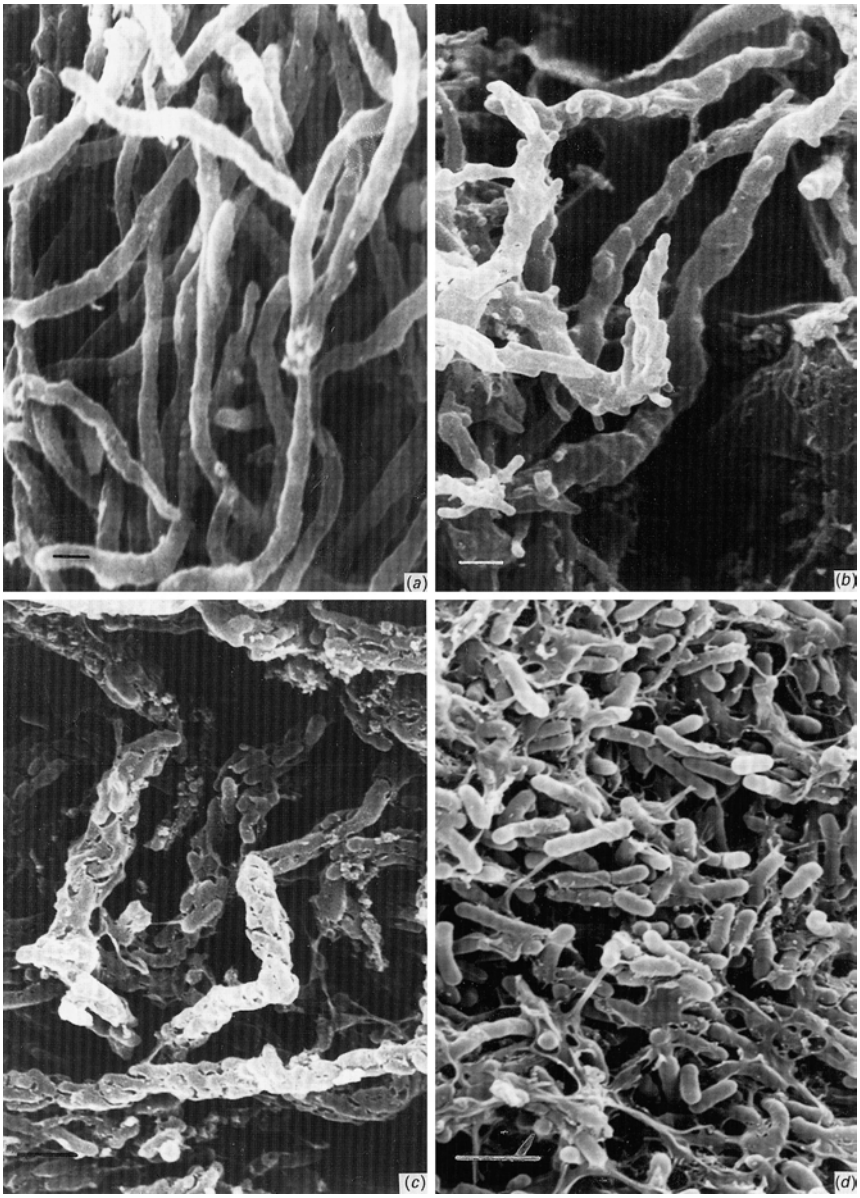

Fig. 2.6. Scanning electron micrographs of infected cells from nodules of four species within the caesalpinioid genus *Chamaecrista*. (a) *C. ensiformis* var. *ensiformis*, showing persistent infection threads. (b) *C. flexuosa*; bacterial outlines are visible through the infection thread sheath. (c) *C. desvauxii* var. *latistipula*; only a thin thread sheath covers the bacteria. (d) *C. nictitans* subsp. *nictitans*; bacteria appear free in the host cell. (From Naisbitt *et al.*, 1992.)

bacteria were named *Bacillus radicicola* by Beijerinck. The generic name *Rhizobium* was formally adopted in 1926 (Buchanan, 1926).

Cross-inoculation groups

Since 1888 there have been perhaps as many attempts at classification as there are strains of rhizobia, most of them based on the observation of host–rhizobial specificity. This led rapidly to the concept of a 'cross-inoculation group' (Fred *et al*., 1932) – a group of legume host species supposedly nodulated specifically by one set of rhizobial strains, and not by rhizobial strains that can induce nodules on legumes of a different cross-inoculation group. Thus, a cross-inoculation group defined not only a group of legumes but the corresponding rhizobial species. For example, under this classification, legumes of the *Melilotus* cross-inoculation group, including *Melilotus alba*, *Medicago sativa* and *Trigonella foenum-graecum*, are all nodulated by strains of *Rhizobium meliloti* and not by any other rhizobial species. Conversely, *R. meliloti* would not be expected to nodulate legume plants from, for example, the *Trifolium* cross-inoculation group.

The concept of cross-inoculation groups is clearly of practical use in choosing which rhizobial strains to inoculate on to particular legume crops, and it will be used on occasion in later chapters. It is also of some scientific value in raising the issue that there are particular groups of legume plants that tend to share common bacterial symbionts. However, the idea that all rhizobial–legume associations could be classified into neat, non-overlapping cross-inoculation groups has long been discredited – a paper published as long ago as 1944 had the glorious title: 'Over five hundred reasons to abandon the cross-inoculation concept' (Wilson, 1944). In point of fact, the range of legumes nodulated by any one rhizobial strain will generally overlap with, but be distinct from, that of another rhizobial strain of similar host-plant origin, and knowledge of host range may be heavily biased by the choice of host plants tested. Rhizobial strains have long been described as 'specific', for strains apparently restricted in their host range, or 'promiscuous', for strains with a very broad host range.

Many *Rhizobium* and *Bradyrhizobium* strains are so promiscuous that their host ranges do not even consist of closely related legumes, but may include legume plants that are so distantly related as to be placed in different subfamilies within the *Leguminosae*. For example, the fast-growing *Rhizobium* strain NGR234 (Trinick, 1980) has been shown to elicit nodules on over 112 genera of legumes, including members of the three subfamilies such as *Lablab purpureus* (*Papilionoideae*, and the plant from which the strain was originally isolated) and *Leucaena leucocephala* (*Mimosoideae*) and *Chamaecrista fasciculata* (*Caesalpinioideae*) (Pueppke and Broughton, 1999).

Another pitfall of the cross-inoculation concept is that it refers only to the capacity to nodulate a host plant, and pays no attention to fixation abilities. It is common to find strains of rhizobia that can elicit nodules on, say, ten different legume host species and yet in association with perhaps five of those host plants fix N_2 only weakly or not at all, a phenomenon that can be referred to as 'host-specific fixation' (Wilson *et al*., 1987). This phenomenon was well recognized from the early

days of *Rhizobium* research (e.g. Fred *et al.*, 1932). It indicates clearly that plant–bacterial recognition and specificity do not end with the initial induction of nodules, but continue right through nodule development, including stages such as bacteroid differentiation and nodule maintenance. All these later stages of specificity, which are crucial from the practical viewpoint of aiming for enhanced symbiotic yields of fixed N, are overlooked by the cross-inoculation concept, at least in its simplest form.

The cross-inoculation concept also fails to encompass the several known examples of specificity within a legume host species. For example, *Sinorhizobium fredii* strains can effectively nodulate wild soybean cultivars found in China, but frequently either are unable to induce nodules, or the nodules formed are ineffective, on the improved soybean cultivars found in North America (Keyser *et al.*, 1982; Scholla and Elkan, 1984). Although the germplasm from which the North American cultivars have been bred was originally imported from the Far East, there is only limited genetic diversity among commercially grown North American soybean varieties, and it is probable that either the original imported lines happened to have low symbiotic capacity with *S. fredii* strains, or the capability to effectively nodulate with these strains was lost during breeding.

Another well-known example concerns peas, and the symbiont *Rhizobium leguminosarum* bv. *viciae*. In this case, strains of this biovar found in western European soils are unable to nodulate wild pea varieties from Central Asia and the Middle East, including the pea cultivar 'Afghanistan'. However, isolates obtained from the same geographical location as these pea cultivars are fully capable of nodulating these genotypes. This has led to the hypothesis that rhizobia and legumes may become mutually adapted and form local, complementary gene pools that enable host genotypes and rhizobia derived from the same geographical region to form an effective symbiosis (Lie *et al.*, 1987).

An interesting evolutionary point is that the different genera of bacteria that nodulate legumes appear to have diverged before the origin of angiosperms, an observation that implies either that the symbiosis must have arisen independently more than once, or that genes required by the bacterial symbiont to induce nodules were passed from one genus to another by lateral transfer, as further discussed below.

Rhizobial taxonomy

Rhizobial taxonomy is currently in great flux with the proliferation of 16S rRNA analysis as well as numerical taxonomy on strains isolated from a diversity of tropical and temperate legumes. Sequence analysis of 16S rRNA (also referred to as small subunit rRNA, or SSU rRNA) has become the standard for defining bacterial groups at the genus level, although other methods, in particular DNA–DNA hybridization, are needed to distinguish species within genera (Young, 1996). Numerical taxonomy has also frequently been applied, in which phenotypic traits (primarily a battery of metabolic tests) are analysed and the results coded in a form amenable to computer analysis. However, in some cases conclusions from numerical taxonomy conflict strongly with more phylogenetic approaches (So *et al.*, 1994). The International Subcommittee for the Taxonomy of *Rhizobium* and *Agrobacterium* recommends

that a combination of these approaches be adopted when describing new genera and species of root- and stem-nodulating bacteria (Graham *et al.*, 1991). This combined approach is referred to as polyphasic taxonomy, in which phenotypic, genotypic and phylogenetic data are integrated (Vandamme *et al.*, 1996). Rhizobial taxonomy is reviewed in detail by Young and Haukka (1996), van Berkum *et al.* (1997) and Wang and Martínez-Romero (2000), and is discussed further below.

Rhizobium

The genus *Rhizobium* contains (by definition) the type species of the genus, *R. leguminosarum* Frank 1889 (Frank, 1889). It is now agreed to comprise three biovars (Jordan, 1984): biovar *viciae* nodulating *Pisum*, *Vicia*, *Lathyrus* and *Lens*; biovar *trifolii* nodulating *Trifolium*; and biovar *phaseoli* nodulating *Phaseolus*. These three biovars are all closely related, the different host ranges being conferred by large symbiotic plasmids, which are freely transferable between them.

Additional species in the genus *Rhizobium* include the *Phaseolus*-nodulating species *R. tropici* and *R. etli*. *R. tropici* forms effective symbioses with several tropical legumes, including *Leucaena* species; it possesses a single copy of the *nifH* nitrogenase subunit gene and is frequently adapted to growth in acid soils (Martínez-Romero *et al.*, 1991). *R. etli* included non-symbiotic isolates from rhizosphere soil of *Phaseolus vulgaris*; nodule isolates (designated bv. *phaseoli*) were originally considered to be restricted to *Phaseolus* in their host range and are characterized by reiterated *nifH* genes (Segovia *et al.*, 1994). Further investigations revealed that some strains of *R. etli* could indeed nodulate *Leucaena* and in fact had a host range as broad as *R. tropici* (Hernandez-Lucas *et al.*, 1995). Wang *et al.* (1999c) proposed a separate biovar, *R. etli* bv. *mimosae*, for strains that harbour a distinct symbiotic plasmid and nodulate *Mimosa affinis* as well as *P. vulgaris*.

Two further *Phaseolus*-nodulating *Rhizobium* species have been proposed: *R. gallicum* and *R. giardinii* (Amarger *et al.*, 1997). These species were isolated from French soils and each is divided further into two biovars, which differ in their host range and in the number of copies of the *nifH* gene (one biovar, *R. giardinii* bv. *giardinii*, does not have any detectable *nifH*-hybridizing sequence and is unable to fix nitrogen with *Phaseolus*). *R. mongolense*, isolated in Mongolia from nodules of *Medicago ruthenica* (van Berkum *et al.*, 1997), appears to be very similar to, and perhaps synonymous with, *R. gallicum.* A combined taxonomic approach indicated that *R. giardinii* is actually more closely related to *R. galegae* (see below) than to the other currently accepted *Rhizobium* species, whereas *R. gallicum* clearly clustered with the other bean-nodulating *Rhizobium* species (Amarger *et al.*, 1997). *R. huautlense,* which was described from a collection of strains from nodules of *Sesbania herbacea* in Mexico, lies even closer to *R. galegae* than *R. giardinii* (Wang *et al.*, 1998).

Another recent addition to the genus *Rhizobium* is a group of 13 tropical rhizobial strains isolated from diverse hosts from Hainan province in China. These were found to cluster together quite independently of other known rhizobial species, based on numerical taxonomy (Gao *et al.*, 1994), but further phylogenetic analysis has placed them firmly in the genus *Rhizobium* as *R. hainanense* (Chen *et al.*, 1997).

Sinorhizobium

This genus has had a slightly chequered history. It was originally proposed to accommodate two different groups of fast-growing strains isolated from soybean, *S. fredii* and *S. xinjiangensis*, largely on the basis of phenotypic analysis (Chen *et al.*, 1988). This new genus was then challenged on the grounds that the 16S rRNA sequences were not sufficiently different from the other *Rhizobium* 16S rRNA sequences then available, particularly that of *R. meliloti*, to warrant a new genus (Jarvis *et al.*, 1992). However, the continual isolation of new fast-growing species of rhizobia has revealed that there is indeed considerable genetic division between different groups of fast-growing strains and hence the genus was subsequently redefined to include *R. meliloti* (now *S. meliloti*) and two new species, *S. saheli* and *S. terangae*, which were isolated from nodules of *Sesbania* and *Acacia* species in Senegal (de Lajudie *et al.*, 1994). Nodules of *Medicago truncatula* grown in French soils yielded isolates described as *S. medicae* (Rome *et al.*, 1996) and a collection of rhizobia from legume trees in Sudan, principally comprising isolates from *Acacia* and *Prosopis*, led to the description of *S. arboris* and *S. kostiense* (Nick *et al.*, 1999a).

Mesorhizobium

The type species of *Mesorhizobium* is *M. loti* (Jarvis *et al.*, 1997), formerly *R. loti* (Jarvis *et al.*, 1982). *M. loti* was first described in the early 1980s, when it was already recognized to be quite distinct from the other *Rhizobium* species then defined. This difference was underscored once 16S rRNA sequences became available, as these indicated the genetic distinctness of *M. loti* (Jarvis *et al.*, 1986). However, it was not until the mid 1990s, when a number of further rhizobial species with clear affinities to *M. loti* were described, that a new genus, *Mesorhizobium*, was created to contain *M. loti* together with the former *R. huakuii* (isolated from *Astragalus*), *R. ciceri* and *R. mediterraneum* (both isolated from chickpea), and *R. tianshanense* (isolated from a range of legumes in China) now also renamed as *Mesorhizobium* species (Jarvis *et al.*, 1997), although the distinctions between some of these species are not clear (van Berkum and Eardly, 1998). Two other species have been described that belong to this genus: *M. plurifarium*, which contains strains from woody legumes isolated from Brazilian, Senegalese and Sudanese soils (de Lajudie *et al.*, 1998b); and *M. amorphae*, from nodules of *Amorpha fruticosa* in China (Wang *et al.*, 1999b).

Azorhizobium

There is still only one species in the genus *Azorhizobium*: *A. caulinodans*, the rhizobial species that induces both root and stem nodules on the legume *S. rostrata* (Dreyfus *et al.*, 1988). In a study of DNA homologies between 191 strains isolated from stems and roots of *S. rostrata* plants growing in Senegal and in the Philippines, 184 were found to belong to the genus *Azorhizobium*. The remaining seven strains were probably *Rhizobium* strains and did not induce effective nodules on *S. rostrata* stems. Among the *Azorhizobium* strains, 175 corresponded to *A. caulinodans*, according to DNA homology and other criteria. The other nine strains showed much lower DNA homology to the *A. caulinodans* type strain ORS571, and suggested the existence of a second genomic *Azorhizobium* species (Rinaudo *et al.*, 1991). One of the more

remarkable features of *Azorhizobium* strains is their ability to fix N_2 in the free-living state and actually to grow on the products of that fixation. Although *Bradyrhizobium* strains can be induced to fix N_2 in microaerobic conditions, they cannot actually use the products of this fixation, due to concomitant shut-down of activity of glutamine synthetase, the enzyme needed to assimilate ammonia (Chapter 3). Interestingly, 16S rRNA analysis places *Azorhizobium* closer to *Bradyrhizobium* than to the root-nodulating fast-growing genera (Young and Haukka, 1996) (Fig. 2.7).

Bradyrhizobium

Bradyrhizobium was the first rhizobial genus to be created in addition to *Rhizobium* (Jordan, 1982). It was erected to accommodate so-called 'slow-growing strains' of rhizobia and for 10 years contained only one named species: the soybean-nodulating *B. japonicum* (Jordan, 1982). The existence of at least two genetically divergent types of *B. japonicum* was recognized early on (Stanley *et al.*, 1985) and the group was subsequently split into two species with the creation of *B. elkanii* (Kuykendall *et al.*, 1992). A third genus of soybean-nodulating bradyrhizobia, *B. liaoningense*, was created in 1995 to accommodate exceptionally slow-growing strains (Xu *et al.*, 1995). Despite the early separation of *B. japonicum* as the soybean nodulating

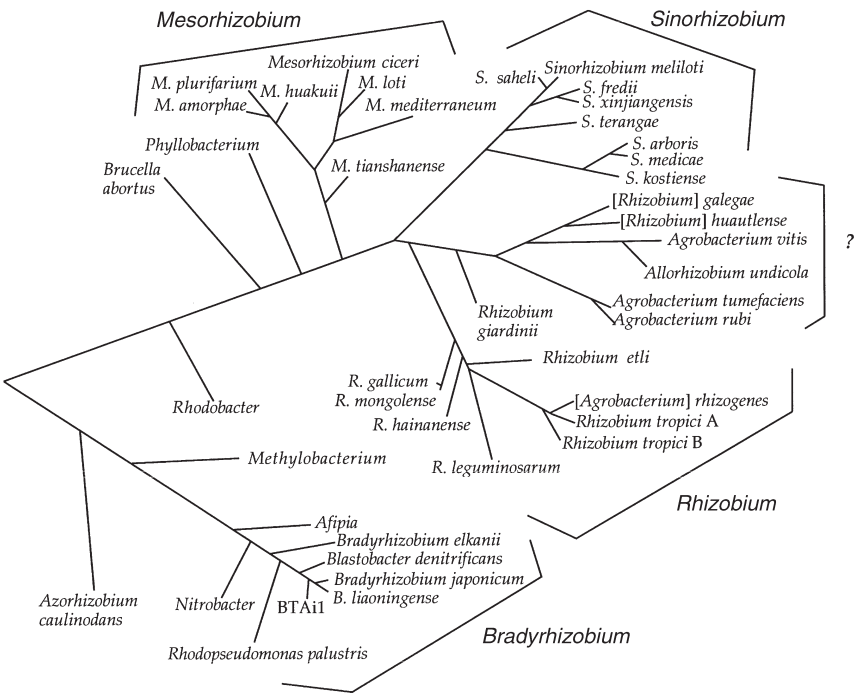

Fig. 2.7. Phylogenetic tree showing probable relationships between different rhizobial species and some related bacteria. (After Martínez-Romero and Caballero-Mellado, 1996; Wang and Martínez-Romero, 2000.)

rhizobia, the ability of some cowpea strains to nodulate soybean effectively has long been recognized (e.g. Sears and Carroll, 1927).

Although *Bradyrhizobium* strains isolated from plants other than soybean can be distinguished into a number of phylogenetic groups (e.g. Doignon-Bourcier *et al.*, 1999), there remain no named species. Such strains are designated *Bradyrhizobium* sp. followed by the name of the host plant from which they were isolated, as in *Bradyrhizobium* sp. (*Arachis*) strain NC92 for the strain depicted in Fig. 2.4 which was isolated from a groundnut plant (*Arachis hypogaea*). In the literature these *Bradyrhizobium* strains are sometimes referred to as members of the 'cowpea miscellany' because cowpea (*Vigna unguiculata*) is a test plant nodulated by many of these poorly characterized *Bradyrhizobium* strains. Some loose cross-inoculation groups of *Bradyrhizobium* strains can be identified (Thies *et al.*, 1991b).

The taxonomy of bradyrhizobia has been studied by 16S rRNA analysis, which reveals that all bradyrhizobia cluster tightly together, and lie on a branch of the α-proteobacteria that is quite distinct from the one containing fast-growing rhizobial genera. However, *Bradyrhizobium* strains are almost indistinguishable on this criterion from other bacteria with quite distinct phenotypes, most notably the photosynthetic bacterium *Rhodopseudomonas palustris* (Young and Haukka, 1996). This is of particular interest in light of the fact that 16S rRNA analysis of rhizobia that form stem-nodules on *Aeschynomene* species, and are also capable of photosynthesis, indicates that they are also bradyrhizobial strains (So *et al.*, 1994). *Bradyrhizobium* strains possess a single copy of the rRNA genes whereas fast-growing rhizobia may have two or three copies (Kündig *et al.*, 1995).

Differentiation between rhizobial genera

Table 2.2 summarizes the currently accepted species of rhizobia and Fig. 2.7 shows a taxonomic tree illustrating the relationships between them and some other bacterial groups, as determined by analysis of RNA genes. Until the 1990s just a few phenotypic characters, most notably growth rate, substrate utilization and free-living N_2-fixation, were used to distinguish between the (then) three accepted genera of rhizobia. However, the criteria for classification of new rhizobial genera and species have since been more tightly defined, and identification of species to genus level now requires detailed genetic as well as phenotypic characterization (Graham *et al.*, 1991). This raises the issue of how this is to be applied by the researcher or agronomist working in the field. This important dilemma is recognized by the Subcommittee on the Taxonomy of *Rhizobium* and *Agrobacterium* (Graham *et al.*, 1991), which

> . . . is in agreement with a phylogenetic focus but wants to ensure that those persons most likely to encounter unusual symbionts (field microbiologists and botanists) do not feel excluded from participation in taxonomic studies and have access to collaborators willing to undertake phylogenetic evaluations. Newly erected species must also be supported by phenotypic differences which can be used in strain identification.

In other words, while a new species can be erected only if there are clear genetic differences based on 16S rRNA sequence analysis and DNA–DNA hybridization,

Table 2.2. The current species of rhizobia, with examples of host plants.

Genus and species	Host plants	Reference
Allorhizobium		
A. undicola	*Neptunia*	de Lajudie *et al*. (1998a)
Azorhizobium		
A. caulinodans	*Sesbania rostrata*	Dreyfus *et al*. (1988)
Bradyrhizobium		
B. elkanii	*Glycine, Vigna*	Kuykendall *et al*. (1992)
B. japonicum	*Glycine*	Jordan (1982)
B. liaoningense	*Glycine*	Xu *et al*. (1995)
Mesorhizobium		
M. amorphae	*Amorpha*	Wang *et al*. (1999b)
M. ciceri	*Cicer*	Nour *et al*. (1994)
M. huakuii	*Astragalus*	Chen *et al*. (1991b)
M. loti	*Lotus*	Jarvis *et al*. (1982)
M. medicae	*Medicago*	Rome *et al*. (1996)
M. mediterraneum	*Cicer*	Nour *et al*. (1995)
M. plurifarium	*Acacia, Leucaena, Prosopis,* etc.	de Lajudie *et al*. (1998b)
M. tianshanense	*Glycrrhiza, Sophora, Glycine,* etc.	Chen *et al*. (1995)
Rhizobium		
R. etli	*Phaseolus, Mimosa*	Segovia *et al*. (1994)
R. galegae	*Galega*	Lindström (1989)
R. gallicum	*Phaseolus*	Amarger *et al*. (1997)
R. giardinii	*Phaseolus*	Amarger *et al*. (1997)
R. hainanense	*Centrosema, Desmodium, Stylosanthes, Tephrosia*	Chen *et al*. (1997)
R. huautlense	*Sesbania*	Wang *et al*. (1998)
R. leguminosarum		Jordan (1984)
bv. *phaseoli*	*Phaseolus*	
bv. *viciae*	*Vicia, Pisum*	
bv. *trifolii*	*Trifolium*	
R. mongolense	*Medicago, Leucaena*	van Berkum *et al*. (1997)
R. tropici	*Phaseolus*	Martínez-Romero *et al*. (1991)
Sinorhizobium		
S. arboris	*Acacia, Prosopis*	Nick *et al*. (1999a)
S. fredii	*Glycine* (and many others)	Scholla and Elkan (1984)
S. kostiense	*Acacia, Prosopis*	Nick *et al*. (1999a)
S. meliloti	*Melilotus*	Jordan (1984)
S. saheli	*Sesbania, Acacia*	de Lajudie *et al*. (1994)
S. terangae	*Sesbania, Acacia*	de Lajudie *et al*. (1994)
S. xinjiangensis	*Glycine*	Chen *et al*. (1995)

there also need to be clear phenotypic features that will allow a degree of identification of rhizobial strains.

Future genera of rhizobia?

The continual discovery of novel rhizobial strains will almost certainly lead to the continued need for new genera of root-nodulating bacteria. One group that has remained unresolved for quite some time is that of *R. galegae*, which was isolated from the root nodules of the perennial temperate legume *Galega* (Lindström, 1989). Based on phenotypic criteria and 16S rRNA sequence, it is quite distinct from the other *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* species (Young, 1996). However, as discussed above, the recently described *R. giardinii* and *R. huautlense* appear to cluster with *R. galegae*, and perhaps this will lead to the creation of a new genus for these species in the future. A new species and genus, *Allorhizobium undicola*, was created for strains from nodules of the aquatic legume *Neptunia natans* in Senegal (de Lajudie *et al*., 1998a), which appears to sit firmly among *Agrobacterium*, together with these three *Rhizobium* species. Unusual isolates from nodules of *Crotalaria* spp. in Senegal appear to belong to another genus, *Methylobacterium* (Sy *et al*., 2000), which lies somewhere between *Azorhizobium* and *Bradyrhizobium*.

The construction of phylogenetic trees based on 16S rRNA analysis has revealed the close relationships between the rhizobia and other soil-inhabiting bacteria with a wide range of functions. Attached to the 'bradyrhizobial branch' are photosynthetic N_2-fixing bacteria (see below), bacteria capable of reduction of nitrate in denitrification and species that can oxidize nitrite to nitrate, as well as mild pathogens such as the cat-scratch bacterium *Afipia felis*. The fast-growing bacteria are intermixed with *Agrobacterium* – over which there has been much debate about renaming of genera – as well as *Phyllobacterium* and the bacterium that causes brucellosis in cattle. Nodule isolates that are indistinguishable from *Agrobacterium* on the basis of their 16S rRNA sequences have been widely reported. Comparison of phylogenies based on the 23S rRNA gene sequences of a range of rhizobia and *Agrobacterium* strains led van Berkum *et al*. (2000) to question the validity of *Sinorhizobium* as a separate genus from *Rhizobium*. Martínez-Romero *et al*. (2000) indicated that the *Rhizobiaceae* is likely to be split up in future, and that new families will be formed to represent branches of the phylogenetic tree.

Many legumes are nodulated by rhizobia by several (if not all) of the main rhizobial genera even though these are clearly far apart in terms of their phylogenetic relationships. The host-range of rhizobia is much more closely mirrored if their similarity is compared on the basis of their nodulation genes (Young and Haukka, 1996; Haukka *et al*., 1998). This is perhaps hardly surprising, but illustrates that specific functions can be exchanged between bacteria, such as the ablity to nodulate and fix N_2 with particular legumes. One stark example of this occurred when a *Mesorhizobium* strain was inoculated into soils in New Zealand. Although the inoculated strain could not be recovered, *Lotus* plants were well nodulated and a range of other non-symbiotic rhizobia had acquired a 'symbiotic island' of DNA from the inoculant strain (Sullivan *et al*., 1995, 1996; Sullivan and Ronson, 1998). Phylogenetic analysis of the glutamine synthetase genes of rhizobia also provides strong

evidence for horizontal or lateral transfer of genes between the different genera of rhizobia (Turner and Young, 2000).

The utility of the phylogenetic classification of rhizobia is questionable in practical terms. To date no clear patterns have emerged that allow the ecology of the bacteria to be predicted from their taxonomic position, and perhaps this is too much to expect. As rhizobia from a wider range of hosts and regions are studied, the comments of Wilson (1944) on the difficulties of finding discrete and satisfactory groups for rhizobia become even more pertinent. It seems that we can broaden the host range even of species that are thought to be highly specific if sufficient legume species are tested, as shown for example with *R. etli*.

Given that probably only the tip of the iceberg has been uncovered as far as the diversity of rhizobia (and other bacteria) is concerned, it seems that the rush to name so many new species of rhizobia is rather hasty. Many of the new rhizobial species are described on the basis of a few dozen strains, or less, and the majority of the world's soils have not been explored for rhizobia at all. No doubt more species will be described in the near future, but the challenge of turning this into a predictive science remains large.

Actinomycetes that Fix N_2: *Frankia*–Plant Symbioses

The second group of bacteria that is known to form N_2-fixing symbioses with higher plants is the genus *Frankia*. These bacteria are taxonomically very different from all rhizobial strains. For a start, while all genera of rhizobia are Gram-negative bacteria, *Frankia* is a Gram-positive genus. One of the major differences between Gram-negative and Gram-positive bacteria is in the chemical composition and ultra-structure of the cell wall, and since surface features of a bacterium are likely to be important in a symbiotic interaction, this difference in itself could be significant in terms of the plant–bacterial symbiosis. The most striking difference, however, is in the growth habit. While rhizobia are all motile unicellular bacteria, *Frankia* strains are filamentous bacteria of the order Actinomycetes. This means that they show a mycelial growth, very similar in general terms to that of a fungal (and hence eukaryotic) mycelium. A *Frankia* strain was only obtained in pure culture for the first time in 1978 (Callaham *et al*., 1978), and even now cultures are very difficult and time-consuming to establish and maintain (Benson and Schultz, 1990). Most observations on the structure of free-living *Frankia* are derived from cultures and are well reviewed in Newcomb and Wood (1987).

In essence, a *Frankia* mycelium consists of a mass of hyphae that branch and anastomose (fuse) to form a dense mat. These hyphae are divided at regular intervals by transverse septa. Sporangia develop from the hyphae, both terminally and, in some strains, also at intervening positions. At these points the hyphae can be seen to enlarge and become divided first by transverse septa. Subsequently there is further division by longitudinal septa, forming a club-like sporangium in which each compartment is filled with spores that, on release and dispersal, can germinate to initiate a new *Frankia* mycelium.

Apart from the hyphae and the reproductive sporangia, the third structure that is characteristic of many *Frankia* strains is the vesicle (see Fig. 3.2). The vesicles are roughly spherical structures that arise from the hyphae on short stalks and are typically surrounded by a multi-layered lipid envelope (Harriot *et al.*, 1991). The development of vesicles in a *Frankia* culture was shown to be correlated with the ability to reduce acetylene (Tjepkema *et al.*, 1980), and subsequently immunochemical techniques were used to demonstrate that the enzyme nitrogenase is specifically located within the vesicles, at least in pure cultures of *Frankia* (Meesters, 1987). Circumstantial evidence also supports the idea that nitrogenase is localized to vesicles in the symbiotic state, although there is no conclusive proof that it does not occur in the hyphae as well (Huss-Danell, 1997). The latter must certainly be the case in *Casuarina* and *Allocasuarina* species where the N_2-fixing *Frankia* symbionts lack vesicles (see below). The main role that has been proposed for the vesicles is in protecting nitrogenase from O_2 damage (Torrey and Callaham, 1982). This hypothesis is further supported by the observation that the lipid content and concentration in the vesicle envelope vary in response to oxygen concentration, both in free-living cultures (Harris and Silvester, 1992) and in the symbiotic state (Kleemann *et al.*, 1994). The major lipid component of the extracellular envelope of vesicles is an unusual group of lipids called hopanoids, which derive from the *Frankia* symbiont but are not found in other, closely related but non-N_2-fixing genera of actinomycetes.

Infection of host plants by *Frankia* strains

Frankia strains can form nodules on host plants from at least eight families of angiosperms, and thus the capacity to form actinorhizal nodules appears to be less of a taxonomic specialization in the plant kingdom than the ability to form rhizobial root nodules. Almost all of the host plants are perennial woody dicots (shrubs and trees) and most actinorhizal plants are temperate species such as Alder (*Alnus* spp.) and various members of the *Myricaceae*, and therefore do not concern us in this book. The main tropical actinorhizal host plants come from the family *Casuarinaceae* – primarily members of the two genera *Casuarina* and *Allocasuarina*. As these species are able to grow in seemingly adverse conditions of high salinity, heavy metal concentrations and extreme pH, they are considered to have potential for agroforestry and land reclamation (Diem and Dommergues, 1990).

As with rhizobia, there are two main pathways of infection of actinorhizal root nodules: via root hairs or by direct intercellular penetration. Again, as with rhizobia, this appears to be host controlled, as the same *Frankia* strain can infect different host plants by the two different mechanisms (Berry and Sunnel, 1990). Similarly, nodule shape is host plant rather than *Frankia* strain dependent (Zhang and Torrey, 1985).

Root hair infection

In the root hair infection pathway, which has been described for *Myrica*, *Comptonia*, *Alnus* and *Casuarina* species (Berry and Sunnel, 1990), extensive deformation and

branching of root hairs is observed. *Frankia* infection occurs at a site where the root hair wall is deformed into a curl or fold, similar to rhizobial infection. Unlike rhizobia, because *Frankia* is a filamentous species, the infecting hyphae remain continuous with the external hyphae in the rhizosphere. Inside the root hair, however, they do become surrounded by the host cell plasmalemma and by host cell wall material – reminiscent of a legume infection thread. As infection from the rhizosphere proceeds, cells of the outer root cortex begin to divide and form a small swelling known as a prenodule. These cells are infected with the *Frankia* hyphae that have penetrated from the outside. Evidence suggests that N_2-fixation commences even within the prenodule, as some relevant plant and bacterial genes have been found to be expressed there (Franche *et al.*, 1998) and vesicles have been observed in the prenodules of *Alnus* spp. (Angulo Carmona, 1974).

Direct epidermal infection

This alternative mode of infection was first observed in *Elaeagnus* species (Miller and Baker, 1985) and has since been described for *Hippophäe*, *Ceanothus* and *Cercocarpus* species (Berry and Sunnel, 1990). In this case, *Frankia* hyphae penetrate the root directly between adjacent epidermal cells and spread through the root cortex in the intercellular spaces. No prenodule is formed and infection therefore occurs directly into cells of the developing primordium (see below).

Nodule development

Irrespective of the infection pathway, development of the mature actinorhizal nodule begins with initiation of a nodule meristem in tissue deep within the root, usually within the pericycle. This is the layer of cells that surrounds the central vascular tissue and is the site where lateral root meristems are initiated in uninfected roots. In fact actinorhizal nodules have a structure that is very similar to that of a lateral root: the vasculature is central in contrast to the peripheral vasculature that is characteristic of a legume root nodule. In some actinorhizal species (e.g. *Casuarina* spp.) a lateral root referred to as a 'nodule root' actually grows out of the end of the nodule.

The nodule primordium grows out from the root, with the active meristem at its tip – rather as in an indeterminate legume nodule – and cells just behind the meristem become infected, whether from the *Frankia* hyphae that lie in cells of the prenodule, or, in the case of direct epidermal infection, from the intercellular hyphae that entered via the root epidermis. As growth of the nodule continues outwards, the meristem may divide and then further subdivide, each separate meristem giving rise to a nodule 'lobe'. The individual lobes may be very distinct, as in the '*Myrica* type' nodules also formed by *Casuarina*, or they may constitute only small knobs, the whole nodule having a coralloid appearance as in the '*Alnus* type' nodules also formed by *Allocasuarina* (Fig. 2.8). In either case the whole nodule structure is believed to arise from a single infection event.

As with an indeterminate legume nodule, the *Frankia* nodule is divided into four zones. From the outer tip of the nodule progressing back to the root pericycle these are: the meristem; the infection zone; the fixation zone; and the senescence zone. Plant genes that appear to have a specific role in actinorhizal nodule formation

Fig. 2.8. (a) *Datisca cannabina* growing in Pakistan and (b) a *Datisca* actinorhizal root nodule (*Alnus* type). (Photographs: A.D.L. Akkermans.)

and the associated processes of N_2-fixation and assimilation have been identified in all zones except the meristem. These include genes encoding products that appear to have roles in the formation of the matrix in which the infection threads are embedded, in provision of photosynthate to *Frankia* and in assimilation of the fixed N by the plant (Franche *et al*., 1998).

Of particular interest is the identification in *Casuarina* of two separate haemoglobins (Appleby *et al*., 1988), the protein with a high affinity for O_2 that is believed to be responsible for providing O_2 to the actively respiring bacteroids in legume nodules while protecting the O_2-sensitive nitrogenase from detrimental levels of dissolved O_2 (Chapter 3). One, which is specifically active in the nodule, is closely related to that found in legume nodules. Remarkably, when the gene for this haemoglobin was introduced into the legume *Lotus corniculatus*, it was found still to be regulated in a nodule-specific manner (Jacobsen-Lyon *et al*., 1995). This suggests that, at least in this case, the genetic signals specifying the regulation of genes in a nodule are conserved between legume and actinorhizal nodules. This may make sense in light of a paper using a molecular taxonomic analysis to suggest that there may in fact be a common phylogenetic origin for the predisposition in angiosperms to form symbiotic N_2-fixing nodules (Soltis *et al*., 1995).

Within the fixation zone of the actinorhizal nodule, *Frankia* hyphae are found intracellularly, but always surrounded by encapsulating wall material and cell membrane, both deposited by the host plant. In most actinorhizal nodules, symbiotic vesicles are formed within the infected cells and, as discussed earlier, it is likely that these are the site of nitrogenase. There is a great deal of circumstantial evidence indicating that these are indeed the site of symbiotic nitrogenase activity (Huss-Danell,

1990), as has been demonstrated for vesicles formed in free-living cultures of *Frankia* (Meesters, 1987). However, one recent attempt to localize nitrogenase in an actinorhizal nodule using specific antibodies directed against nitrogenase, while clearly demonstrating that nitrogenase does occur in the vesicles, also indicated the presence of cross-reacting antigens outside the vesicles in the hyphae (Sasakawa *et al.*, 1988).

Nodules induced on *Casuarina* and *Allocasuarina* spp. are unusual in that only filamentous hyphae are seen within the nodules – no sporangia or vesicles have been observed, despite the fact that these nodules are clearly capable of N_2-fixation and despite the fact that strains isolated from *Casuarina* nodules do form sporangia and vesicles in pure culture (e.g. Zhang and Torrey, 1985). It has been observed that the walls of infected host plant cells and of adjacent uninfected cells in *Casuarina* nodules thicken and change in composition, and this may serve to keep the O_2 concentration sufficiently low that no additional mechanism of O_2 protection is necessary (Berg and McDowell, 1988). However, the discovery of haemoglobin in *Casuarina* nodules, and the fact that it appears to be expressed at high levels in *Casuarina* nodules in particular (Fleming *et al.*, 1987; Huss-Danell, 1997), suggests that this may play the major role in O_2 protection.

Stem nodulation

Nodules have been found at heights up to 7 m on the trunks of the tree species *Casuarina cunninghamiana* and *C. glauca*, growing on the island of Réunion in the Indian Ocean. These nodules had acetylene reduction activity of 0.77 μmol C_2H_4 h^{-1} g^{-1} dry weight (see Chapter 4), compared with values of 2.88 μmol C_2H_4 h^{-1} g^{-1} dry weight for root actinorhizal nodules on the same trees. An organism with the appearance of a filamentous bacterium was visible inside the stem nodules and the overall structure was very similar to that of typical root nodules on *Causuarina* species (Prin *et al.*, 1991). Crushed nodules were successfully used to induce aerial nodules on plants grown in the glasshouse, and some aerial nodulation even occurred when a purified *Frankia* strain was used as inoculum, confirming that these nodules are indeed aerial actinorhizal nodules (Prin *et al.*, 1992; Huss-Danell, 1997).

Classification and identification of *Frankia* strains

The genus *Frankia* was first named in 1886 (Brunchorst, 1886), at about the same time as the first identification of rhizobia as the N_2-fixing symbionts of legume nodules. The monogeneric family *Frankiaceae* was defined much later (Becking, 1970) and several new species of *Frankia* were proposed in addition to the original *Frankia alni*. At that time, however, no *Frankia* isolates had been obtained in pure culture and the taxonomy was based on the use of crushed nodule suspensions to inoculate test plants and hence to define new species according to cross-inoculation groups. The idea of defining *Frankia* species was later abandoned until more reliable distinguishing criteria could be identified (Lechevalier, 1984); instead, it was agreed

that all strains should be named *Frankia* sp. with a clearly defined catalogue number (Lechevalier, 1983).

Other useful criteria for taxonomic purposes gradually emerged using *Frankia* strains in pure culture. These included the chemical composition of the cell wall (Lechevalier and Lechevalier, 1990), total fatty acid composition (Simon *et al.*, 1989), and protein electrophoretic patterns and isozyme profiles (e.g. Gardes *et al.*, 1987). These criteria, combined with infection tests, showed that identified *Frankia* strains fell into two broad groups, the *Alnus* and the *Elaeagnus* groups, differing in host range and in many biochemical features (St-Laurent *et al.*, 1987). Lalonde *et al.* (1988) proposed naming two species, *Frankia elaeagni* and *Frankia alni*, with the latter divided into subsp. *pommerii* and *vandijkii*. These species are now broadly accepted, but probably do not cover all of the diversity within the genus *Frankia* (Lechevalier, 1994).

Molecular techniques have been applied widely to classification of *Frankia*-like microorganisms (e.g. Fernandez *et al.*, 1989; Hahn *et al.*, 1989b; Nazeret *et al.*, 1991). DNA–DNA hybridization has led to the identification of at least 23 independent genomic species of *Frankia* (that is, species that share more than 70% DNA homology) (see Table 1 in Lechevalier, 1994). Analysis of 16S rRNA sequences initially led to the non-symbiotic genera *Geodermatophilus* and *Blastococcus* being grouped into the family *Frankiaceae* (Hahn *et al.*, 1989b); subsequent more extensive 16S analysis led to these two genera being excluded from the *Frankiaceae* again (Normand *et al.*, 1996). The region of the 16S rRNA gene chosen for analysis in the genus *Frankia* has been critical, due to an unexpectedly high diversity of sequences among *Frankia* strains (Hahn *et al.*, 1989b; Harry *et al.*, 1991). In some cases 16S rRNA analysis has supported the groupings of genomic species obtained by DNA–DNA hybridization, whereas in others it has provided contradictory data (Lechevalier, 1994). This diversity has proved useful in enabling the design of DNA probes that can be used to discriminate between different strains of *Frankia* (Hahn *et al.*, 1989a, 1990).

An extensive 16S rRNA study, which included analysis of strains from nodules that cannot be obtained in pure culture, suggested four different groupings of *Frankia* strains. The first includes *F. alni* and related strains, including *Casuarina* and a *Myrica* symbiont; the second comprises unisolated symbionts of *Dryas*, *Coriaria* and *Datisca*; the third is *Elaeagnus*-infective strains; and the final group contains 'atypical' strains, e.g. one that infects *Alnus* but does not fix nitrogen in symbiosis (Normand *et al.*, 1996).

Cross-inoculation groups of Frankia

As mentioned above, attempts have been made to define *Frankia* species according to the host species that they infect (Becking, 1970), following the (rather problematic!) precedent set in the world of rhizobia. One of the more surprising difficulties hindering these attempts has been the fact that strains isolated from the nodules of a given species often do not even reinfect that species. For example, a majority of cultures isolated from nodules of *Casuarina* species cannot be induced to infect the same *Casuarina* species in the greenhouse, although many of them will readily infect

Elaeagnus species (Torrey and Racette, 1989). In some cases strains that show typical *Frankia* morphology have been isolated from nodules, but have not been induced to reinfect any host plant in controlled culture (Hahn *et al.*, 1989a; Mirza *et al.*, 1991). While such observations may initially seem to cast doubt on the identification of an isolate as a *Frankia* strain, suggesting that they could be *Frankia*-like contaminants, there are other possible explanations. For example, infection by a *Frankia* strain may require the action of some helper microorganism such as a fungal or bacterial species that is present in soil but not in axenic culture where the test is carried out.

Another observation that complicates the concept of cross-inoculation groups is that, no matter what taxonomic criteria are used, it is clear that a remarkable diversity of *Frankia* strains can be isolated from a single nodule (Rouvier *et al.*, 1996). On the other hand, these taxonomic approaches, while generally failing to distinguish clear species, indicate that strains tend to cluster together according to the host plant of origin, lending some weight to a loose cross-inoculation concept. An observation that is of relevance here is that nodulation of *Casuarina* and *Allocasuarina* species tends to be relatively specific, as they are only infected by strains that have been isolated from these hosts (Reddell and Bowen, 1986; Torrey and Racette, 1989). Thus strains and sources of inoculum for use with *Casuarina* species in the field should be selected with considerable care.

Cyanobacteria

Cyanobacteria are Gram-negative eubacteria with the distinguishing characteristic of carrying out oxygenic photosynthesis; that is, they possess not only photosystem I, like all photosynthetic organisms, but also photosystem II, which is otherwise found only in eukaryotic chloroplasts. It is PSII that obtains electrons for re-reduction of oxidized chlorophyll by splitting water and thereby generates O_2; non-cyanobacterial photosynthetic eubacteria obtain the necessary electrons from sources other than water (e.g. H_2S).

Cyanobacteria are also unusual among photosynthetic bacteria in possessing chlorophyll *a*, exactly the same light-harvesting pigment as that found in photosynthetic eukaryotes, in contrast to the bacteriochlorophyll *c* present in other photosynthetic bacteria. Because of these similarities to photosynthetic eukaryotic organisms, cyanobacteria were once considered to be an intermediate group between eukaryotes and prokaryotes – and this is reflected in their common name, the blue-green algae.

The main classification system now used for cyanobacteria was devised by Rippka *et al.* (1979), with the aim of providing a more rigorous basis than the field-based system that had earlier been used to classify cyanobacteria under the botanical code (Stafleu *et al.*, 1972). This publication is highly recommended for anyone wishing to identify particular cyanobacteria, because of the wealth of detail on methods, and the extensive illustration provided in both diagrams and photographs.

Rippka's classification divided 178 living strains into 22 genera, based on morphological and developmental features that could readily be determined in cultured material and that were, as far as possible, constant for a given strain (because the previous classification had been botanical in nature, it had been based on descriptions of dead type-specimens). These 22 genera were placed in five main sections, as described in Table 2.3. As indicated, one of the primary morphological criteria used in this classification is whether the strains are unicellular or filamentous in nature.

Most cyanobacteria are obligate photoautotrophs; that is, they must fix their own carbon by photosynthesis and cannot grow by heterotrophic metabolism of existing sources of organic carbon. This obligate photoautotrophy is probably due to an inability of cyanobacteria to take organic carbon sources into the cell, rather than to a lack of enzymes for the metabolism of these compounds. Facultative

Table 2.3. The major taxonomic groups of cyanobacteria (after Rippka *et al.*, 1979; see also Castenholz and Waterbury, 1989).

Cell arrangement	Group	Reproduction	Heterocysts	Division	Genera
Unicellular forms	I				*Gloeothece* *Gloeobacter* *Gloeocapsa* *Synechococcus* *Synechocystis* *Chamaesiphon*
	II	Multiple fission, possibly also with binary fission			*Dermocarpa* *Xenococcus* *Dermocarpella* *Myxosarcina* *Chroococcidopsis* *Pleurocapsa* group
Filamentous forms (cells form a trichome)	III	Intercalary cell division and trichome breakage	No	One plane	*Spirulina* *Oscillatoria* *Pseudanabaena* *Lyngbya* *Phormidium* *Plectonema*
	IV	As above, plus may form homogonia	Yes	One plane	*Anabaena* *Nodularia* *Cylindrospermum* *Nostoc* *Scytonema* *Calothrix*
	V	As section IV	Yes	More than one plane	*Chlorogloeopsis* *Fischerella*

heterotrophy in certain strains is one of the taxonomic criteria used by Rippka *et al*. (1979), who presented data on individual strains.

N_2-fixing species are found among members of all five taxonomic sections of the cyanobacteria. This widespread distribution of N_2-fixation ability is initially surprising because, as discussed above, cyanobacteria carry out oxygenic photosynthesis. This creates a problem for N_2-fixation, as O_2 is invariably damaging to nitrogenase activity, and mechanisms adopted by cyanobacteria for protecting nitrogenase from O_2 are discussed further in Chapter 3. The ability to carry out N_2-fixation in anaerobic conditions was analysed and is reported for all strains described by Rippka *et al*. (1979). A number of free-living species of cyanobacteria are of possible importance in tropical cropping systems and these are discussed further in Chapter 6. Symbiotic species are found in group IV and are primarily, if not exclusively, *Nostoc* species. A key characteristic of group IV strains is the ability to differentiate specialized cells for N_2-fixation, known as heterocysts (described further in Chapter 3). The key cyanobacterial symbiosis of importance in tropical cropping systems is that with *Azolla*.

The *Azolla*–cyanobacterial symbiosis

Azolla Lam. is a genus of aquatic ferns, members of which occur throughout the world. The ferns are usually found free-floating on the surface of the water. The seven recognized species of *Azolla* are divided into two subgenera (Stergianou and Fowler, 1990; Saunders and Fowler, 1993). Subgenus *Azolla* contains two sections: section *Azolla* containing *Azolla caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla* and *A. rubra*; and section *Rhizosperma*, which contains only *A. pinnata*, within which two susbspecies are recognized, *A. pinnata* subsp. *pinnata* and *A. pinnata* subsp. *imbricata*. The second subgenus, *Tetrasporocarpia*, contains only *A. nilotica*. A combination of morphological and molecular data indicates that *A. caroliniana*, *A. mexicana* and *A. microphylla* are very close taxonomically, as are *A. filiculoides* and *A. rubra*, although there has been no formal reclassification (Van Coppenolle *et al*., 1995a,b). All species of *Azolla* contain a heterocystous N_2-fixing cyanobacterial symbiont, referred to as *Anabaena azollae* Strasburger and placed in the order *Nostocales*, family *Nostocaceae*.

The *Azolla* cyanobacterial symbiont is still formally referred to as an *Anabaena* species, *Anabaena azollae*. However, there is now considerable evidence supporting the contention that the *Azolla* symbiont is in fact a *Nostoc* species proper, the genus to which all other symbiotic cyanobacteria are assigned. The primary taxonomic criterion used to assign cyanobacteria to this genus is the formation of homogonia filaments (Rippka *et al*., 1979), and the *Azolla* symbiont does form such structures at specific developmental stages of the symbiosis (Peters and Meeks, 1989). In addition, DNA probe techniques were used to analyse cyanobacterial symbionts isolated from *Azolla* species and it was found that the symbionts were indeed more closely related to a free-living *Nostoc* species than to a free-living *Anabaena* species by these criteria (Plazinski *et al*., 1990). However, the fact that the symbionts of *Azolla* appear to be

obligate, i.e. it has never been possible to culture them and use the cultures to reinfect *Azolla*, makes taxonomic classification very difficult. Thus, there has been no formal taxonomic reclassification of the *Azolla* symbiont and the name *Anabaena azollae* continues to be used.

Molecular techniques have been used to look at genetic variation in the symbionts associated with the different *Azolla* species. These experiments are conducted by extracting microbial symbionts/endophytes from the *Azolla* fern and analysing these directly without any culture. Hence there is no guarantee that a single microbial species is being examined – in fact this is highly unlikely. Nevertheless, DNA and fatty acid composition analysis methods have given remarkably concordant results, indicating that there are characteristic differences in the symbionts associated with each species. In particular, symbionts from *A. nilotica*, *A. pinnata* (section *Rhizosperma*) and *A. rubra* cluster together and those from *A. filiculoides*, *A. microphylla*, *A. mexicana* and *A. caroliniana* cluster together, symbionts from the latter three forming a very tight cluster consistent with the idea that they may in fact constitute a single species. These data have led to the suggestion that there may have been coevolution of host and symbiont (Caudales *et al.*, 1995; Rasmussen and Svenning, 2000).

The symbiont remains associated with *Azolla* throughout the life cycle of the fern, being located in specialized cavities in the upper surface of the leaves. The *Azolla* leaves themselves are only 1–3 cm in diameter and occur in tight clusters, this dense packing enabling formation of the typical dense mats of *Azolla* seen on the still surfaces of ponds, drainage ditches and paddy fields (Fig. 2.9). These mats may be reddish or purple in colour due to the presence of anthocyanins. *Azolla* is not able to colonize turbulent waters because the mats become fragmented and cannot grow vigorously enough.

The structure of the fern is based around the floating stem or rhizome. On the surface of the water are borne alternately arranged leaves, and adventitious roots arise from the rhizome beneath the water surface. During growth of new leaves the *Anabaena* colonization is maintained from a colony of undifferentiated *Anabaena* filaments that is located at the tip of each stem. As a new leaf differentiates, some of this apical colony becomes transferred into the cavity of the developing leaf. This process is facilitated by a structure called the primary branched hair, a multicellular epidermal trichome that extends from the new leaf primordium into the apical *Anabaena* colony.

The mature leaf cavity contains two such branched hairs and about 25 simple hairs, all with ultrastructure as seen in transfer cells, and these may function both in provision of carbon to the symbiont and in assimilation of ammonia by the host (Calvert *et al.*, 1985). The *Anabaena* filaments within the mature leaf cavity show differentiation of 20–30% of cells into heterocysts – a higher proportion than observed in the free-living strains – and have high nitrogenase activity.

While most reproduction of *Azolla* is by vegetative means, and thus poses no problem for maintenance of the cyanobacterial symbiont, *Azolla* does sporulate, if unpredictably. When this occurs, filaments of the *A. azollae* microsymbiont are incorporated into the developing micro- and megasporocarps, and their presence

Fig. 2.9. The *Azolla–Anabaena* symbiosis. (a) A dense mat of *Azolla pinnata* fronds in Rwanda. (b) A single *A. pinnata* frond. (c) Transverse section through an *A. pinnata* frond showing symbiotic *Anabaena* in the leaf cavity. (d) Scanning electron micrograph of *Anabaena azollae* filaments with heterocysts in *Azolla* leaf cavity. (Photographs b, c, d: J.H. Becking.)

in the megasporocarps on fertilization and subsequent germination of a fresh sporophyte ensures continuity of the symbiosis. This life cycle makes reinfection by free-living cyanobacteria unnecessary, and, as discussed above, it is not certain whether the true *Azolla* symbiont is capable of free-living growth.

Free-living and Endosymbiotic Bacteria

There are several free-living bacteria, in addition to the free-living cyanobacteria described above, that have been implicated in making significant contributions to plant productivity in tropical cropping systems. The extent of this contribution, and indeed whether such a contribution is actually due to N_2-fixation, is the subject of considerable dispute and will be debated in Chapter 6. This section simply describes the organisms.

In recent years attention has focused on a group of bacteria that are clearly endophytic and are also capable of N_2-fixation. These are Gram-negative bacteria of the genera *Acetobacter*, *Azoarcus*, *Azospirillum* and *Herbaspirillum*. Although the contribution of such bacteria to the nitrogen economy of the host plants remains to be proved, a number of scientists consider the contribution to be substantial, particularly in the case of endosymbiotic bacteria of sugarcane (e.g. Boddey, 1995; Döbereiner *et al.*, 1995).

The term endophytic bacteria has been used in a number of different ways, but the generally accepted definition now is the most straightforward – that is, 'bacteria found within tissues internal to the epidermis' (Kloepper *et al.*, 1992). This definition takes no account of whether such bacteria are beneficial to the host plant, of neutral effect or pathogenic. The endophytes can then be further defined as to the tissues that they inhabit; for example, the term 'xylem-limited bacteria' or 'XLB' is used to refer to bacteria found only within xylem tissue. It should be emphasized that, unlike rhizobia, these bacteria are not necessarily intracellular – a good number are found only in the apoplast or intercellular spaces. Endophytic bacteria can be further divided into facultative endophytes, which are also quite capable of surviving in the free-living state in the soil, and obligate endophytes, which appear to require the host-plant environment for their survival.

Azospirillum

Perhaps the most well-known genus of such bacteria is *Azospirillum* (Tarrand *et al.*, 1978). *Azospirillum* species are Gram-negative, aerobic, highly motile bacteria found in close association with the roots of various plant species (in a few cases even endophytically, see above). To date five different species have been described: *A. lipoferum* ('fat-bearing'); *A. brasilense* ('pertaining to Brazil') (Tarrand *et al.*, 1978); *A. amazonense* ('pertaining to the Amazon') (Falk *et al.*, 1985); *A. halopraeferans* ('salt preferring') (Reinhold *et al.*, 1987); and *A. irakense* ('pertaining to Iraq') (Khammas *et al.*, 1989; Bally *et al.*, 1992). Although *Azospirillum* species are aerobic, they can only fix N_2 in microaerobic conditions because they have no specific mechanism for protecting nitrogenase from O_2 damage. Because of this, when grown in semi-solid

N-free medium they show growth of a characteristic 'pellicle' – a balloon-like pattern of growth at the area below the surface where the concentration of O_2 is sufficiently low to allow N_2-fixation.

Strains from the first three species of *Azospirillum* have been isolated from the rhizosphere of roots of all the major cereal crops in Africa and Latin America. These include maize, sorghum, wheat, rice and millet (reviewed in Döbereiner and Pedrosa, 1987). *A. halopraeferans* was found associated with the roots of Kallar grass, a salt-tolerant grass grown in saline soils in Pakistan (Reinhold *et al.*, 1987). Although azospirilla are primarily discussed in the context of cereal crops, there is also evidence concerning the colonization of non-cereal plants (Bashan *et al.*, 1991).

Most *Azospirillum* strains colonize the plant exterior but there are some that are endophytic in root tissue. The location of specific strains within plant tissues has been confirmed with the use of monoclonal antibodies (Schloter *et al.*, 2000) and specific fluorescent rRNA probes (Assmus *et al.*, 1995). This phenomenon appears to be strain- rather than species-specific and has been observed with a variety of *Gramineae*, including sugarcane.

Herbaspirillum

H. seropedicae was the first diazotrophic endosymbiont identified. It could be isolated from rhizosphere soil and surface sterilized roots of maize, sorghum and rice, but not from uncropped soil (Baldani *et al.*, 1986a). It shares several characteristics in common with *Azospirillum*, including formation of pellicles in semi-solid media, but is differentiated from *Azospirillum* by being smaller in size with more than one flagellum, and DNA homology experiments have confirmed that it should be placed in a separate genus (Falk *et al.*, 1986). It appears to be primarily a root-inhabiting endophyte, having been isolated from the roots of 13 different graminaceous species, and has also been isolated from the stems and leaves of maize and rice and from the stems of sugarcane (James and Olivares, 1998). Curiously, it is able to fix N_2 in the presence of high concentrations of sucrose (up to 10%) but is unable to use sucrose as a carbon source, hence the benefit that *Herbaspirillum* obtains by inhabiting plant tissues remains unclear. Indeed one theory suggested that *Herbaspirillum* grows on organic acids produced by other endosymbionts, such as *Acetobacter diazotrophicus* (James and Olivares, 1998).

Two further species of *Herbaspirillum* have been identified: *H. rubrisubalbicans*, also an N_2 fixer; and a third, non-fixing species referred to only as *Herbaspirillum* 'species 3' (Baldani *et al.*, 1996). The latter is found primarily in clinical isolates and only very rarely in plants. *H. rubrisubalbicans*, formerly known as *Pseudomonas rubrisubalbicans*, is a mild plant pathogen of certain cultivars of sugarcane and sorghum. Both endophytic *Herbaspirillum* species are found in the xylem and *H. rubrisubalbicans* can also be found in the adjacent apoplast (intercellular spaces) of host plants. *H. seropedicae* can produce mottle stripe disease on sorghum and *Pennisetum* (Pimentel *et al.*, 1991). The pathways of infection are not certain, but it appears that *Herbaspirillum* species infect the roots of host plants either from the rhizosphere or they are carried within the seed coat, entering the roots via cracks in the epidermis at lateral root junctions (Chapter 6). Entry into the xylem can also

occur at these lateral root junctions where the endodermis surrounding the stele (the pericycle) is disrupted, allowing passage from the root cortex into the vascular tissue. The bacteria can then be carried to the aerial parts of the plant in the transpiration stream, and it is possible that they may become incorporated into the seed, a common means of transmission of plant-associated bacteria (McInroy and Kloepper, 1995; James and Olivares, 1998).

Acetobacter

Acetobacter is a genus of Gram-negative microaerobic bacteria characterized by an ability to grow at low pH and to form acetic acid from ethanol. There is only one N_2-fixing member of this genus known, which is hence called *A. diazotrophicus*. *A. diazotrophicus* was isolated from sugarcane and was first thought to constitute a new bacterial genus, '*Saccharobacter nitrocaptans*' (Cavalcante and Döbereiner, 1988), but further molecular analysis showed that it was an *Acetobacter* sp. (Gillis *et al*., 1989). Its host range is much more limited than *Herbaspirillum*, being thus far isolated only from sugarcane, the grass *Pennisetum purpureum* and sweet potato, all of which are very rich in either sugar or starch. Like *Azospirillum* and *Herbaspirillum*, *A. diazotrophicus* is best grown in semi-solid medium, where it forms a pellicle. It can tolerate sucrose concentrations up to 30% and is capable of using sucrose as a carbon substrate, although it turns out that this is due to secretion of an enzyme that hydrolyses the sucrose to glucose and fructose extracellularly (Alvares and Martinez-Drets, 1995). *A. diazotrophicus* does not contain nitrate reductase, and is able to fix N_2 in the presence of levels of nitrate as high as 25 mM. This may mean that it can continue to fix N_2 in the host plant, even while the host is assimilating nitrate directly from the soil, thus potentially providing the host plant with two sources of N. Within the plant, *A. diazotrophicus* has been observed both in the xylem and in the adjoining apoplast. Its presence in the xylem may seem unexpected, given the low sucrose levels available there, but it is thought that the low pO_2 in the xylem may favour this tissue as a location for diazotrophic bacteria (James and Olivares, 1998). The main method of transmission of the bacteria from generation to generation of host plant is within the sets of sugarcane, due to the fact that vegetative propagation is the norm for cane. However, evidence also suggests that *A. diazotrophicus* can infect plants by much the same means as *Herbaspirillum* (see above), including the possibility of being seed borne. An additional possible route of transmission for *A. diazotrophicus* is apparently via sap-feeding insects, including mealy bugs and leaf hoppers, as the bacteria have been isolated from a number of members of both groups of insects.

Other heterotrophic N_2-fixing bacteria that associate with plants

Several other groups of N_2-fixing bacteria have been described in association with the roots of different crop plant species. Members of the genus *Azotobacter* are free-living bacteria with the remarkable ability to fix N_2 aerobically (Chapter 3). The two best-known species, *A. chroococcum* and *A. vinelandii*, have a rather strict requirement for neutral pH conditions and thus are not abundant in tropical soils except in a few near-neutral soils in the humid tropics (Döbereiner and Pedrosa, 1987). One species,

A. paspali, has been found in association with the roots of one specific ecotype of *Paspalum notatum*, a subtropical invasion grass (Döbereiner *et al.*, 1972), where the requirements for high pH appear to be satisfied specifically in the rhizosphere. A further species has been described: *Azotobacter salinestris*, which was isolated from saline soils in Canada and Egypt (Page and Shivprasad, 1991).

Studies of bacteria associated with the roots of Kallar grass (*Leptochloa fusca*) led to the discovery of *Azoarcus*, of which two species have been described: *A. indigens* and *A. communis* (Reinhold-Hurek *et al.*, 1993). *Azoarcus* is found deep within the grass tissues, where it colonizes the vascular system and aerenchyma (Hurek *et al.*, 1993, 1994), and produces nitrogenase (Egener *et al.*, 1998; Reinhold-Hurek and Hurek, 1998). *Azoarcus* species also occur as endophytes in rice (Engelhard *et al.*, 2000).

Members of the aerobic N_2-fixing genus *Beijerinckia* are more tolerant of low pH and are therefore more common in tropical soils, where acid conditions often prevail. They occur much less abundantly in temperate regions and this may be due either to the pH conditions or to other aspects of mineral nutrition provided by tropical soils (Becking, 1961a,b). They show characteristic slow growth on N-free agar media, with colonies appearing only after 8–10 days incubation. Four species are recognized: *B. indica* and *B. mobilis* (Derx, 1950), *B. derxii* (Tchan, 1957) and *B. fluminensis* (Döbereiner and Ruschel, 1958). Both *B. indica* and *B. fluminensis* have been found associated with the rhizosphere of sugarcane and other plants (Döbereiner and Pedrosa, 1987).

A further group of aerobic N_2-fixing bacteria of possible significance in tropical soils is the *Derxia* species, such as *D. gummosa*. These were first isolated from a soil in western India (Jensen *et al.*, 1960) and have been found in soils throughout the tropics, but there is no evidence suggesting that they form specific associations with any plant roots. Moreover, although they are described as aerobic N_2-fixing bacteria, in culture they do not fix N_2 until very large (20 mm diameter and 10 mm height) gelatinous colonies form, inside which the environment is presumably microaerobic.

Several species of truly microaerobic N_2-fixing bacteria have also been described as associating with plant roots. These include other diazotrophic members of the Spirillaceae (the family that contains *Herbaspirillum*) which have been found associated with roots, such as *Aquaspirillum fasciculus*, *A. perigrenum* and *Campylobacter nitrofigilis*. The latter was isolated from roots of *Spartina alterniflora* in a salt marsh in Canada, and is tolerant of 7% NaCl (McClung *et al.*, 1983).

Strains that are classified as *Pseudomonas* species are known to be very common rhizosphere organisms, and some strains assigned to this genus possess the ability to fix N_2 (e.g. Bally *et al.*, 1983; Barraquio *et al.*, 1983; Watanabe *et al.*, 1987b; Jenni *et al.*, 1989). On the other hand, it is widely acknowledged that the genus *Pseudomonas* is a 'dumping ground' for aerobic, polarly flagellated, Gram-negative, rodlike bacteria of uncertain affinity (de Vos *et al.*, 1989; Young, 1992; Chan *et al.*, 1994). Anyone who has tried to identify a new bacterial strain they have isolated may well have shared the experience of identifying it as a '*Pseudomonas*' species. Clearly extreme caution has to be exercised before assigning an unknown bacterium to this genus. The reclassification of the plant pathogen *Pseudomonas rubrisubalbicans* as *Herbaspirillum* presents one example, as already discussed. Other N_2-fixing bacteria

isolated from cereal rhizospheres were identified as *Pseudomonas cepacia* but have since been transferred to the genus *Burkholderia*, which contains a number of species not known to fix N_2. Isolates from rice rhizospheres in Vietnam were named *B. vietnamiensis* (Gillis *et al*., 1995), and a further group of isolates from Vertisols in Martinique have been named as *B. carribensis* (Achouak *et al*., 1999b). Strains of *B. cepacia* have been used successfully in biocontrol of fungal 'damping off' with maize (Hebbar *et al*., 1998), but others are better known as causal agents of the debilitating human disease, cystic fibrosis. In fact some of the most highly transmissible pathogenic clones of *B. cepacia* are grouped by RFLP analysis of their 16S rRNA genes with *B. vietnamiensis* (Segonds *et al*., 1999). Given the severity of cystic fibrosis, use of *Burkholderia* as crop inoculants seems unlikely to be worth the potential risks.

Some members of the *Enterobacteriaceae* also possess the ability to fix N_2, the most notable example in the scientific literature being *Klebsiella pneumoniae*. N_2-fixing bacteria reported as being *Klebsiella* or *Enterobacter* species have been isolated from the rhizosphere of rice (Ladha *et al*., 1983), and an N_2-fixing enteric bacterium, *Rahnella aquatilis*, has been isolated from the rhizosphere of rice and maize (Berge *et al*., 1991), but, in general, members of the *Enterobacteriaceae* do not appear to be of major significance in agriculture. Of 18 strains of *Erwinia herbicola* and 16 strains of *Enterobacter agglomerans* tested, only four of the former and two of the latter were found to possess nitrogenase activity in an acetylene reduction assay (Papen and Werner, 1979). No species of *Escherichia* or *Salmonella* have been shown to possess endogenous nitrogenase activity, although *E. coli* strains with the ability to fix N_2 have been constructed by the transfer of the *nif* gene cluster from *K. pneumoniae* (Dixon and Postgate, 1972).

There are very few reports of free-living Gram-positive N_2-fixing bacteria found associated with plants. A new species of *Bacillus*, *B. azotofixans*, was isolated from the rhizosphere of several different grasses and was also found as a free-living species in the soil in Brazil (Seldin *et al*., 1984). Previously, N_2-fixing *Bacillus* strains had only been isolated from soil, and were assigned to one of two species: *B. polymyxa* or *B. macerans* (Witz *et al*., 1967). These three species were transferred to a new genus, *Paenibacillus* (Ash, 1993), together with other species in which N_2-fixation has yet to be assessed (Achouak *et al*., 1999a).

Aside from these bacteria that have been isolated from the rhizosphere of plants, N_2-fixing bacteria can also be found in the phyllosphere of many plants. In a review by Ruinen (1974), a compendium of N_2-fixing phyllosphere bacteria included representatives of most of the genera listed above. As discussed earlier, even rhizobia can be found epiphytically. Large populations of stem- and root-nodulating bacteria were found on the leaves of host (*Aeschynomene* species or *S. rostrata*) and non-host plants (Adebayo *et al*., 1989; Robertson *et al*., 1995).

This brings us to conclude this section with an important point. It might seem from the above, and from the accompanying literature, that Brazil is a country particularly favoured with rhizospheric diazotrophic bacteria, and that the only other major location for such bacteria is in rice paddies in the Philippines, in the same way as most rhizobial species seem to arise in China, Mexico or Senegal. It should be remembered that in fact these two locations are favoured with scientists who

have shown remarkable interest in and dedication to the detection of such micro-organisms, and that, yet again, it seems that we learn as much about the ecology of scientists as about the ecology of bacteria from perusing the literature.

Conclusions

The genera, species and strains of bacteria that are capable of N_2-fixation are extraordinarily diverse. The primary groups are the symbiotic bacteria (rhizobia, *Frankia* and symbiotic cyanobacteria), free-living cyanobacteria, and other free-living diazotrophs that are found in soil and in the rhizospheres of certain plants.

Advances in molecular taxonomy have provided a more rigorous means of classifying bacteria. However, this may say little about the practical competence of a bacterial strain to, say, fix N_2 or nodulate a specific host plant. Thus, while advances in bacterial taxonomy are of great importance in providing a true systematic description of bacterial species, variation on a fine scale within bacterial 'species' is such that no conclusions can ever be drawn about phenotypic properties without rigorous testing.

Chapter 3

The Process of N_2-fixation

This chapter reviews aspects of the process of N_2-fixation in order to give adequate background for the material in the rest of the book. However, it will not give a very detailed account of any of the biochemical processes involved and references to further work can be found in the review articles cited here.

What Is N_2-fixation?

All organisms require nitrogen in order to live. A majority of organisms can only use 'combined nitrogen' – that is, N that is already complexed with some other atoms to form an ion such as ammonium (NH_4^-) or nitrate (NO_3^-). However, the greatest concentration of N that is available on the earth is atmospheric dinitrogen gas (N_2). This highly stable gas constitutes almost 80% of the earth's atmosphere, an abundant supply to those organisms that can access it.

The process of N_2-fixation is the reduction of this N_2 to a biologically useful, combined form of N – ammonia. Because N_2 is very stable, the reaction is costly energetically and it is perhaps for this reason that N_2-fixation capability is not universal (Chapter 2). The equation for the reaction is as follows:

$$N \equiv N + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Concomitant reduction of at least two protons to hydrogen is an inevitable part of the reaction (Simpson and Burris, 1984), and thus the equation given above is the correct one rather than the more simplified version sometimes seen in which six protons and six electrons are allocated to dinitrogen to produce two molecules of ammonia, with no mention of the simultaneous evolution of hydrogen.

Nitrogenase structure

The reaction is carried out by an enzyme known as nitrogenase. Until recently, there was thought to be only one type of nitrogenase that was possessed by all N_2-fixing organisms, and was highly conserved between species. This is the molybdenum (Mo) nitrogenase, which is indeed in almost every known N_2-fixing organism. However, two alternative nitrogenase enzymes have been found in *Azotobacter* and other bacterial species in recent years, and these will also be discussed. In addition a totally different form of nitrogenase has reportedly been isolated from *Streptomyces thermoautotrophicus* (Ribbe *et al.*, 1997). This enzyme is very unusual and apparently uses superoxide as its reductant and contains a molybdo-pterin cofactor active site. Since it seems to be distributed very sparsely, it will not be considered further.

The Mo nitrogenase has been most extensively studied in *Klebsiella pneumoniae*, *A. vinelandii* and *Clostridium pasteurianum*. The enzyme consists of two components, each referred to in several ways. Component 1 contains the active site where N_2 is actually reduced, and is also known as the MoFe protein or dinitrogenase. Component 2, amongst other roles, provides electrons to Component 1 for N_2 reduction and is known as the Fe protein or dinitrogenase reductase. The three-dimensional X-ray crystallographic structures of both proteins from *A. vinelandii* and *C. pasteurianum* and the MoFe protein from *K. pneumoniae* have been determined, as has the complex formed between the Fe protein and the MoFe protein with aluminium tetrafluoride-MgADP. The aluminium complex is believed to mimic a transition state in the hydrolysis of MgATP.

The MoFe protein is a tetramer of molecular weight around 230 kDa composed of two α subunits encoded by the *nifD* gene and two β subunits encoded by the *nifK* gene. The Fe protein is a homodimer of molecular weight around 60 kDa, the subunits being encoded by the *nifH* gene. There is a 4Fe4S cluster in the Fe protein held symmetrically between the two subunits at one end of the interface. This protein also binds two MgATP molecules, which are thought to bind at the interface of the two subunits. The MoFe protein contains two each of two unique metal sulphur clusters. The P clusters bind symmetrically at the interfaces of the α and β subunits and have the stoichiometry 8Fe7S. Structurally they appear as two 4Fe4S cubes which share a sulphur atom at one corner. They are bound to the polypeptide through cysteine ligands to the Fe atoms with two cysteine residues, one from each subunit, each forming bridges between two iron atoms whereas the other iron atoms are bound to single cysteines. The other cluster in the MoFe protein is known as the iron molybdenum cofactor, often referred to simply as FeMoco. These cofactors are believed to be the site of N_2-reduction. FeMoco has a stoichiometry of Mo7Fe8S.homocitrate and is bound to the α subunit through a cysteine residue to one iron atom and a histidine ligand to the molybdenum which also binds the homocitrate molecule. Although no other amino acid residues bind directly to FeMoco, some hydrogen bonds are formed particularly to the sulphur atoms and are apparently essential for effective activity. In the putative transition state complex between the Fe protein and the MoFe protein the structure of the Fe protein is considerably distorted. The Fe protein binds at the interface of the α and β subunits and undergoes a conformational change which

places the 4Fe4S cluster within 10 Å of a P cluster, which in turn is approximately 14 Å from the FeMoco centre. This juxtaposition of the metal centres in the complex is thought to indicate the electron transfer chain from the Fe protein through to FeMoco. Further detailed reviews of the structure and synthesis of nitrogenases can be found in Howard and Rees (1996) and Smith (1999).

Mechanism of N_2-reduction

To reduce a molecule of N_2, the nitrogenase activity begins with acquisition of electrons by the Fe protein from a strong electron donor. In all N_2-fixing organisms studied, these nitrogenase-specific electron donors seem to belong to one of two groups of proteins: ferredoxins and flavodoxins. Ferredoxins belong to the class of iron–sulphur proteins discussed above, containing iron–sulphur clusters capable of transferring single electrons. Flavodoxins, in contrast, are proteins containing the prosthetic group flavin mononucleotide (FMN) and each FMN group can transfer two electrons.

So, nitrogenase activity integrates with normal cellular metabolism in part by diverting reducing equivalents – electrons – to the ferredoxin or flavodoxin that donates electrons to the Fe protein. These electrons are transferred in turn from the Fe protein to the MoFe protein. The Fe protein, in addition to the iron–sulphur cluster, has two molecules of MgATP associated with it. The reduced protein (i.e. carrying an electron to be donated) binds to the MoFe protein. There is one binding site on each half of the the MoFe protein tetramer, and these two sites are believed to function independently. Following binding, an electron is transferred from the Fe protein to the MoFe protein with concomitant hydrolysis of both bound ATP molecules to ADP. Finally the two components of nitrogenase dissociate.

As illustrated in the equation above, in addition to the electrons whose provision we have discussed, the reaction requires an equal number of protons. Putative proton transfer pathways have been identified but the observed hydrogen evolution results

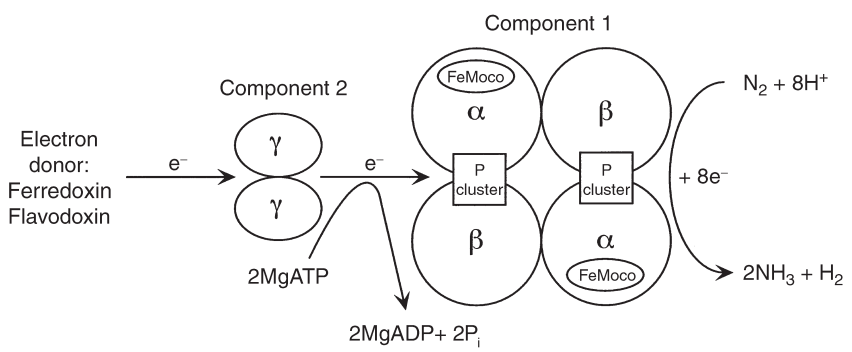

Fig. 3.1. The subunit structure of the Mo nitrogenase and the reaction of N_2-reduction.

from a proportion of these electrons and protons being used 'wastefully' rather than being allocated to N_2-reduction. H_2 evolution seems to be an essential part of the mechanism of N_2-reduction although its precise role has yet to be determined. Mechanisms to recover the reducing equivalents 'lost' in this H_2 evolution are discussed below.

The final result of the reaction is, of course, reduction of N_2 to NH_3. Because the whole reaction requires eight electrons and these are transferred one at a time from the Fe protein to the MoFe protein, it is believed that a number of enzyme-bound intermediates are formed during the reaction – rather than all eight electrons being stored and then used at once. The exact nature of the intermediates, and of the reaction, remains uncertain. The subunit structure of nitrogenase and the reaction of N_2-reduction is illustrated in Fig. 3.1.

Acetylene reduction by nitrogenase

As well as the reduction of N_2 to NH_3, nitrogenase will reduce a number of other substrates that contain triple bonds. The most important of these alternative substrates is acetylene, which is reduced to ethylene, as this reaction forms the basis of the widely used acetylene reduction assay (ARA) (Chapter 4). The reduction of acetylene is dominant over the reduction of N_2 by nitrogenase, due to the higher water solubility of actylene and the higher enzyme affinity for acetylene. Thus acetylene is an effective inhibitor of N_2-reduction. Hydrogen is another important inhibitor of nitrogenase, although it only inhibits reduction of N_2 and not of acetylene.

Alternative nitrogenases

For a long time the accepted dogma was that all nitrogenases contained Mo as a constituent of FeMoco. Since 1980 two alternative nitrogenase enzymes have been described in *Azotobacter* spp.: one that incorporates vanadium (V) rather than Mo, and one that has no requirement for any metal other than Fe. These are referred to as vanadium nitrogenase and the third or 'Fe-only' nitrogenase, respectively. There are strong similarities between the structures of each nitrogenase, but the subunits are encoded by entirely separate sets of genes – *nif* genes for Mo nitrogenase, *vnf* for V nitrogenase and *anf* for the third or alternative nitrogenase. The first evidence of an alternative N_2-fixation system was obtained in *A. vinelandii* (Bishop *et al.*, 1980) and subsequent work has concentrated on this species, which possesses all three nitrogenase systems, on *A. chroococcum*, which has only the Mo and V nitrogenases, and on the Fe nitrogenase of *Rhodobacter capsulatus* (Eady, 1996).

Both additional nitrogenases are comprised of two component proteins with a similar subunit structure to Mo nitrogenase, except that there is an extra (δ) subunit associated with the Component 1 (Eady, 1996). The stochiometry of Component 1 is thus $\alpha 2\beta 2\delta 2$. The function of the δ subunit is not known, but there is considerable sequence homology between the V nitrogenase and the third nitrogenase δ subunits,

so presumably they play a similar role in each. There is also significant sequence homology between the Fe protein polypeptides (encoded by *nifH*, *vnfH* and *anfH*, respectively) and also the α and β subunits of Component 1 (encoded by *nifDK*, *vnfDK* and *anfDK*) of all three enzymes. Probably the most important difference between the three nitrogenases lies in the cofactor structure, since this is the site of substrate reduction. In the V nitrogenase the cofactor contains V rather than Mo, and in the Fe nitrogenase the cofactor contains Fe (Eady, 1996).

Consistent with the idea that the cofactor forms the actual site of substrate reduction, there are marked differences in the reactions of all three nitrogenases that could be attributed in part to these different cofactors. Two differences are of importance here. One is that the reduction of N_2 to ammonia is less efficient in the two alternative nitrogenases, almost 50% of electrons going to production of H_2, in contrast to 25% in Mo nitrogenase. The second is that both reduce acetylene (C_2H_2) not only to ethylene (C_2H_4), but also produce a small proportion (2–4%) of ethane (C_2H_6). Production of ethane from acetylene is very characteristic of the two alternative nitrogenases and can be detected using gas chromatography (Dilworth *et al.*, 1987).

Neither alternative nitrogenase is normally expressed in *Azotobacter* in the presence of Mo (Bishop *et al.*, 1980; Eady, 1996). In the absence of Mo, V nitrogenase is expressed if V is present, and the third nitrogenase is expressed only if neither metal is present. The need for additional nitrogenases remains obscure. The most obvious explanation is to enable N_2-fixation to continue even in the absence of Mo (and V), but Mo-deficient soils occur only in some regions (Chapter 13), and many N_2-fixing organisms have very efficient mechanisms of Mo scavenging and storage (Shah *et al.*, 1984). Another possible rationale results from an observed difference in temperature responses for the three enzymes. While Mo nitrogenase is the most efficient at N_2-reduction at 30°C, at 5°C the V nitrogenase is about six times more efficient than the Mo nitrogenase (Eady, 1996). Non-Mo nitrogenases have been identified in *Azotobacter* spp., *C. pasteurianum*, *Anabaena variabilis*, *R. capsulatus* and *Rhodospirillum rubrum*. A better understanding of the role and distribution of alternative nitrogenases is required to assess their importance in nature.

Hydrogen

Evolution of H_2 during N_2-fixation is commonly assessed using the electron allocation coefficient (EAC) which is defined as follows:

$$\text{EAC} \quad \frac{\text{electrons used in } N_2\text{-fixation}}{\text{total electron flux to nitrogenase}}$$

The maximum possible value of the electron allocation coefficient is 0.75, as a minimum of two of the eight electrons allocated to nitrogenase always go to H_2 evolution during N_2-reduction (see above). In practice, more H_2 is generally evolved, such that measured values for the electron allocation coefficient are often in the range of

0.4–0.65 (Minchin *et al.*, 1996). Whether this is due to artefacts in measurement of nitrogenase activity (Chapter 4) or other physiological limitations is unclear (Layzell and Moloney, 1994).

In many organisms, H_2 can be oxidized by an enzyme known as hydrogenase. This reaction is by no means always associated with N_2-fixation, but interest here derives specifically from the possible role of such hydrogenases in recapturing the H_2 that is inevitably evolved by nitrogenase during N_2-reduction. The simplified overall reaction carried out by hydrogenase is as follows:

$$H_2 + X \rightarrow 2HX$$

where X is an electron acceptor, usually O_2. This reaction is achieved by the hydrogenase splitting H_2 into protons and electrons and the electrons then being passed down the respiratory chain to the final electron acceptor, i.e. O_2 in aerobic organisms (Evans *et al.*, 1987). From the chemiosmotic gradient created by the respiratory chain, ATP can be generated.

Although hydrogenases also occur in free-living N_2-fixing organisms (e.g. *A. chroococcum*), the greatest interest has centred on their possible role in enhancing the efficiency of N_2-fixation by rhizobial strains in symbiosis with legumes. In rhizobial strains that express an uptake hydrogenase, the H_2 evolved by nitrogenase is recaptured within the nodule and consequently measurement of the electron allocation coefficient is problematic. It has been postulated that expression of hydrogenase might contribute to increased symbiotic efficiency in several ways:

- The hydrogenase activity provides a source of reductant which may be metabolically useful, e.g. in the generation of ATP, and thereby reduces 'wastage' of energy.
- Because H_2 oxidation is coupled to O_2-consumption at least in aerobic organisms such as rhizobia, this will help in keeping the partial pressure of O_2 below the 'danger level' for nitrogenase (see below).
- H_2 can act as a powerful inhibitor of N_2-reduction by nitrogenase, and thus hydrogenase activity may serve to keep N_2-reduction efficiency high by removing a potential inhibitor of N_2-reduction.

There has been much debate about whether possession of an active hydrogenase does in practice confer a significant benefit on legumes growing in the field. Increases, decreases or no effects of hydrogenase activity on yield have been measured, and whether advantages are observed appears to depend on whether nitrogenase activity is restricted by H_2 inhibition or O_2 or carbohydrate limitation (Layzell and Moloney, 1994).

The distribution of hydrogenases is sporadic among rhizobia, although it appears to be more common in *Bradyrhizobium* than in *Rhizobium* strains. Hydrogenase has also been found in *Azorhizobium caulinodans* strain ORS571 (de Vries *et al.*, 1988). In at least some *Bradyrhizobium* strains, expression of hydrogenase is controlled by the host plant (van Berkum, 1990).

Oxygen Sensitivity of Nitrogenase

Nitrogenase is a highly O_2-sensitive enzyme. The mechanism of O_2 sensitivity is not known, but both components become irreversibly inactivated on exposure to atmospheric levels of O_2. This may be an accidental relic of the evolution of nitrogenase at a time when the earth's atmosphere still contained very little O_2, or it may be a fundamental feature of the dinitrogen reduction reaction. In any case, protection of nitrogenase from O_2 damage is a very important aspect of N_2-fixation, and a surprising variety of mechanisms have been adopted by N_2-fixing organisms.

The first solution is to avoid the problem altogether, as occurs in obligate anaerobes such as *Clostridium pasteurianum*. Similarly, many facultative anaerobes, such as *K. pneumoniae*, have a system of genetic control that ensures that nitrogenase is only synthesized when the ambient O_2 tension is very low (Gussin *et al.*, 1986), the optimum oxygen concentration for N_2-fixation in *K. pneumoniae* being 0.03 μM in comparison with the concentration of 225 μM found in air-saturated water (Hill, 1992).

All other organisms face a paradox. They have an absolute requirement for O_2 to generate the ATP and reducing equivalents required by nitrogenase, and yet a high pO_2 will inactivate the enzyme. The optimum conditions for nitrogenase activity in an aerobic organism therefore occur when the supply of O_2 exactly matches respiratory demand: if the O_2 supply is insufficient, then respiration, and consequently many other metabolic reactions, including nitrogenase activity, will be reduced; on the other hand, if the oxygen tension rises above the rate of respiratory consumption, nitrogenase activity will be damaged. The simplest way for obligate aerobes to cope with this dilemma is to fix N_2 only in microaerobic conditions – that is, when the pO_2 is well below atmospheric concentrations.

Microaerobiosis can be achieved in a number of ways, almost all of which require exploitation or creation of a physical barrier that restricts O_2 diffusion in combination with respiratory consumption of O_2. Some organisms, such as *Azospirillum brasilense*, use a 'behavioural' strategy by migrating to regions of intrinsically low pO_2. This movement results in the characteristic pellicle formation observed when they are grown in culture (Chapter 2). Other organisms show a different behaviour, aggregating to form colonial structures in the centre of which the pO_2 is lowered. Such behaviour is seen in a number of unicellular cyanobacteria, and in the characteristically huge colonies of *Derxia gummosa* (Chapter 2).

Another strategy to obtain microaerobic conditions is to restrict nitrogenase to specialized cells that have a wall with very low permeability to O_2 – for example, the vesicles formed by *Frankia* (Fig. 3.2) and the heterocysts of filamentous cyanobacteria. Cyanobacteria have a particularly tricky problem in that they actually produce O_2 during oxygenic photosynthesis, and they solve this by either spatial or temporal separation of photosynthesis and N_2-fixation.

Spatial separation occurs among members of sections IV and V of the filamentous cyanobacteria (Chapter 2). Cells at regular intervals along the filament (commonly about every ten cells) differentiate into heterocysts. These are cells in which photosynthesis ceases – photosystem II (PSII) becomes inactive – so enabling

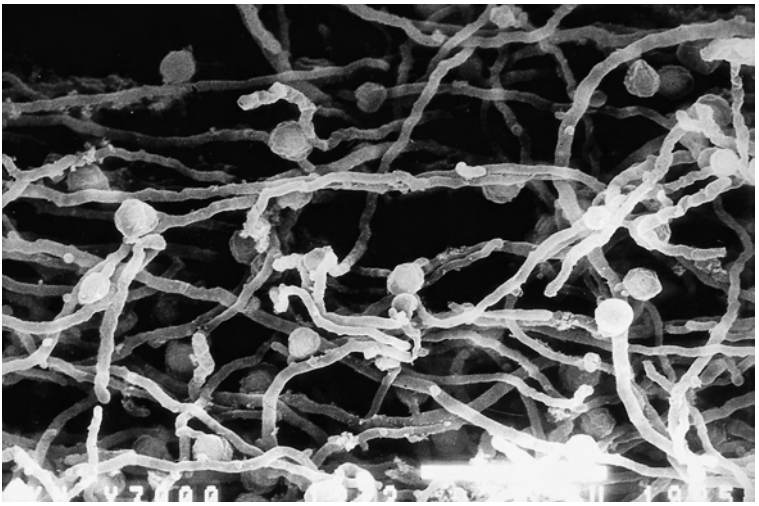

Fig. 3.2. Vesicles formed by *Frankia* growing in pure culture provide protection for nitrogenase against oxygen damage. (Photograph: A.D.L. Akkermans.)

high levels of nitrogenase activity without the problem of evolution of O_2 within the same cell. They are usually larger than their adjacent vegetative cells in the cyanobacterial filament, and are enclosed within a double-layered envelope that includes novel polysaccharide and glycolipid components which render them almost impermeable to O_2. Heterocysts are terminally differentiated cells in that they can neither divide nor de-differentiate – in this respect they are perhaps analogous to rhizobial bacteroids.

There are also N_2-fixing species among most of the non-heterocystous filamentous and among the unicellular genera of the cyanobacteria. In these species, there is temporal separation of photosynthesis and N_2-fixation. In *Gloeocapsa* this was shown to be related to light–dark cycles, photosynthesis occuring in the light and nitrogenase activity in the dark (Mullineaux *et al.*, 1981). In another unicellular cyanobacterium, *Synechococcus* sp., synchronized cell cultures were used to show that photosynthesis and nitrogenase activity were in fact separated in two phases of the cell cycle. Maximal O_2 evolution, and hence photosynthesis, occurred just before cell division and it declined thereafter to reach a minimum value halfway through the cell cycle. Patterns of nitrogenase activity were exactly the inverse of this, reaching a maximum when photosynthesis was at a minimum and vice versa. This temporal separation was maintained for at least one cell division cycle in continuous light (Mitsui *et al.*, 1986). It was later shown that, for *Synechococcus*, nitrogenase is synthesized *de novo* every cycle of activity (Huang *et al.*, 1988).

N_2-fixation within a symbiotic association is another very successful means to address the O_2 problem. The O_2 physiology of legume nodules has been particularly well studied and comprises two main components. One is a strong and variable physical barrier to O_2 diffusion in the nodule cortex (Hunt and Layzell, 1993; Witty and Minchin, 1996). The existence of such a variable barrier was deduced from the

observation that external O_2 concentrations can be increased up to almost 100% without a significant decline in nitrogenase activity or a significant increase in nodule respiration. Direct evidence for the barrier was subsequently obtained from direct measurements of O_2 concentrations in the nodule using O_2-selective microelectrodes (Witty *et al.*, 1987). This barrier is located just outside the infected zone of a nodule, possibly in a 'boundary layer' where thin-walled cells are very tightly packed without intervening gas spaces (Fig. 3.3). The variable diffusion resistance of this barrier appears to operate, at least partly, through a rapid osmotic response to environmental stimuli which causes cells to collapse or expel water into intercellular spaces. The second main component of O_2 protection in the legume nodule is the presence within the infected cells of leghaemoglobin, an O_2-binding molecule that serves to provide an adequate supply of O_2 for respiration, while keeping it sequestered away from nitrogenase. Other mechanisms are almost certainly involved in the overall reversible response to changes in external O_2 concentrations within the infected region of the nodule, including enhanced mitochondrial respiration and glycoprotein occlusions (Minchin, 1997). There is also indirect evidence for conformational protection of nitrogenase (see below) in bacteroids of soybean nodules (Denison *et al.*, 1992).

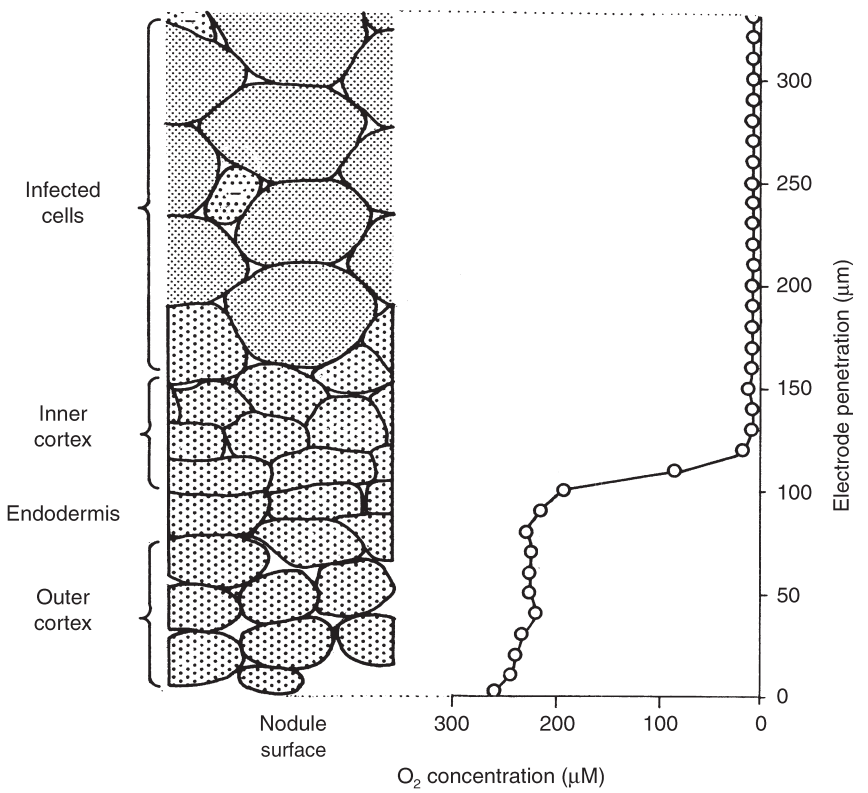

Fig. 3.3. The change in oxygen concentration across a legume root nodule. (After Witty *et al.*, 1986.)

A few organisms are able to fix N_2 at levels of O_2 close to atmospheric partial pressures. *Azotobacter* is the best studied and has three protection systems (Kennedy and Toukdarian, 1987). One is known as 'respiratory protection', which means that cultures of *Azotobacter* can adapt their rate of respiration in step with the external pO_2. This is achieved by use of a branched respiratory chain, with different branches generating different amounts of ATP. When the pO_2 is high, but the requirement for ATP is not correspondingly increased, *Azotobacter* can divert its respiratory electron flow along a path that leads to minimal generation of ATP, thus burning O_2 rapidly without overburdening itself with ATP. Conversely, if the pO_2 falls, *Azotobacter* can increase its respiratory efficiency – i.e. generate more ATP per electron transferred – simply by using a different respiratory pathway (Hill, 1992).

Azotobacter has a further temporary means of preventing O_2 from reaching nitrogenase, sometimes referred to as 'conformational protection'. It is mediated by a low molecular weight iron–sulphur protein that binds to the entire nitrogenase complex and thereby protects it from O_2 damage. In the process, nitrogenase is temporarily inactivated (Scherings *et al.*, 1983). It is thought that this mechanism is used by *Azotobacter* to protect its nitrogenase following a sudden change in external pO_2, while the organism has time to adapt its respiratory system.

The third mechanism for protection of nitrogenase has been termed 'auto-protection' as it is directly mediated by the Fe protein acting to reduce O_2 to H_2O_2, which is subsequently converted to H_2O by superoxide dismutase. This occurs when the molar ratio of reduced (electron-bearing) Fe protein to O_2 is four or greater (Hill, 1992). Yet another form of metabolic protection lies in the activity of uptake hydrogenases which recapture H_2 evolved by nitrogenase and, in the process, use O_2 (see above).

Assimilation of Biologically Fixed N

All N derived from N_2-fixation is obtained first in the form of ammonia (see above). Generally this will be protonated to form ammonium (NH_4^+) ions, and it is these that must be assimilated. This assimilation may be by the N_2-fixing organism itself, in which case the organism can feed directly on N_2 and can correctly be termed a 'diazotroph'. In legume symbiotic associations, however, the fixed N is assimilated not by the prokaryotic symbiont but by the host plant. There is considerable evidence indicating that neither *Rhizobium* nor *Bradyrhizobium* species can grow on the products of N_2-fixation, and thus strictly they are not diazotrophs (Ludwig, 1984). Recent reports indicate that alanine and not ammonium is the form in which fixed N is excreted from bacteriods (Waters *et al.*, 1998), though this is a topic of current debate.

Assimilation of ammonium ions into glutamate

The predominant assimilation pathway for ammonium is a two-step process (Fig. 3.4). In the first step, glutamine synthetase (GS) adds ammonium to glutamic acid

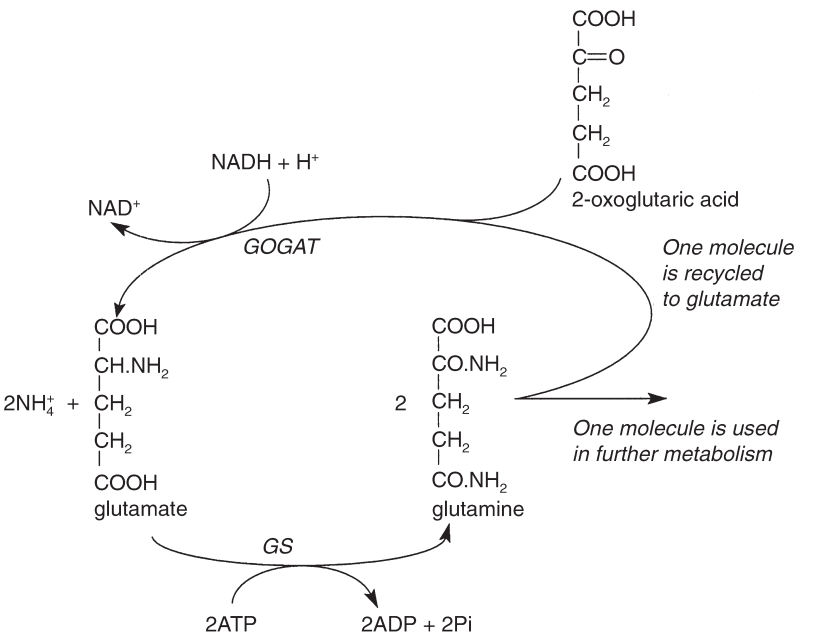

Fig. 3.4. Incorporation of ammonium into glutamate by the action of GS and GOGAT.

with concomitant hydrolysis of ATP. This glutamine is then used by glutamine-2-oxoglutarate-amino-transferase (glutamate synthase or GOGAT) to aminate one molecule of 2-oxoglutaric acid, the net effect being generation of two molecules of glutamate. In general one of these glutamate molecules recycles as an acceptor molecule for ammonium, whereas the other can be used as an amino donor in further metabolism. This pathway is often referred to as the GS/GOGAT pathway or the glutamate synthase cycle. GS has a K_m for ammonium ions of approximately 20 μM and is the key enzyme of ammonia assimilation in prokaryotes and in higher plants (Lea *et al.*, 1990).

Further metabolism of fixed N

Once the ammonia has been incorporated into glutamate and glutamine, these two amino acids serve as the nitrogen donors (amine and amide donors, respectively) for the synthesis of all other nitrogen compounds, including other amino acids, nucleic acids and secondary metabolites, and, of course, they may be directly used in the synthesis of proteins. The relative rates of the different nitrogen metabolic pathways depend on the needs of the organism.

In most prokaryotes, except *Frankia* and some cyanobacteria, all further metabolism naturally takes place in the same cell, and thus these compounds can be used

directly. In symbiotic associations with higher plants, the situation is quite different. Firstly, at least in the case of rhizobial–legume symbioses, the bacterial GS activity is repressed (Brown and Dilworth, 1975) and the ammonia is exported to the host cell cytoplasm for assimilation by the plant GS and GOGAT. Secondly, N is likely to be in excess in the nodule (a 'source') and to be required elsewhere in the plant (a 'sink'). Thus the transport of the products of N_2-fixation must also be addressed.

Legumes can be grouped into two classes, depending on the form in which biologically fixed N is exported from the nodule: the amide producers and the ureide producers (see Table 4.3). Amide exporters transport this N as either asparagine (ASN) or glutamine (GLN), whereas ureide producers transport it as allantoin (ALN) and allantoic acid (ALC). Asparagine is synthesized mainly in the infected cells by the action of aspartate aminotransferase (AAT) and asparagine synthetase (AS). There is a subcellular compartmentation of the pathway with GS and AS being located in the cytosol and NADH-GOGAT and AAT in the plastids, thus necessitating the transport of amino and organic acids into and out of the plastids. The substrate–product interdependence of these four enzymes suggests that their activities are highly coordinated.

Synthesis of ureides is more complex, both biochemically and logistically, and is based on *de novo* synthesis and then partial catabolism of purines (Atkins and Smith, 2000). In essence, glutamine derived from GS activity is translocated into the plastids of infected cells, and the newly fixed N moiety ends up in inosine monophosphate by one route or other. This is converted to xanthine monophosphate, then to xanthine in the plastid, and then to uric acid in the cytoplasm. These steps all take place in the infected cell and the uric acid is then transferred to adjacent uninfected cells, where the final synthesis of the ureides, allantoin and allantoic acid, takes place in the peroxisomes and endoplasmic reticulum, respectively. Nodules of ureide-producing legumes have approximately equal numbers of infected and uninfected cells.

The form in which newly fixed N is transported in actinorhizal plants also varies in a host-dependent manner (Schubert, 1986; Huss-Danell, 1990). In the majority of actinorhizal plants, it appears to be in the form of amides, predominantly asparagine, but in *Alnus* species citrulline (technically a ureide as it is an acyl derivative of urea, but also an amino acid and an amide) is transported. Among *Casuarina* species, no citrulline was detected in *C. cunninghamia* (Wheeler and Bond, 1970), whereas in *C. equisetifolia* citrulline was found to be the dominant form of N transported in the xylem (Walsh *et al.*, 1984). Citrulline was detected in nodules of three species of *Casuarina* but its presence was not related to transport of fixed N (Sellstedt and Atkins, 1991). The enzymology and physical location of enzymes of nitrogen assimilation have not been so well worked out in actinorhizal nodules.

There has been some speculation on the relative advantages to the plant of transporting N as amides or as ureides, and these hinge on the relative ratio of N : C atoms in the two types of transport compounds, and on the differences in solubility. In allantoin and allantoic acid the N : C ratio is 1 : 1 whereas in asparagine and glutamine it is 1 : 2 and 2 : 5, respectively. Thus ureides constitute a more efficient means of transporting N in terms of C usage, but when overall costs of synthesis and subsequent reassimilation are considered there appears to be little difference in

energy costs between the two types of transport compounds (Sprent and Sprent, 1990). There are also differences in solubility, ureides being less soluble than amides in solution, but this does not appear to present problems for ureide-exporting plants grown at low temperatures (Neves and Hungria, 1987).

Detoxification of ammonia

Teleologically speaking, GS serves two roles. The first is to assimilate ammonia and thus to provide useable N for general metabolism. The second results from the observation that free ammonia is actually toxic, and thus the action of GS can also be viewed as a detoxification mechanism. In animals, excess N is generally excreted, e.g. as a component of urea. In plants, however, where N is often a limiting factor, the potentially toxic NH_4^+ is sequestered in the form of storage compounds that are non-toxic and are also available for later provision of N. When N is required from this reservoir, it is generally released by catabolic breakdown of the storage compound followed by reassimilation of the ammonia so released (Schubert, 1986). The storage of high amounts of N in legumes makes them especially valuable as fodder with a high protein content in pasture systems (Chapter 10).

Energy Costs of N_2-fixation

An agronomically important aspect of N_2-fixation is that it is an energetically unfavourable reaction. The exact costs of N_2-fixation in the legume–rhizobial symbiosis in terms of ATP molecules are a matter of dispute. Since transfer of each electron from Fe protein to the MoFe protein requires hydrolysis of two ATP molecules, this means that a minimum of 16 ATP molecules is required per molecule N_2 reduced. In practice the costs are far higher, due to inefficiency in nitrogenase activity, and because of all the ancillary costs such as nodule development and maintenance, and the costs of NH_4^+ assimilation and transport. The estimates in the literature vary widely, but typical estimates would be a cost of up to 28 mol ATP mol^{-1} N (N_2) (Saari and Ludden, 1986), excluding all ancillary costs, or up to 33% of plant photosynthate being required when the total cost of symbiotic N_2-fixation and transport is considered (Minchin *et al.*, 1981). Although significant, these costs may not be much greater than the costs of assimilating nitrate (Pate and Layzell, 1990).

Regulation of N_2-fixation

As we have seen, there are several potential problems with N_2-fixation. One is the energy cost of reducing N_2. Another is the sensitivity of the enzyme to oxygen. Thus, as might be anticipated, all organisms capable of N_2-fixation appear to have systems of regulating the onset and persistence of N_2-fixation activity.

Inhibition by fixed N in free-living N_2-fixing organisms

As discussed above, at least 16 ATP molecules are needed to reduce one molecule of dinitrogen. The net result is generally provision of an amide group on glutamate. Thus, if nitrogen is supplied in this or any other form of combined N, as certainly happens in many laboratory-grown cultures, fixation of N_2 would be a waste of ATP, and it is not surprising that all free-living N_2-fixing organisms studied to date appear not to show nitrogenase activity if sufficient combined nitrogen is already available in the cell.

Regulation in response to combined nitrogen has been most intensively studied in *K. pneumoniae*. It has been shown that the signal that indicates the intracellular availability of combined nitrogen is the ratio between glutamine and 2-oxoglutarate. If it is low, this indicates a low cellular concentration of NH_4^+ (the logic behind this is clear from the GS/GOGAT pathway of ammonia assimilation; Fig. 3.4), and leads, through a complex cascade of genetic regulation, to synthesis of a series of enzymes involved in N metabolism. Some of these enzymes are involved in catabolism of nitrogen-rich amino acids (histidine, proline and arginine). The other enzyme of nitrogen metabolism that is regulated in this manner is nitrogenase. By this means nitrogenase is only synthesized if nitrogen is sufficiently limiting to warrant fixation of N_2. It should be noted that a deficiency of combined nitrogen is not the only factor regulating induction of nitrogenase activity in *K. pneumoniae*. A system of genetic control also exists to prevent synthesis of nitrogenase if the concentration of O_2 is too high (see above).

Another form of regulation of nitrogenase activity that has been identified in some free-living N_2-fixing organisms is temporary inactivation of the enzyme by covalent modification in response to availability of ammonia. It is mediated by two enzymes, one of which, Fe protein ADP-ribosyl transferase (DRAT), covalently modifies Fe protein by addition of an ADP molecule in the presence of ammonium. This temporarily inactivates nitrogenase. When the ammonium concentration falls, the ADP-ribosylation is reversed by a second enzyme called dinitrogenase reductase activating glycohydrolase (DRAG) and nitrogenase activity is restored. This phenomenon was originally observed in *R. rubrum* and has since been found in other organisms, including *R. capsulatus*, *Azospirillum* spp. and, most probably, *A. caulinodans* (Roberts *et al.*, 1990). However, it is by no means universal amongst N_2-fixers, not being found in *Azotobacter* spp. or in *K. pneumoniae*.

Regulation of nodulation and N_2-fixation in symbioses with plants

In the case of legume symbioses, regulation of N_2-fixation in response to available fixed N is mediated by the host legume rather than by the bacterial symbiont. It has long been known that a large concentration of nitrate will inhibit the development of root nodules (Fred and Graul, 1916). However, the exact significance of this is not entirely clear, as the different energy requirements of nitrate assimilation and N_2-fixation plus associated costs are not so great.

The effect of nitrate on a number of stages of the nodulation process has been examined, and all, from induction of rhizobial nodulation genes through root hair development and penetration and infection thread formation, are inhibited to a greater or lesser extent by the presence of nitrate (Carroll and Mathews, 1990). The end result is that the actual number of nodules formed on legume roots is reduced, leading eventually to complete suppression of nodulation if concentrations of nitrate exceed a certain threshold value. This may vary for different species, but is in the range of 2–20 mM (Harper and Gibson, 1984). At intermediate concentrations of nitrate, partial inhibition of nodulation will occur and this observation is used in the calibration of the ureide method for measurement of N_2-fixation (Chapter 4). At such intermediate concentrations the effect may be manifested in the developing nodules being smaller, such that the nodule mass per plant is reduced while the total number of nodules remains almost unaltered (Streeter, 1988). It should be pointed out that low concentrations of nitrate (1–2 mM) actually promote nodulation by ensuring early, rapid growth of the plant and development of a healthy root system able to nodulate profusely. This is termed the 'starter effect'.

A third effect of nitrate is actual inhibition of fixation in active nodules. This has been demonstrated both in greenhouse (e.g. Streeter, 1985; Minchin *et al.*, 1989) and in field-grown plants (Eardly *et al.*, 1984). Many of these results were obtained using ARA as a measure of nitrogenase activity, and it has since been shown that acetylene itself induces major changes in nodule nitrogenase activity (Chapter 4). Nevertheless, the phenomenon of nitrate inhibition of symbiotic N_2-fixation is a real one (Streeter, 1988) that appears to occur in three stages. In the first stage, nitrate is restricted to the cytoplasm of nodule cells and the immediate decrease in nitrogenase activity is due to an increase in the resistance of the O_2 diffusion barrier. This is then followed by a reduction in C metabolism (Arrese-Igor *et al.*, 1997). Irreversible nodule senescence follows, perhaps encouraged by toxic effects of nitrite on nitrogenase and leghaemoglobin (Becana and Sprent, 1987; Escuredo *et al.*, 1996).

A phenomenon that is almost certainly related to the above observations is autoregulation. This means that a legume root system will develop only a certain number of active nodules, and then no further new infection occurs. It appears that early infection events in the root produce a signal that is translocated to the shoot and the shoot in turn produces a signal that is transported to all parts of the root, thus suppressing further development of nodules (Carroll and Mathews, 1990). However, this autoregulation must somehow be sensing the N_2-fixing activity of the nodules, since if the nodules are induced by an ineffective rhizobial strain, new nodules continue to be formed until the nitrogen requirements of the plant are met.

Parsons *et al.* (1993b) suggested that further regulation of nodule growth and activity is triggered by the concentration of reduced N compounds in the phloem. This N feedback mechanism is supported by an array of direct and indirect evidence (Hartwig, 1998; Serraj *et al.*, 1999), and the effects of nitrate on nodulation and N_2-fixation described above (including autoregulation) may be mediated through signals from the shoot. The N feedback mechanism provides an elegant explanation of how the plant host may regulate both the degree of infection and the activity of

N_2-fixation in nodules, which probably also extends to actinorhizal symbioses (Baker *et al.*, 1997) and *Parasponia* and *Gunnera* symbioses.

Conclusions

N_2 is reduced by the enzyme nitrogenase to ammonia. In most N_2-fixing organisms there appears to be one form of nitrogenase, the Mo nitrogenase, but two other forms of nitrogenase have been identified in *Azotobacter*. The Mo nitrogenase reduces two protons to one molecule of H_2 for every molecule of N_2 reduced. In some species there is an uptake hydrogenase that oxidizes this hydrogen to water, thereby generating ATP and salvaging some of the energy 'wasted' in the evolution of H_2 by nitrogenase.

The ammonia generated by nitrogenase is assimilated as the NH_4^+ ion into glutamate. In both bacteria and plants this occurs via the joint action of the enzymes glutamine synthetase (GS) and glutamine-2-oxoglutarate-amino-transferase (GOGAT). In legumes the fixed nitrogen is further assimilated and transported predominantly as the amino acids asparagine and glutamine in amide-exporters, or as the ureides allantoin and allantoic acid in the ureide-exporters.

N_2-fixation is inhibited by a supply of combined N in all systems. In free-living diazotrophs, this occurs through inhibition of nitrogenase synthesis or by temporary inactivation of nitrogenase through covalent modification of the enzyme. In legume–rhizobial symbioses, inhibition by combined nitrogen can occur at three levels: nodulation may be suppressed completely; the total nodule mass may be reduced; or the nitrogenase activity of mature nodules may be inhibited. Legume symbioses also show autoregulation whereby further nodule formation is inhibited once a sufficient number of effective nodules have developed.

Chapter 4

Assessment of the Role of N_2-fixation

An accurate method of measuring N_2-fixation is essential for any evaluation of the usefulness of different N_2-fixing plants or different technologies, yet such a method has remained remarkably elusive. Despite active research on this subject since the discovery of N_2-fixation, many measurements of the amount of N_2 fixed in the field remain little better than informed guesses. This is largely because any such method must, by definition, be capable of differentiating N gained from the atmosphere from that absorbed from the soil, something that is very hard to do.

In some cases measurement of the *benefit* derived from N_2-fixation may be sufficient. It can be argued that if a response in growth or crop yield is observed following inoculation with N_2-fixing bacteria, the contribution of N_2-fixation will be adequately reflected in a simple measurement of the yield increase. But, as explained in Chapter 5, such conclusions can be surprisingly misleading. Careful consideration should be given to the aims and priorities of research before the methods for measurement of N_2-fixation are selected. Is accuracy of measurement the main criterion? Or will relative measurements of N_2-fixation between, for instance, different legume genotypes be more important than knowing the exact total amount of N_2 fixed?

As an extensive literature on the subject already exists, including many thorough reviews (e.g. Boddey, 1987; Peoples *et al.*, 1989a; Witty and Giller, 1991; Chalk and Ladha, 1999), only the main principles and assumptions of the different methods will be described here.

Direct Methods

Rates of activity

The best method of measuring N_2-fixation is to use an isotope of N other than ^{14}N, the isotope that makes up virtually all of the N_2 in the atmosphere. Two isotopes of N are useful as tracers in N_2-fixation experiments: the radioactive isotope ^{13}N, which has a half-life of only 10.05 min; and the stable isotope ^{15}N. The short half-life of ^{13}N restricts its use to experiments lasting only a few hours, and so it is not generally useful for measuring N_2-fixation, but it has been used for studies on the assimilation of the products of N_2-fixation in cyanobacteria (e.g. Thomas *et al.*, 1977).

By contrast, ^{15}N has been widely adopted. As it is a stable isotope it can be used without special safety precautions, and the measurement of incorporation of ^{15}N from $^{15}N_2$ into biological materials has become a standard technique used to prove unequivocally the presence of active N_2-fixation in an organism. The bacterial culture or plant tissue is incubated in an enclosed atmosphere which is enriched with $^{15}N_2$. After a period of incubation the N in the biological material is purified by digestion and distillation, and the proportion of ^{15}N atoms present is determined using mass spectrometry. The amount of N_2 fixed can be calculated very precisely from measurements of the total N and the proportion of ^{15}N in the material, if the ^{15}N-enrichment of the experimental atmosphere is known (see Table 4.5). The incubation time and the ^{15}N-enrichment of the atmosphere required for the experiment depend on the rate of N_2-fixation relative to the amount of N already present in the organism (Bergersen, 1980). The size and sophistication of the incubation chamber needed depend on the duration of the experiment, as care must be taken that the oxygen is not exhausted when aerobic systems are studied. Such measurements of rates of $^{15}N_2$ incorporation are also commonly used to calibrate the acetylene reduction assay, one of the main indirect methods of measuring nitrogenase activity in physiological studies.

Integrated measurements

The simplest method of quantifying the amount of N_2 fixed over a period of time, such as a growing season, is to measure the concentration of N in the tissue and to multiply by the weight of the plant material produced. This is, of course, only precisely equivalent to the N_2 fixed when no other sources of N are available. For example, if legume plants are grown in N-free media (e.g. perlite or acid-washed sand) in the glasshouse and watered with N-free nutrient solutions, then virtually all of the N present in the plant at final harvest must have come from N_2-fixation. There will always be some N present at the beginning of an experiment, if only the N in the seed or bacterial inoculum (Table 4.1), but if the amount of N_2 fixed is large this will be negligible in comparison.

Table 4.1. Measurement of N_2-fixation by the N-difference method in three tropical legumes inoculated with *Bradyrhizobium* sp. (*Arachis*) strain NC92 and grown in perlite in the glasshouse (all values are means of four plants and are uncorrected for seed N).

Host plant	Inoculum	Shoot weight (g)	Nodule mass (mg)	Total N (mg)	Fixed N (mg)
Siratro (*Macroptilium atropurpureum*)	+	0.56	52	19	18
	–	0.09	0	1	–
Groundnut (*Arachis hypogaea*)	+	3.11	103	113	63
	–	3.19	0	50	–
Pigeonpea (*Cajanus cajan*)	+	1.62	201	57	52
	–	0.45	0	5	–

This method, known as the N-difference method, has the advantage of giving a measure of the total amount of N_2 fixed over the length of the experiment and is indispensable for many laboratory-based studies. For example, Table 4.1 illustrates such an experiment, conducted in the glasshouse, comparing the abilities of rhizobial strains to fix N_2 with different host plants. In this example, differences between the contribution of seed N in the three species are apparent. The seed of groundnut contributes a large amount of N so that the uninoculated plants can grow without visible N deficiency for 3 weeks, whilst the seeds of pigeonpea and siratro contain much less N. This example serves to illustrate the value of the N-balance method in comparison with simple measurement of weight, since in some plants (e.g. groundnut) dry matter production may not be a good indication of the amount of N_2-fixation and analysis of the N content is necessary. Screening of rhizobial strains has traditionally been conducted using this method in the 'Leonard jar' assay (see Chapter 14).

Problems also occur when this method is employed under conditions thought to be free of mineral N but which are in fact contaminated with N. For example, vermiculite was used as an 'N-free' growth medium for the study of associative N_2-fixation (Rennie and Larson, 1979) but later research showed that significant quantities of mineral N can be released from vermiculite when it is incubated under warm, moist conditions (Giller *et al.*, 1986). As with seed N, the degree to which contamination is a problem depends on the relative size of the pools of fixed N and combined nitrogen.

An extension of this approach is to carry out similar experiments in soil where some N is available for plant uptake, and to try to estimate the amount of N absorbed from the soil. This can be done either by drawing up a detailed budget for gains and losses of N (an N-balance experiment) or by using another plant that cannot fix N_2 as a reference plant to estimate the amount of mineral N absorbed by the test plant (an N-difference experiment). In N-balance experiments the amount of N in each of the various pools (i.e. in the soil and in the plants) is measured both at the beginning and at the end of the experiment. Any gains unaccounted for are then attributed to N_2-fixation. Losses of N that are not measured (e.g. due to denitrification) will result

in an underestimate of the amount of N_2 fixed. As the total amount of N in the soil is generally large compared with the amount of N_2 fixed, any error in the estimation of the amount of soil N will result in a large discrepancy in the estimate of N_2-fixation.

In the N-difference method this particular problem is avoided by measuring the uptake of N in the N_2-fixing plant and in a non-fixing plant grown in the same soil. Subtraction of the N in the non-fixing plant from that in the N_2-fixing plant will then give an estimate of N_2-fixation. However, this rests on the assumption that the two plants take up the same amount of soil N, an assumption which is unlikely to be satisfied, not least because the N demands of N_2-fixing plants from the soil are likely to be less. When there is more soil N available for plant uptake, and soil N contributes a larger part of the N in the plants, errors are likely to be correspondingly greater because the assessment is based on measurement of a small difference between two large numbers. At best these methods can be rough indications of the amounts of N_2-fixation and at worst the results can be misleading. For example, these types of experiment were used to support claims that large amounts of N_2 are fixed by free-living heterotrophic bacteria, but further research indicated that cyanobacteria were actually more important (Witty *et al.*, 1979).

Indirect Methods

Rates of activity

Alternative substrates for nitrogenase

Many compounds that contain triple bonds can be reduced by nitrogenase. Acetylene is the most useful of these alternative substrates for the measurement of nitrogenase activity as ethylene is produced (Dilworth, 1966) and this can readily be quantified by gas chromatography. Whilst fixation of N by nitrogenase always results in the concomitant formation of hydrogen (Chapter 3), this does not occur during reduction of acetylene to ethylene:

$$\begin{array}{ll} N \equiv N + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 & \\ HC \equiv CH + 2H^+ + 2e^- \rightarrow H_2C = CH_2 & \\ \text{acetylene} & \text{ethylene} \end{array}$$

The theoretical conversion ratio for acetylene (C_2H_2) reduced to N_2 fixed is thus 4 mol to 1 mol.

The use of acetylene for welding means that it is widely available, or can easily be made by reacting calcium carbide (CaC_3) with water. The acetylene reduction assay of nitrogenase activity is carried out by incubating the test material in an atmosphere containing 10% acetylene in a closed vessel. The amount of ethylene (C_2H_4) produced after a period of incubation is then measured by gas chromatography. Frequently the nitrogenase activity, or acetylene reduction activity (ARA), is expressed directly as μM C_2H_4 produced per plant (or per nodule) h^{-1}. Alternatively the amount of N_2 that would have been fixed (since reduction of acetylene always precludes reduction of N_2 by nitrogenase) is calculated either by using the theoretical

conversion ratio of 4 : 1 or by calculating the actual conversion ratio using a direct measurement of the rate of N_2-fixation by $^{15}N_2$ incorporation as described above, in parallel with an acetylene reduction assay.

Measurement of ARA is undoubtedly very useful as a rapid method for detection of nitrogenase activity and for measurement of enzyme rates in simple laboratory systems with free-living bacteria. As no N_2 is actually being fixed by nitrogenase when acetylene is present, prolonged incubation periods can lead to N starvation in cultures, which in turn can cause further synthesis of nitrogenase and artificially high rates of N_2-fixation (David and Fay, 1977). Much greater problems are associated with using ARA to quantify amounts of N_2 fixed and these problems warrant detailed description.

ARA measurements of 'associative' N_2-fixation

Application of acetylene reduction assays for measurement of N_2-fixation in soil is complicated by the effects of acetylene on other microbial processes. For example, acetylene blocks the last steps of denitrification (Balderston *et al.*, 1976) and autotrophic nitrification (Hynes and Knowles, 1978) so efficiently that it is used in experiments designed to measure these processes.

In addition, acetylene blocks bacterial oxidation of ethylene in soil so that 'endogenous' ethylene accumulates (de Bont, 1976; Nohrstedt, 1976). Thus control treatments used to estimate background concentrations of ethylene in soil, in which ethylene accumulation is measured in the absence of acetylene, greatly underestimate the accumulation of endogenous ethylene that occurs in the presence of acetylene. Witty (1979) demonstrated this clearly by using ^{14}C-labelled acetylene for ARA measurements of N_2-fixation in soil cores. Only half of the ethylene that accumulated over the incubation period was ^{14}C-labelled; the remaining unlabelled ethylene must have come from the soil.

Other methods for estimation of endogenous ethylene accumulation were suggested by Nohrstedt (1983). A small concentration (0.05%) of acetylene can be used in the control treatments; this is sufficient to block ethylene oxidation and thus produce the increase in ethylene that is not due to nitrogenase activity and yet is too low to divert a significant proportion of nitrogenase activity from N_2-fixation to acetylene reduction. A further method is to include controls in which 10% acetylene is added together with 2% carbon monoxide. This inhibits nitrogenase activity whilst having little effect on other microbial processes, thus allowing N_2-fixation and endogenous ethylene accumulation to be distinguished.

The ^{14}C-labelled acetylene method has been demonstrated to be more accurate than the carbon monoxide inhibition method, although the latter method is more suitable for routine use (Tann and Skujins, 1985).

Other problems in the application of acetylene reduction assays for measurement of low rates of nitrogenase activity in soil result from the differences in solubility and rates of diffusion of acetylene, ethylene and N_2 (van Berkum and Bohlool, 1980). Given these problems it is suggested that the acetylene reduction assay should not be used for estimating N_2-fixation with non-legumes.

ARA measurements of N_2-fixation in legume nodules

The acetylene reduction assay has frequently been used to quantify N_2-fixation in field- or glasshouse-grown legumes, but there is considerable evidence which suggests that few of these measurements bear much relation to reality (Witty and Minchin, 1988). The most common method of measuring ARA in legume nodules is to uproot the plants, separate the shoot and roots and then incubate the roots plus attached nodules in a closed container under 10% acetylene. When this 'static' assay of ARA was developed, apparently linear rates of ethylene production were measured over incubation periods of 30 min to 1 h.

The development of more sophisticated flow-through incubation systems, where the nodules are held in a constantly flowing gas stream into which acetylene can be introduced (Witty *et al.*, 1983), has allowed more detailed investigation of the time course of ARA. On introduction of acetylene the rate of ethylene production rises quickly to a maximum value and then begins to decline after a few minutes, reaching a lower steady rate after 30 min (Fig. 4.1a). This 'acetylene-induced decline' in nitrogenase activity is accompanied by a similar decline in nodule respiration and is thought to be brought about by changes in the diffusion resistance of the nodule to oxygen (Chapter 3). Ethylene production measured in this way can be plotted as the total cumulative ethylene produced over the period of the assay just as in the 'static' assay (Fig. 4.1b), but the more detailed analysis reveals that the true rate (i.e. the initial rate) of nitrogenase activity is much greater than that measured in the static assay in an enclosed incubation vessel.

There are several additional sources of error in ARA measurements of nitrogenase activity. One is the simple difficulty of ensuring that all nodules are recovered from the soil, particularly in field-grown plants. A second is the fact that ARA measurement reflects nitrogenase activity only at a particular point in time – over the duration of the assay – which is far from being a measurement of the amount of N_2

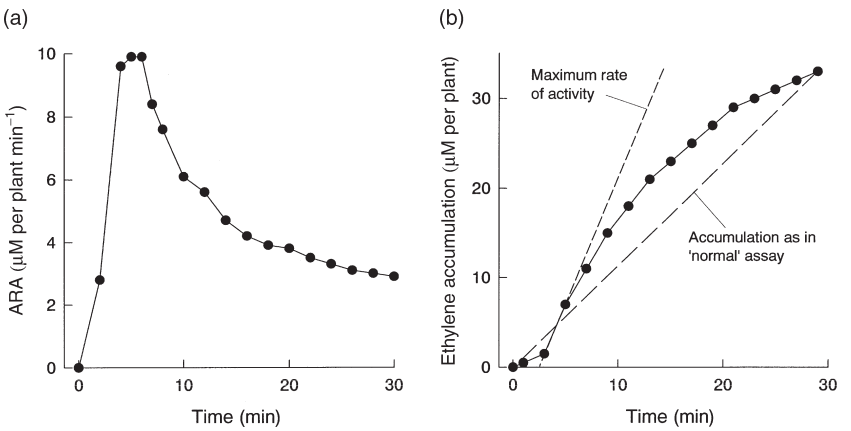

Fig. 4.1. The acetylene reduction assay in a flow-through system. (a) The change in rate of ethylene production (μM C_2H_4 per plant min^{-1}) showing the acetylene-induced decline. (b) The cumulative ethylene production (μM C_2H_4 per plant).

fixed over an entire growing season. Given the large diurnal fluctuations, and the wide variations in rates of nitrogenase activity (as assessed by ARA) commonly found between harvest intervals, calculation of the total N_2 fixed from spot measurements of ARA (or other methods that give an instantaneous rate of N_2-fixation) is bound to be inaccurate unless estimated very frequently. Moreover, as the acetylene reduction assay on detached roots is a destructive assay, the same plants can never be assayed twice. A third problem is that it has now been shown that virtually any disturbance, from one as mild as shaking soil off the roots to one as dramatic as removing the shoots, can cause a marked reduction in the rate of nitrogenase activity (Minchin *et al.*, 1986). Finally, the theoretical conversion factor of 4 : 1 mol C_2H_4 reduced to N_2 fixed is often assumed to be valid, and is used directly in calculations. However, experimental measurements of this ratio show that the true conversion factor can vary widely and is often between 5 : 1 and 7.5 : 1 (Witty and Minchin, 1988).

It can be tempting to assume that such errors will be uniform across different experimental treatments and thus do not affect the validity of purely comparative measurements. Unfortunately this is not the case. The magnitude of each of these errors can be influenced by treatments that are commonly compared using ARA measurements (Table 4.2), including the effect of different rhizobial strains or legume genotypes. In the light of these errors, use of the 'static' acetylene reduction assay can be justified only for simple demonstration that nodules are fixing N_2 and never for accurate measurement or even just relative comparison of nitrogenase activity between treatments.

For physiological studies of N_2-fixation, flow-through incubation chambers can be used to measure and compare rates of nitrogenase activity by acetylene reduction. This can be done on detached root systems, or on whole plants if the plants are grown in special pots that can be sealed into a flow-through gas system. Such experiments on detopped root systems have yielded useful information (e.g. Witty *et al.*, 1983) but measurements should be made on undisturbed plants if true *in situ* rates of N_2-fixation are to be observed (Minchin *et al.*, 1986). Flow-through ARA chambers have been developed for use in the field (Denison *et al.*, 1983; Sheehy *et al.*, 1991) but problems of restricted root growth and other technical difficulties prevent their widespread use.

Vessey (1994) argued that the static ARA assay still has valuable applications, despite all of the problems listed above, for measuring relative differences between nodulated legumes in the field. Others disagree (e.g. Minchin *et al.*, 1994) and suggest that there are few circumstances where ARA has benefits over simple measurements of yield or N accumulation. It is important to remember that much of the knowledge on N_2-fixation accumulated before the 1980s was obtained using the static acetylene reduction assay. Given present understanding of the limitations of this method, some caution in accepting conclusions from earlier research is certainly justified.

Analysis of N-transport compounds

Newly fixed N is assimilated in the legume nodule and then transported to other parts of the plant either as amides or as ureides (Chapter 3). Nitrogen is also absorbed

Table 4.2. Sources of error in acetylene reduction assays of N_2-fixation. (After Witty and Minchin, 1988.)

Error in measurement of absolute rates	Causes of variation	Treatments responsible
Decrease in activity caused by disturbance and by the acetylene-induced decline in nitrogenase activity	Alteration of the oxygen diffusion resistance of the nodule In comparative assays, differences in the initial diffusion resistance can alter the extent of the error	General: any plant disturbance washing of roots Treatment dependent: defoliation water stress temperature plant species or genotype rhizobial strain
Lack of calibration of C_2H_2 : N_2 conversion ratios	C_2H_2 : N_2 ratios greater than 4 : 1	Treatment-dependent differences in electron allocation between N_2 and H_2 due to: temperature irradiance plant species or genotype rhizobial strain uptake hydrogenase
Incomplete recovery of nodules	Failure to excavate all of root system	Treatment-dependent differences in nodule distribution and soil structure as a result of: plant spacing or age fertilizer applications plant species or genotype rhizobial strain

from the soil, predominantly as nitrate but also as ammonium. Nitrate is either reduced to ammonium in the roots (by the sequential action of the enzymes nitrate reductase and nitrite reductase) and assimilated into amino acids before being transported, or is transported directly as nitrate to the stem before reduction and assimilation. Ammonium ions, on the other hand, are always rapidly assimilated before being transported, as they are toxic. Ureide production is restricted to certain taxonomic groups of legumes (Table 4.3). Of the papilionoid legumes, all members of the tribes *Phaseoleae* (except perhaps *Erythrina* spp. which has a somewhat anomalous taxonomic position) and *Desmodieae* examined so far transport the products of N_2-fixation as ureides, whilst all members of the *Aeschynomeneae*, *Robinieae*, *Trifolieae* and *Vicieae* transport amides.[1] (Footnote on p. 81.) Among the *Caesalpinioideae* and *Mimosoideae* there are as yet no confirmed reports of species that transport the products of N_2-fixation as ureides. Although the xylem sap of actinorhizal plants

Table 4.3. Tropical legumes that transport the products of N_2-fixation mainly as ureides or amides. (After Ledgard and Peoples, 1988; Peoples *et al.*, 1989a, 1991a; Peoples and Herridge, 1990; Yoneyama and Kondo, 1990; Herridge *et al.*, 1996.)

Type of legumes	Ureide producers	Amide producers
Grain legumes	*Phaseoleae*: *Cajanus cajan, Glycine max, Lablab purpureus, Macrotyloma geocarpum, Phaseolus lunatus, P. vulgaris, Psophocarpus tetragonolobus, Vigna aconitifolia, V. angularis, V. mungo, V. radiata, V. subterranea, V. trilobata, V. umbellata, V. unguiculata* *Indigofereae*: *Cyamopsis tetragonoloba*	*Aeschynomeneae*: *Arachis hypogaea* *Cicereae*: *Cicer arietinum* *Genisteae*: *Lupinus mutabilis* *Vicieae*: *Lathyrus sativus, Lens culinaris, L. esculenta, Vicia faba*
Forage legumes	*Desmodieae*: *Desmodium discolor, D. uncinatum* *Phaseoleae*: *Calopogonium caeruleum, Centrosema pubescens, Macroptilium atropurpureum, Macrotyloma uniflorum, Pueraria javanica, P. phaseoloides*	*Aeschynomeneae*: *Arachis glabrata, A. pintoi, Zornia* spp. *Crotalarieae*: *Crotalaria* spp. *Trifolieae*: *Trifolium pratense, T. repens, T. subterraneum*
Shrub and tree legumes	*Desmodieae*: *Codariocalyx gyroides, Desmodium rensonii*	*Aeschynomeneae*: *Aeschynomene indica* *Acacieae*: *Acacia alata, A. auriculiformis, A. extensa, A. pulchella, A. unisauvis* *Ingeae*: *Calliandra calothyrsus* *Mimoseae*: *Leucaena diversifolia, L. leucocephala, L. macrophylla, Prosopis juliflora* *Robinieae*: *Gliricidia sepium, Sesbania grandiflora, S. sesban, S. rostrata*

such as *Alnus* and *Casuarina* have been shown to contain the ureide citrulline, this appears not to be related specifically to N_2-fixation (Walsh *et al.*, 1984).

Ureide transporters tend to have a low activity of nitrate reductase in the roots and so the majority of the nitrate absorbed is transported directly to the shoots. The majority of the N in the xylem sap of a ureide-producing legume that is fixing most of its N will therefore be in the form of ureides, whilst if the same species is absorbing most of its N from the soil, the majority of N in the sap will be in the form of nitrate or, to a lesser extent, amides. The proportion of N in the xylem sap that is present as ureides has therefore been developed as an index of N_2-fixation (McClure *et al.*, 1980; Pate *et al.*, 1980).

To carry out ureide assays, xylem sap is obtained by decapitating plants and collecting the bleeding sap from the cut stump. The contents of ureides, total α-amino N and nitrate in the sap are estimated by colorimetric assays and the results are expressed as the relative % ureide content (Peoples *et al.*, 1989a). To obtain actual measurements of N_2-fixation, the relative ureide content must be calibrated against the % N_2 fixed by using another method for the measurement of N_2-fixation. This is usually done by growing the test legumes in pots in the glasshouse, feeding them with increasing nitrate concentrations to progressively suppress nodulation and N_2-fixation (Chapter 3), and measuring the ureide content of plants that are dependent on N_2-fixation to different degrees (Fig. 4.2a). This assay does not measure the total N_2 fixed – it only provides an estimate of the proportion of plant N derived from N_2-fixation. Sequential measurements of N accumulation together with repeated estimates of xylem sap composition are required to estimate total amounts of fixed N (Herridge *et al.*, 1990).

Limitations of the technique include the variation in composition of the sap with the age of the plants and the difficulty in extraction of sap (Peoples *et al.*, 1989b). Bleeding sap can be collected from cut stumps of plants only if there is sufficient available soil moisture. Sap can also be extracted from segments of shoot tissue by application of a mild vacuum or, alternatively, soluble N compounds can be extracted in aqueous solution, although the composition of such extracts may differ from that of the bleeding sap and may require separate calibration (Herridge and Peoples, 1990). The method does not appear to be valid once seed filling begins due to remobilization of N within the plant (Aveline *et al.*, 1995). Nevertheless, the method has been shown to give estimates of N_2-fixation that correlate very well with estimates obtained using other methods (Fig. 4.2b). A simpler approach is to collect leaf or preferably petiole samples and extract the ureides with water for analysis (Peoples *et al.*, 1989a; Alves *et al.*, 2000).

Whilst the above method can only be used with legumes that transport the products of N_2-fixation as ureides, the relative concentrations of nitrate and amide-N

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[1] Ureides have been detected as minor components of the xylem sap in several species (e.g. *Arachis hypogaea, Erythrina variegata, Flemingia macrophylla, Gliricidia sepium, Paraserianthes falcataria, Sesbania* spp., *Stylosanthes hamata*) where they are not related to N_2-fixation. In addition unrelated coloured products form when the sap of some species are assayed for ureides which has probably led to incorrect reports of some species as ureide producers (Peoples *et al.,* 1991a).

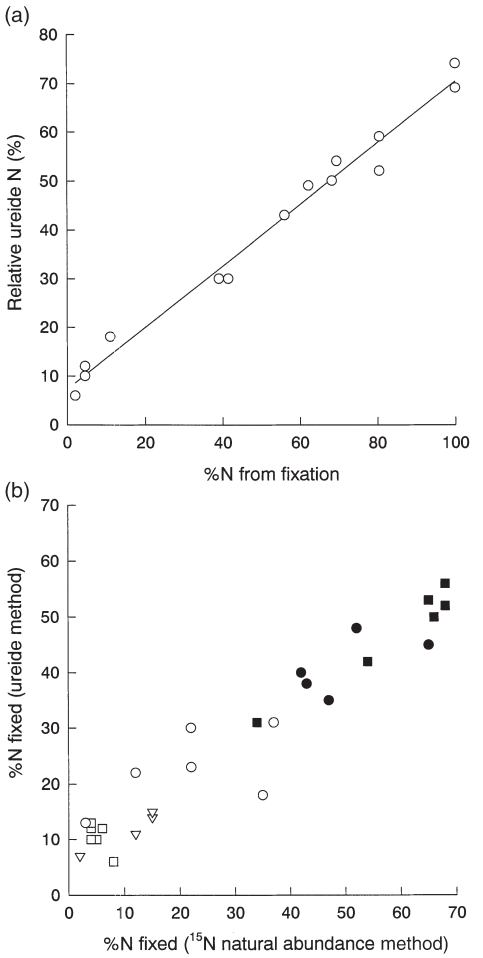

Fig. 4.2. (a) Glasshouse calibration of the ureide method for measuring N_2-fixation in soybean against ^{15}N isotope dilution measurements for soybean (Herridge and Peoples, 1990). (b) Comparison of field measurements of N_2-fixation in soybean made using either the ureide method or the ^{15}N natural abundance method. The different symbols represent measurements from different fields within the same experimental station. (From Herridge *et al.,* 1990.)

were also shown to be useful indicators of dependence on N_2-fixation in chickpea, groundnut, lentils and pea (Peoples *et al.*, 1986, 1987). Clearly this depends on nitrate absorbed from the soil not being assimilated in the roots, and this relationship was found not to hold in faba bean (*Vicia faba*), which had a large proportion of its nitrate reductase activity in the roots.

Although there are obvious limitations to the widespread use of the analysis of N-transport compounds for estimating N_2-fixation, there may be situations in which the method has particular advantages. If a method could be developed for tapping the xylem sap of tree legumes so that sap could be sampled frequently without killing the tree, then this could provide a way of assessing seasonal contributions from N_2-fixation in the field. Unfortunately, of the legume trees that have been investigated, few of those widely used in agroforestry transport the products of N_2-fixation as ureides (Table 4.3 and Chapter 11).

Integrated measurements

The ^{15}N isotope dilution method

One of the most widely used methods for integrated measurements of N_2-fixation is based on the principle of ^{15}N isotope dilution. An understanding of the terminology involved in the description of stable isotope use is necessary background for an explanation of this method and a number of definitions are presented in Table 4.4.

The content of the N present in a substance that consists of the stable isotope ^{15}N is normally expressed as the proportion of ^{15}N atoms present (i.e. atom % ^{15}N). Nitrogen in the atmosphere is virtually all $^{14}N_2$ and the natural ^{15}N content or natural abundance of the atmosphere has been shown to be a constant 0.3663 atom % ^{15}N throughout the world (Mariotti, 1983). The remaining 99.6336% are therefore ^{14}N atoms. Any substance that has an atom % ^{15}N greater than that of the atmosphere is said to be enriched with ^{15}N and the ^{15}N-enrichment is expressed as the atom % ^{15}N above that of the atmosphere, or atom % ^{15}N excess. Similarly a material depleted in ^{15}N has an atom % ^{15}N below that of the atmosphere. When discussing the ^{15}N enrichment of natural materials, that is their natural abundance which is usually a small value of atom % ^{15}N excess, then the term $\delta^{15}N$ is used (Table 4.4).

If a plant is grown in conditions where its sole source of N is fertilizer N that is entirely composed of ^{15}N (100 atom % ^{15}N), then all of the N in the plant (apart from the amount that is originally present in the seed or bacterial inoculum) will be ^{15}N. If the plant is able to fix dinitrogen ($^{14}N_2$) from the atmosphere, then the plant will have an atom % ^{15}N that is less than that of the fertilizer (i.e. less than 100 atom % ^{15}N). This difference can be used to calculate the proportion of N derived from N_2-fixation and is the underlying principle of the isotope dilution method for measurement of N_2-fixation. The exact calculation of the amount of N from N_2-fixation is shown in equation 2a in Table 4.5. For example, if the N_2-fixing plant has a final ^{15}N-enrichment of 75 atom % ^{15}N, then:

Table 4.4. Terms used in the description of ^{15}N stable isotope methods.

Term	Definition
Atom % ^{15}N	Abundance of ^{15}N atoms as a percentage of the total = $(^{15}N/(^{15}N + {}^{14}N)) \times 100\%$
Natural abundance	Atom % ^{15}N present in natural materials
Natural abundance of the atmosphere	Atom % ^{15}N of the atmosphere = 0.3663 atom % ^{15}N
Atom % ^{15}N excess	The percentage of ^{15}N above that in the atmosphere = atom % ^{15}N –0.3663
^{15}N-enriched nitrogen	Nitrogen with an atom % ^{15}N above that of the atmosphere
^{15}N-depleted nitrogen	Nitrogen with an atom % ^{15}N below that of the atmosphere
$\delta^{15}N$ (in parts per thousand, or ‰)	$((R_{sample} - R_{reference})/R_{reference}) \times 1000$, where $R = (({}^{15}N + {}^{14}N)/({}^{14}N + {}^{14}N))$

Table 4.5. Equations for calculating N_2-fixation using ^{15}N isotope methods.

Method	Equation	Where
1. Isotope enrichment methods using $^{15}N_2$ gas	$N\text{-fixed} = (E_{organism}/E_{gas}) \times N_{plant}$	E = atom % ^{15}N excess
2. Isotope dilution		
(a) Fertilizer as sole source of N	$N \text{ from fixation} = N_{fixing\ plant} \times [1 - (E_{fixing\ plant}/E_{fertilizer})]$	
(b) Using reference plant and adding equal amounts of fertilizer to each plant	$N \text{ from fixation} = N_{fixing\ plant} \times [1 - (E_{fixing\ plant}/E_{reference\ plant})]$	
(c) Using reference plant to which a larger amount of fertilizer is added (*A* value method)	N from fixation = $N_{fixing\ plant}$ − (%FR/100) × (soil N pool + fertilizer-N)	%FR = % fertilizer recovery soil N pool = amount of available soil N estimated using reference plant (Witty, 1983)
3. Natural abundance	$N \text{ from fixation} = N_{fixing\ plant} \times [(\delta^{15}N_{reference\ plant} - \delta^{15}N_{fixing\ plant})/\delta^{15}N_{reference\ plant} - B)]$	*B* = the $\delta^{15}N$ of the same N_2-fixing plant when grown with N_2 as the sole source of N

$$\%N \text{ from } N_2 - \text{fixation} = [1 - (75/100)] \times 100\%$$
$$= (1 - 0.75) \times 100\%$$
$$= 25\%$$

Experimental conditions under which all the combined N available to a plant is ^{15}N are hard to achieve but some attempts have been made to do this. For example, Lethbridge and Davison (1983) fed $Ca(^{15}NO_3)_2$ fertilizer with a ^{15}N content of 99 atom % ^{15}N to wheat and clover plants grown in sterile sand. The seed had been labelled with ^{15}N, as had the bacterial inoculum. N_2-fixation was detected by isotope dilution only where clover was inoculated with live *Rhizobium* cells (Table 4.6). This carefully controlled experiment demonstrated that no N_2-fixation could be detected in wheat inoculated with *Azotobacter* or *Azospirillum* under these conditions.

Generally the ^{15}N isotope dilution method is used to estimate N_2-fixation in plants growing in soil, where the N available for uptake by the plant is clearly much less than 100 atom % ^{15}N, and so the problem arises of differentiating between N_2 fixed from the atmosphere and N absorbed from the soil. In this case a reference plant that does not fix N_2 is used to measure the ^{15}N-enrichment of the available soil N. It is not necessary that the reference plant absorbs the same total amount of N as the N_2-fixing plant but it is assumed that both of the plants sample soil and fertilizer N in proportion to the amounts available in the soil (i.e. that they both absorb N from the soil with the same ^{15}N-enrichment). Unfortunately this is not always the case. The reasons why this basic assumption of the ^{15}N isotope dilution method is often violated were elucidated clearly by Witty (1983) with the aid of a simple mathematical model. The problems are largely due to the difficulty in ensuring that ^{15}N uptake is not affected by variation in (1) rooting depth, or (2) in the timing of N uptake:

1. If the ^{15}N-enrichment of the soil N available for plant uptake varies with depth, any differences in the rooting patterns of the test and reference plants may lead to absorption of soil N of different enrichments. The effect on estimates of N_2-fixation

Table 4.6. Atom % ^{15}N in 6-week-old plants of wheat inoculated with free-living, N_2-fixing bacteria and white clover inoculated with *Rhizobium*. The seed, bacterial inoculum and fertilizer N added were all labelled to 99 atom % ^{15}N and the inoculum was either live or heat-killed. (From Lethbridge and Davison, 1983.)

		Shoot inoculum		Root inoculum	
Plant	Inoculum	Live	Dead	Live	Dead
Wheat	*Azotobacter*	97.6	93.8	90.4	97.6
Wheat	*Azospirillum*	95.0	94.3	92.2	89.7
Wheat	*Bacillus*	95.7	95.8	95.1	93.7
Clover	*Rhizobium*	81.4[a]	95.0	76.1[a]	94.6

[a]Significantly different ($P < 0.001$) from heat-killed control.

can be most easily explained by reference to Fig. 4.3. In Fig. 4.3a the N_2-fixing plant (the legume) has roots that extend deeper into the soil than the ^{15}N-fertilizer has penetrated, whilst the non-fixing reference plant (shown as a cereal in these examples) has a more superficial rooting system. The legume will have access to N from deeper horizons, which is not enriched with ^{15}N, and will thus absorb soil N with a smaller atom % ^{15}N excess than that absorbed by the cereal. This will lead to an overestimate of N_2-fixation. The opposite is true in Fig. 4.3b. Only if the root systems have an identical distribution with depth will an uneven distribution of ^{15}N through the soil have no effect on the estimate of N_2-fixation (Fig. 4.3c).

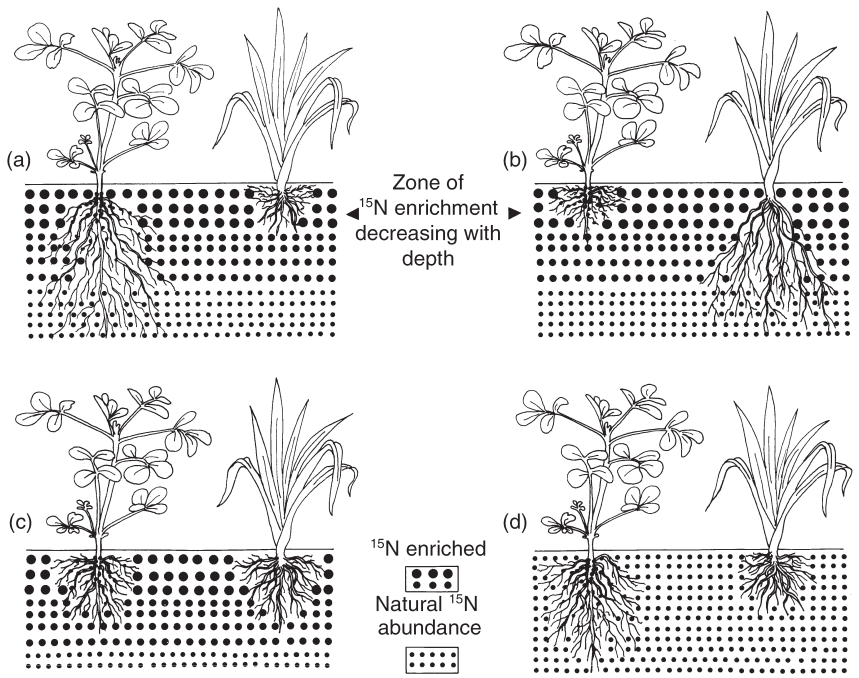

Fig. 4.3. Potential errors in ^{15}N isotope dilution estimates of N_2-fixation caused by differences in rooting patterns between the N_2-fixing plant (shown here as a legume) and the non-fixing reference plant (the cereal). (a) The legume roots explore deeper soil horizons, where the ^{15}N-enriched fertilizer has not penetrated, and are thus likely to absorb proportionately more unlabelled soil N than the cereal, so N_2-fixation will be overestimated. (b) The opposite case, where the cereal has access to a larger pool of unlabelled soil N, so N_2-fixation is likely to be underestimated. (c) The rooting depths are identical and so differences in the ^{15}N-enrichment of the available N with depth make no difference to the estimates of N_2-fixation. (d) For natural abundance estimates of N_2-fixation, if the natural abundance of the available soil N is uniform with depth, then differences in the depth of rooting between the legume and the cereal plants will make no difference to the estimates of N_2-fixation. (From Peoples *et al.*, 1989a.)

2. Addition of ^{15}N-enriched fertilizer to soil normally results in an initial large enrichment of available soil N, which rapidly declines (Fig. 4.4a). The reduction in ^{15}N enrichment is due to plant uptake and to other losses of plant-available N from the soil, coupled with dilution due to mineralization of N with a smaller enrichment from soil organic matter. If the reference plant takes up soil N more rapidly in the initial stages of growth, as is often the case where small-seeded grasses or cereals are used as reference plants for legumes with large seed reserves, then it will absorb soil N with a larger ^{15}N-enrichment than that absorbed later by the legume (Fig. 4.4b). This will lead to an overestimate of the amount of N fixed. Other possibilities are also described in Fig. 4.4. Only if the N uptake patterns of the test and reference plants are precisely matched, or if the enrichment of plant-available soil N does not vary with time, will both plants absorb ^{15}N from the soil with the same enrichment. This is difficult to achieve in practice but adding the ^{15}N to the soil in forms that only

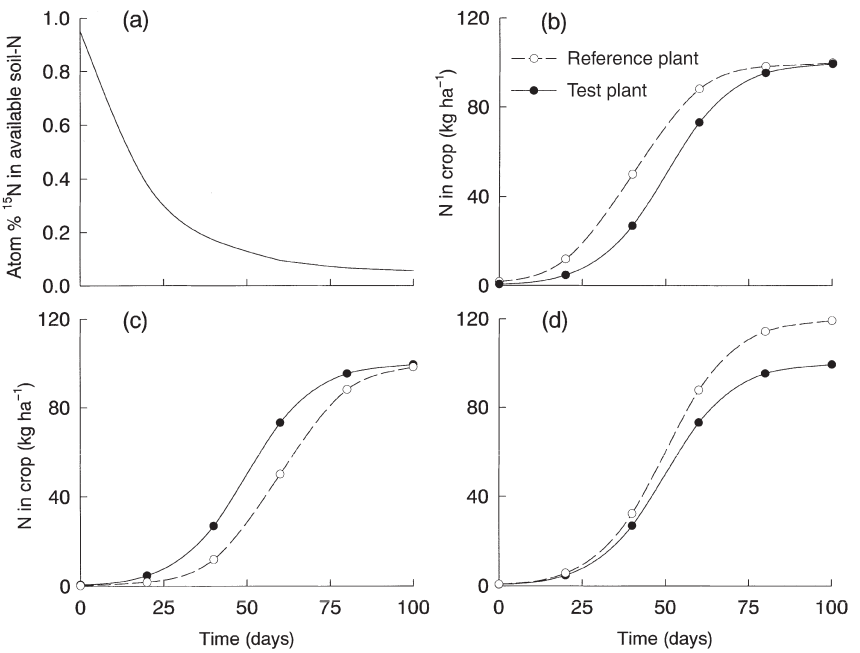

Fig. 4.4. Potential errors in ^{15}N isotope dilution estimates of N_2-fixation caused by differences in the N uptake profiles of the N_2-fixing plant and the non-fixing reference plant with time. (a) The ^{15}N-enrichment of the available soil N tends to decline rapidly after addition of ^{15}N-labelled fertilizers. (b) If the reference plant absorbs N from the soil more rapidly than the legume, it will absorb N with a larger ^{15}N-enrichment than the legume and thus N_2-fixation will be overestimated. (c) If the converse is true and the legume absorbs N from the soil more rapidly than the reference plant, N_2-fixation will be underestimated. (d) If the timing of N uptake by the two crops is identical (irrespective of whether different total amounts of N are absorbed), changes in the ^{15}N-enrichment of the available soil N will have no effect on the estimates of N_2-fixation. (After Witty, 1983.)

become available slowly can reduce the rate of the decline in ^{15}N-enrichment of available soil N and hence reduce these errors (Witty and Ritz, 1984; Giller and Witty, 1987). Several different methods of labelling the available soil N with ^{15}N have been used and these are considered in a detailed review by Chalk (1985). Errors can also be minimized by careful selection of a reference crop that has a root system and N uptake pattern as closely matched to the N_2-fixing crop as possible. In the case of legumes, the best choice of reference crop is often either uninoculated plants, which can be used when the homologous rhizobia are absent from the soil, or non-fixing or non-nodulating genotypes of the legume, where available (Table 4.7).

If the soil in which the experiments are to be conducted is very poor in N, then the non-fixing reference plant may grow extremely badly, due to N deficiency. This can only be remedied by increasing the rate of fertilizer application to the reference plant. However, it is not possible to apply a higher rate of fertilization to both the reference and legume plants, as large N fertilizer additions to the legume will tend to suppress nodulation and N_2-fixation (Chapter 3). The equations for calculating N_2-fixation by the ^{15}N isotope dilution method can allow for different rates of fertilizer N applied to each crop (Table 4.5, equation 2c). This is commonly called the '*A*-value' method (Fried and Broeshart, 1975) and is in principle the same as the ^{15}N isotope dilution method. However, a further assumption is made when the '*A*-value' method is employed – that the addition of a large amount of N fertilizer to the reference plant has no effect on the availability of native soil N – and there is evidence that this assumption is not always valid (Witty, 1983; Witty and Giller, 1991; Chalk, 1996a).

Table 4.7. Legume species for which genotypes that are unable to nodulate (Nod^-) or can form only ineffective nodules (Nod^+Fix^-) are available.

Species	Genotype	References
Arachis hypogaea	Nod^-	Gorbet and Burton (1979); Nigam *et al.* (1980)
Cajanus cajan	Nod^-	Rupela and Johansen (1995)
Cicer arietinum	Nod^-, Nod^+Fix^-	Davis *et al.* (1986)
	Nod^-	Rupela (1992); Singh *et al.* (1992); Singh and Rupela (1998)
Glycine max	Nod^-	Williams and Lynch (1954)
	Nod^+Fix^-	Vest and Caldwell (1972)
Medicago sativa	Nod^-, Nod^+Fix^-	Peterson and Barnes (1981)
Phaseolus vulgaris	Nod^-	Davis *et al.* (1988)
Pisum sativum	Nod^-	Jacobsen (1984)
Stylosanthes guianensis	Nod^+Fix^-	Miles and Sylvester-Bradley (1989)
Trifolium incarnatum	Nod^+Fix^-	Smith and Knight (1984)
T. pratense	Nod^-, Nod^+Fix^-	Nutman (1956, 1984)
T. subterraneum	Nod^+Fix^-	Gibson (1963)
Vicia faba	Nod^+Fix^-	Duc and Picard (1986)
Vigna unguiculata	Nod^+Fix^-	Pemberton *et al.* (1990)

Whatever variation of the ^{15}N isotope dilution method is employed, it is always important to examine whether or not the test plant and reference crop have sufficiently similar rooting patterns (by excavation) and N uptake profiles with time (by sequential measurements of N accumulation). Unless sufficiently detailed experimentation is conducted with each new combination of test plant and reference crop to determine whether or not the assumptions of the method are satisfied, the researcher is faced with a dilemma. Should the results be accepted? The danger is that there will be a tendency to accept the results when they seem plausible, but when the results are not possible (e.g. when a nodulated legume is more enriched than the non-fixing reference plant, which would indicate negative N_2-fixation) they will be rejected readily. In neither case is interpretation of the results justified unless the researcher has tested whether the experiment has satisfied the assumptions of the method.

Due to the differential responses of different crops or varieties to both the climatic and the soil environment, a reference crop that is shown to be suitable for a legume crop in one experiment cannot be claimed to be an universally appropriate reference plant. Detailed studies of the suitability of the reference crop may divert much of the research effort to methodology rather than measurements of experimental treatments. But the reference plant is potentially the greatest source of error in estimates of N_2-fixation using ^{15}N-based methods (Witty and Giller, 1991). One simple and practical approach to this problem is to use several reference plants in each experiment (Boddey *et al.*, 1990), though the final choice of which reference value to accept, or whether to take a mean of the reference values, may be rather arbitrary. The range of estimates calculated using the different reference plants can also be reported.

A further approach that has been advocated recently (Chalk and Ladha, 1999) is to dispense with the reference plant altogether by measuring the ^{15}N-enrichment of available soil N directly. The problem with this approach derives from the dynamic nature of N mineralization/immobilization processes in soil, which mean that frequent sampling and analyses of mineral N are required. Indeed, this is precisely why McAuliffe *et al.* (1958) suggested using a reference plant to provide an integrated measurement of the ^{15}N-enrichment of available soil N throughout the season. The intensive nature of sampling required may be overcome by use of simple equations to calculate the decline in ^{15}N-enrichment of available soil N similar to the model of Witty (1983) shown in Fig. 4.4. Two different approaches have been suggested: one that requires only measurements of the ^{15}N-enrichment of available soil N (Pareek *et al.*, 1990); and one that also requires estimates of legume yield and ^{15}N recovery over time (Hamilton *et al.*, 1992; Smith *et al.*, 1992). Although such methods mean that a reference plant is not required, this approach does not overcome the problem of knowing the precise depths of active N uptake by the legume. This knowledge is required to allow design of an appropriate soil sampling regime to determine the ^{15}N-enrichment of plant-available N. This, coupled with the additional effort required for soil extraction and analysis, indicates that it is unlikely that this method will gain widespread adoption.

Some of the ways of labelling the soil with ^{15}N for isotope dilution experiments that have been used are almost certain to invalidate these assumptions. Rennie (1986) recommended application of labelled fertilizer solutions in aliquots around the roots of each plant 2 weeks after germination, by use of a large syringe. This method cannot ensure that the enrichment of the soil is stable throughout the growth of the crop and certainly will not give a uniform enrichment in the soil. The fact that tests showed that the method gave results consistent with other means of estimating N_2-fixation can only be regarded as fortuitous.

As with all agronomic experimentation, it is necessary to have border rows for the microplots to which the ^{15}N-labelled fertilizers are applied. This is well established for experiments using ^{15}N to measure efficiency of fertilizer use (e.g. Stumpe *et al.*, 1989), but has sometimes been overlooked in the search for cost savings. Amounts of ^{15}N-labelled fertilizers required for isotope dilution measurements of N_2-fixation with agroforestry trees, where large plots are essential, make it particularly tempting to cut plot sizes. Yet agroforestry trees have been shown to have very extensive root systems, which can sometimes extend several metres from the plot so that large borders would be essential to minimize border-row effects. Given that tree roots can also often extend very deep into the soil, the basic assumptions of the isotope dilution method are difficult to satisfy.

The ^{15}N 'natural abundance' method

Many soils are naturally enriched with ^{15}N compared with the atmosphere. Enrichment of soil N occurs due to isotopic discrimination during processes such as ammonia volatilization, denitrification and other transformations of N in soil (for a detailed discussion see Shearer and Kohl, 1986). As the ^{15}N isotope is heavier than ^{14}N, compounds containing ^{15}N tend to react more slowly, particularly in reactions that lead to gaseous losses of N from the soil. The net effect is that the soil, over long periods of time, becomes slightly enriched with ^{15}N. This is not always the case. The ^{15}N content of soil has been shown to vary from −6 to +16‰ $\delta^{15}N$ (Shearer and Kohl, 1986) but many of the soils examined, including soils in the tropics (Yoneyama *et al.*, 1990b), have sufficient enrichment to allow the ^{15}N natural abundance method to be used to measure N_2-fixation.

The principle is the same as that of the ^{15}N isotope dilution method except that ^{15}N-enriched fertilizers are not applied to the soil. Differences in enrichment of the N_2-fixing test and non-fixing reference plants reflect the dependence of the plant on atmospheric N_2, and are used to calculate N_2-fixation (Table 4.5). The natural abundance of the N_2-fixing plant grown on an N-free growth medium (i.e. wholly dependent on N from N_2-fixation) must also be determined, as isotopic fractionation of atmospheric N_2 can occur during N_2-fixation and this can differ between species. This method of estimating N_2-fixation depends on a non-fixing reference plant in the same way as the isotope dilution method. The requirements for selection of the reference plant are also the same: the rooting depths from which soil N is absorbed and the timing of N uptake from the soil should be precisely matched, as even the natural abundance of soil N can vary with soil depth and with time (Shearer and Kohl, 1986). However, as variation with depth in the $\delta^{15}N$ of available soil N is

usually small (Ledgard *et al.*, 1984; Boddey *et al.*, 2000), the closeness with which the rooting patterns of the N_2-fixing plant and reference plant are matched is not as critical as when dealing with ^{15}N-enriched fertilizers (Fig. 4.3d).

The main concerns when using the natural abundance method are threefold:

1. It is preferable to sample whole plants, as different plant parts may vary in $\delta^{15}N$ (Peoples *et al.*, 1991b). This is obviously feasible for crop and forage legumes, but not for N_2-fixing trees.
2. The $\delta^{15}N$ of non-fixing plants growing in the same soil may vary, presumably due to isotopic discrimination during uptake and assimilation of N. In some cases this has been shown to be related to infection with arbuscular mycorrhizas or ectomycorrhizas (Högberg, 1990; Handley *et al.*, 1993). This makes it particularly desirable to use several reference plants as suggested above for the ^{15}N isotope dilution method. It is also essential that the non-fixing reference plants are sampled close to the N_2-fixing plant, which means that grouped comparisons of data collected from samples across many fields are not particularly useful (see Yoneyama *et al.*, 1993).
3. The estimate depends on determination of the $\delta^{15}N$ of the same N_2-fixing plant when grown with N_2 as the sole source of N (the *B* value) to account for isotopic discrimination during N_2-fixation (Table 4.5). *B*-values for any given legume vary depending on the rhizobial strain and environmental conditions (Ledgard, 1989). Therefore *B* values determined in the glasshouse may not reflect isotopic discrimination during N_2-fixation in the field, where promiscuous legumes may be nodulated by a wide range of rhizobia and where different environmental conditions prevail. Inoculation of the legumes with a mixed 'soil' inoculum may help to avoid bias in determination of *B* values. The closer the $\delta^{15}N$ of soil to that of the atmosphere, the greater are the errors associated with an inappropriate *B* value (Ledgard *et al.*, 1985b). In soils with small natural $\delta^{15}N$ enrichments, an inappropriate *B* value may indicate that the N_2-fixing plant is obtaining more than 100% of its N from N_2-fixation – which is, of course, impossible. Under such circumstances, it has been suggested (somewhat arbitrarily) that the *in situ* value of *B* may be assumed to be equivalent to the smallest $\delta^{15}N$ measured, which is required to correct these overestimates to 100% (Peoples *et al.*, 1997).

It was suggested that a $\delta^{15}N$ of 6‰ or more in the plant-available soil N is required for the natural abundance method to be sensitive (Ledgard and Peoples, 1988), but a more recent proposal is that much smaller soil $\delta^{15}N$ values of 2‰ or less may be adequate (Unkovich *et al.*, 1994; Peoples *et al.*, 1997). Theoretically, there is no loss of accuracy at such small enrichments, simply loss of precision (Unkovich *et al.*, 1994), but all of the potential problems are magnified the closer the soil enrichment is to that of air. It is clear that the sensitivity of the natural abundance method decreases as the $\delta^{15}N$ of available soil N tends towards zero (Ledgard and Peoples, 1988; Boddey *et al.*, 2000), due to the latter two problems indicated above. In some soils the $\delta^{15}N$ of the soil N, and that of non-fixing plants, can be strongly negative. For example, Vitousek (1999) measured $\delta^{15}N$ of −11‰ in leaves of non-fixing trees growing on young volcanic soils. The natural abundance

method is equally applicable under such conditions, as fixed N will still have a $\delta^{15}N$ close to zero.

Despite the problems discussed above, the natural abundance method can provide estimates of N_2-fixation that are just as (if not more) reliable as those using ^{15}N-enriched fertilizers (Bergersen *et al.*, 1990). The natural abundance method also has the advantage of not having to worry over the expense and methods of application of ^{15}N-enriched fertilizers. This means that it can be used to assess N_2-fixation in farmers' crops and not just in researcher-designed experiments.

Conclusions

There is no simple way to measure N_2-fixation and it is especially difficult in the field. Careful consideration should be given in designing experiments as to whether accurate measurements of N_2-fixation, comparative assessments of N_2 fixed in different treatments or simply measurements of the benefit from N_2-fixation (i.e. increase in yield or total N content) are required. In the case of nodulated plants, evaluation of nodulation can provide a useful adjunct to measurements of N_2-fixation but it must be borne in mind that there may be problems in recovering all of the nodules, particularly on deep root systems, and there is often no simple relationship between nodule mass and N_2-fixation. Nodulation can be assessed as counts of nodule number, nodule mass or volume, or simply a visual scoring of relative nodulation success (Sylvester-Bradley and Kipe-Nolt, 1988).

Despite the many pitfalls, isotope-based methods provide the best approach for integrated measurements of the amount of N_2-fixation in plants, provided that care is taken to ensure that the assumptions of the methods are fulfilled. However, for programmes of crop improvement, measurement of the N accumulation in the growing crops may be the most practical method for estimating N_2-fixation, and this is an accurate method on soils that have a poor capacity to supply mineral N. Given all of the assumptions made in estimating N_2-fixation, I concur with the comment of van Kessel and Hartley (2000) that all methods are semi-quantitative at best. It is thus useful to keep in mind the various limitations of the different methods when reading research articles on N_2-fixation, as an understanding of the problems can aid in evaluating conclusions that are often drawn from rather dubious data.

Chapter 5

Cycling of Fixed N in Tropical Cropping Systems

In the subsequent sections of this book, the many different ways that N_2 can be fixed in cropping systems of the tropics are discussed. As there are many features that the different cropping systems share, such as the major pathways through which fixed N becomes available for use by other plants and animals, these are described in this chapter.

Legumes have long been recognized as important components of crop rotations and intercrops. Apart from the direct benefits from N_2-fixation in the grain or fodder produced, any N contributed to the soil can be used by subsequent or companion crops. There are several ways in which legumes can contribute N in cropping systems: from grain legume crops, legume green manures, legume/grass leys or improved fallows to subsequent crops in rotations; or from legumes to companion crops in intercrops. The principles that govern the availability of N from legumes are common to other cropping systems where N_2 is fixed by *Azolla*, cyanobacteria, actinorhizal trees, or by grasses or cereals.

The simplest evidence for such a benefit from N_2-fixation is to compare the N budget of systems with or without the legume component (e.g. Searle *et al.*, 1981). Yet this does not actually tell us that there is a net contribution of N from N_2-fixation. The legume may in fact be removing more N from the soil than it is contributing but if it is removing less overall than the non-legume crop with which it is compared, a net benefit from including the legume will be seen. Similarly, if a legume is grown in a mixture with a cereal, it can improve the N economy of the cereal both by contributing N to the soil for uptake by the cereal (often called nitrogen transfer) (Table 5.1) or simply by the legume removing less N than if the cereal was grown in a pure stand. This is sometimes referred to as the 'sparing effect' (Vallis *et al.*, 1967), which simply means that if the legume removes only a small amount of soil N, more is then available for use by the companion crop. It is perhaps

Table 5.1. Mechanisms by which nitrogen from N_2-fixing plants can be made available to other plants. (Modified from Giller and Wilson, 1991, by Ledgard and Giller, 1995.)

Mechanism	Rate of transfer	Likely importance as an N source
Below ground		
Root and nodule senescence and mineralization	Slow	Major
Rhizodeposition	Rapid	Minimal
Transfer between roots by interconnected mycorrhizal hyphae	Rapid	Minimal
Above ground		
Mineralization of severed or senesced plant material	Slow	Major
Consumption by grazing animals or insects and return in excreta or as carcasses	Slow/rapid	Major
Foliar leachates	Rapid	Minimal
Transfer of ammonia to associated plants	Rapid	Minimal

rather an academic problem to distinguish these mechanisms as the apparent effect is the same, but we need a better understanding of the role of the legume within complex cropping systems if we are to be able to optimize the contribution from N_2-fixation. The evidence and arguments concerning this topic will therefore be considered in some detail, but first we must describe the major distinctions between different types of cropping systems.

Legume-based Cropping Systems

The multiple-cropping systems in which legumes are commonly found in the tropics can be divided into two major classes: 'simultaneous' systems, in which the components are grown in mixtures, and 'sequential' systems or rotations. The simultaneous systems include all types of intercropping, alley cropping and mixed swards for grazing; examples of sequential systems are grain legumes or green manures in rotation with cereals or other staple food crops, ley pastures or improved fallows. Many systems have features of both types. For example, strip intercropping, trees on boundaries between fields, grasses and trees on erosion control strips, and savannah parklands with mature trees all have zones of interaction between actively growing legumes and other plants, as well as effects on subsequent crops. As most cropping systems involve a mosaic of crops on different fields, which of course interact when they share a common border, simultaneous systems have been termed 'spatially mixed' and sequential systems as 'spatially zoned' (Chapter 12) (van Noordwijk, 1999).

The primary interactions between plant species in simultaneous systems are direct (interspecific competition or facilitation) whereas interactions in sequential

systems are principally due to modification of the environment for the succeeding plants, often termed 'residual effects'. A further type of interaction, allelopathy, which is the negative effect of one species on another due to release of toxic substances, can occur between plants growing together or in sequence (Rizvi *et al.*, 1999). Thus residual effects can be either positive, boosting growth of a subsequent crop, or negative, where growth of a subsequent crop is depressed. Legumes are best known for their 'residual benefits' in agriculture, and methods for monitoring these are described below. Whether it is best to mix species rather than grow them separately depends on the balance between complementarity in growth and competition, as well as the priorities given to yields from the different plants.

Factors Governing Amounts of N_2 Fixed and Contributions to the Cropping System

The principal factors governing the amounts of N_2 fixed in cropping systems are threefold. Probably the most important factor, which is surprisingly often overlooked, is the amount of land sown to legumes or other N_2-fixing plants (Giller and Cadisch, 1995). The second factor is the ability of the N_2-fixing plants to establish their symbioses and achieve their potential rates of N_2-fixation, which is often restricted by environmental constraints (Chapter 13). The third factor is the relative ability of the established symbioses to fix N_2, which is determined by the genetic potential of the N_2-fixing bacteria, of the plant, and of the symbiosis.

The main environmental factors that constrain N_2-fixation in the tropics are discussed in Chapter 13 and include limitations of water, nutrients (particularly phosphorus) and toxicities. One further limiting factor for N_2-fixation in the field is related to the two phenomena of autoregulation and N feedback regulation, which are discussed in Chapter 3. Rates of N_2-fixation are also regulated at the scale of the ecosystem or cropping system by the amount of combined N available for plant growth (Hartwig, 1998). This is particularly pertinent to any discussion of inputs from N_2-fixation, as the conditions under which measurements are made will have major, and often overriding effects on both the %N from N_2-fixation and the amount of N_2 fixed. By definition, the %N from N_2-fixation is the plant N not taken up from soil or fertilizer and any N_2-fixing plant can be made to derive all of its N from N_2-fixation by growing it under N-free conditions in the glasshouse. Values for %N from N_2-fixation are often presented in the literature as if they are purely genetic features of, say, particular legume crops or plant/strain combinations. But these values can only be measures of the crop's *potential* for N_2-fixation, as the environment may override this potential in many cases. Such genotype/environment interactions are commonplace and well recognized by breeders, but often ignored in the case of N_2-fixation.

There are further significant dangers of misinterpretation if only percentages are quoted. A large proportion of the N may come from N_2-fixation, but this may be a large proportion of a very small amount. If 90% of the N is fixed but the total amount of N is only 10 kg ha^{-1} the amount of N_2 fixed is 9 kg N ha^{-1}, but if only

20% of 100 kg N ha^{-1} is fixed this amounts to 20 kg N ha^{-1}. Another common mistake is to present correlations between the amount of N_2 fixed and the total N in the plant; as the first parameter is based on the second, it is hardly surprising that they show a strong positive relationship. These points are perhaps patently obvious, but they emphasize the need to consider both proportions and amounts in discussion of inputs from N_2-fixation.

The contribution of any N_2-fixing plant to the sustainability of agricultural systems depends on how much of the fixed N is harvested and removed from the system. For grain legumes the amount of N contributed is the amount of N_2 fixed less the amount of N harvested in the seed (Myers and Wood, 1987). This can be simply restated as 'the %N from N_2-fixation must be greater than, or equal to, the percentage of total legume N removed in the grain', which is a useful 'rule of thumb' to indicate whether there are net benefits to the system of growing a legume (Giller *et al.*, 1994). Such calculations ignore inputs below ground (see below) but serve to indicate that many grain legumes, particularly those that are most efficient at packaging their N into the grain (such as soybean), may actually remove more soil N in the grain than they contribute to the soil through N_2-fixation (Peoples and Craswell, 1992; Giller and Cadisch, 1995). If all of the above-ground plant material is removed, including the crop residues, there are few cases where a net contribution from N_2-fixation will be made.

This approach does not have to be confined to examining effects on N alone. A lot of research effort has been directed to calculation of budgets for major nutrients in farming systems in the tropics in recent years (Smaling, 1998). As the soil contains so much N in organic form, often 5000–10,000 kg N ha^{-1} or more, small changes in the N status of the soil are difficult to measure or take into account in N balances. Other problems with this approach derive from the complexities of measuring losses of N from systems through leaching, gaseous transfers and eroding soil. Long-term field experiments comparing cropping sequences with and without the N_2-fixing plant are the most powerful way of employing N balances, but there are few such experiments in the tropics (Leigh and Johnston, 1994).

Inputs from N_2-fixation in Simultaneous Systems

The advantages of growing N_2-fixing plants in mixtures result from many factors in addition to possible benefits from N_2-fixation, including more efficient capture and use of resources for growth, such as light and water (Reddy and Willey, 1981; Marshall and Willey, 1983), and pest control due to avoiding a monoculture (Altieri *et al.*, 1978). The advantage of intercrops over sole crops is commonly expressed in terms of the land equivalent ratio (LER), which is simply an expression of the land required for production of the same yield in the sole crops compared with the intercrop (Willey, 1979). If more land is required when plants are grown as sole crops, then the LER is > 1 and the intercrop is advantageous. The LER is also sometimes calculated and expressed as the relative yield total (RYT), which is mathematically the same as the LER. An extension of the concept of the LER has been to take account of

the length of time during which the land is occupied by the crops (Hiebsch and McCollum, 1987), which is important when the growing season is sufficiently long for more than one crop to be grown. When using these approaches, care must be taken to grow the crops at their optimal densities in both sole stands or mixtures, otherwise the advantages of intercropping may be overestimated (Ong *et al.*, 1996). Of course, the economic value of crops must also be considered when evaluating benefits from intercrops.

The interactions between crop species can be divided into: (i) competitive interactions, in which the crops compete for the same resource; and (ii) facilitative interactions, in which one crop alters the environment of the other in a positive way so as to benefit the growth of the other species (Vandermeer, 1990). Most of the benefits of growing crops in intercrops come from the way that they complement each other in their exploitation of the environment – for instance, by rooting to different depths and thus exploiting different parts of the soil, or by having leaf canopies at different heights, which might increase the total amount of light intercepted, leading to greater overall resource capture. In such examples the benefits of intercropping are due to weak competition for resources. As we shall see, there is considerable controversy as to where the benefits from N_2-fixation fit within this framework; that is, whether the benefits are due to the N_2-fixing plant 'sparing' soil N, or due to a direct contribution of fixed N for use by the other crop. Virtually all the research that has examined N transfer from N_2-fixing plants in plant mixtures has been focused on mixtures of legumes and grasses or cereals.

N transfer in legume/cereal intercrops or mixed legume/grass swards

Several researchers have claimed a significant role for direct nitrogen transfer of fixed N from the legume to the intercropped cereal (e.g. Agboola and Fayemi, 1972). Early experimental work such as that of Virtanen *et al.* (1937), using N balance studies, indicated large amounts of N transfer in pea/cereal mixtures. Later evidence for significant transfer of N from cowpea to maize was based on comparison of the ^{15}N enrichments of the sole and intercropped cereal crops following application of ^{15}N-labelled fertilizer (Eaglesham *et al.*, 1981). The intercropped maize had a larger N yield and a smaller ^{15}N-enrichment, showing that it had taken up more unlabelled N than the sole maize crop. It was concluded that this unlabelled N had been excreted by the cowpea, which was depleted in ^{15}N due to fixation of atmospheric $^{14}N_2$. However, it is equally possible that it was due to a sparing of soil N, perhaps from deeper in the soil where cowpea roots were not active and where the applied ^{15}N-enriched fertilizer did not penetrate. In agreement with the latter interpretation, other workers concluded that it was not possible to calculate N transfer in intercrops by isotope dilution, due to doubts over the matching of N uptake patterns between cereals and legumes which would invalidate the estimates (Papastylianou, 1988). Despite this, the isotope dilution method continues to be used in such studies.

If benefits from N_2-fixation can be explained in terms of sparing of soil N, then this is effectively an example of weak competition due to the legume having an

alternative source of available N (Vandermeer, 1990). There are few studies in which direct facilitative transfer of N from the legume to the cereal has been unequivocally demonstrated, and none carried out in the field. Van Kessel *et al.* (1985) used a split-root system and fed half of the soybean roots with ^{15}N-enriched nitrogen. Maize was grown together with the roots not fed ^{15}N and so the ^{15}N-enrichment found in the maize roots and shoots must have come from the ^{15}N-labelled soybean N. The ^{15}N-enrichment of the maize was more than doubled when the plants were inoculated with vesicular–arbuscular mycorrhiza, but again the amounts of N transferred were small.

A direct method of measuring N transfer was proposed by Ledgard *et al.* (1985a) in which ^{15}N-labelled N is applied to the legume leaves and N transfer is detected by analysing for ^{15}N in the associated grass or cereal. Transfer of N between *Phaseolus* beans and maize was easily demonstrated using this sensitive technique but less than 5% of the bean N was found in the maize plants after several weeks – even under conditions of severe N deficiency (Giller *et al.*, 1991). In the same study, experiments using ^{15}N isotopic dilution only demonstrated significant N transfer from beans to maize when the plants grew poorly due to a severe pest attack, a similar finding to Wilson and Wyss (1937), who only found N transfer when the plant growth was reduced by shading. These results are surprising in that it might be expected that more N would be available for underground transfer when the growth of the legume is more vigorous. A possible explanation is that the pest attack or shading caused premature nodule senescence, so releasing significant amounts of N to the intercropped cereal. Thus there is little direct evidence that facilitated transfer of N from legumes is generally important in the N nutrition of cereals in intercrops (Chalk, 1996b).

Much more attention has focused on mixtures of grasses and legumes (Chapter 10). Most evidence for significant benefits of N transfer has been based on the N difference method – that is, on comparisons of the N economy of pure or mixed grass swards (e.g. Birch and Dougall, 1967) – or on an isotope dilution method in which a comparison of isotope enrichment of pure or mixed grass swards is made after the soil has been labelled with ^{15}N-enriched fertilizer. Estimates of the amount of N transferred vary enormously, from no transfer being found in clover/ryegrass swards (Haystead and Lowe, 1977) to estimates of 20–50% (Ta and Faris, 1987a; Burity *et al.*, 1989) or over 80% of the grass N being derived from the legume (Broadbent *et al.*, 1982). All of these estimates were made using isotope dilution and are subject to the assumption that the uptake of soil and fertilizer N by the grass is not altered in the presence of the legume. There is evidence to suggest that rates of mineralization of organic N in soil may be greater in the presence of N_2-fixing legumes (Ismaili and Weaver, 1986; Jensen and Sørenson, 1988; see above), which would mean that this assumption is not valid in such experiments. Perhaps more importantly, N uptake patterns may differ between the sole grass and the grass in the mixture, particularly where ^{15}N fertilizers have been sprayed on to the surface of a sward. Although these errors will be relatively small if rates of fixation are high (Boller and Nosberger, 1988), the isotope dilution method rarely seems to give accurate measurements of N transfer (Chalk and Smith, 1994).

Using the ^{15}N foliar labelling method, Ledgard (1991) showed that underground transfer of N between white clover and ryegrass was greatest during dry, warm conditions. In the productive grazed pasture studied, the clover fixed 270 kg N ha^{-1} annually and there was an underground transfer of 70 kg N ha^{-1} compared with 60 kg N ha^{-1} transferred above ground via cow excreta. The repeated defoliation under grazing is likely to stimulate senescence and turnover of fine roots and nodules and to increase the availability of legume N for the grass. Indeed, significant transfer of N in mixtures of the forage legume *Stylosanthes guianensis* and the grass *Brachiaria decumbens* was detected only when the legume was killed by cutting and removing the whole shoots (Trannin *et al.*, 2000).

Mechanisms for underground 'transfer' of N

The degree to which N transfer is due to 'excretion' of N from legume roots is a matter of some controversy. It has been known for a long time that exudates from legume roots contain N in the form of simple amino acids (Rovira, 1956) but such demonstrations are necessarily limited to carefully controlled laboratory experiments. Experiments in which movement of N from a legume to a grass was measured over periods of 1 week (Ta *et al.*, 1989) or longer (Ta and Faris, 1987b) were purported to provide evidence for a role of excretion in N transfer on the grounds that these were relatively short time periods. Whether this evidence can be taken to demonstrate excretion of N depends on the rate at which nodule and root tissues senesce and are lost to the soil.

The use of the term excretion is perhaps unfortunate as it implies an active loss of nitrogenous compounds, and it is hard to envisage any direct evolutionary benefit for the legume. Many authors have preferred to use the general term 'rhizodeposition' to include exudation and passive losses, such as loss of structural tissues, because of the difficulty of separating the processes experimentally, and because of the relatively small contribution of exudation to total loss of carbon from the roots. Jensen (1996) labelled pea and barley plants with ^{15}N by growing part of the root system in a separate chamber and feeding with a labelled N solution. Rhizodeposition was estimated to amount to 7% of the N in the pea plants, and roughly half of the below-ground N at plant maturity.

If one adopts the term rhizodeposition in this discussion, the controversy over 'direct transfer' evaporates, and the majority of the evidence does indicate that significant losses of N from legumes to the soil occur in the form of dead and senescent material. An exception is the evidence for the involvement of mycorrhizas in enhancing the transfer of N between legumes and grasses (Van Kessel *et al.*, 1985; Haystead *et al.*, 1988), which may possibly take place by hyphal interconnections between plant roots (Francis *et al.*, 1986; Heap and Newman, 1986), although this again is controversial (Newman and Ritz, 1986). The amounts transferred by this mechanism are in any case unlikely to be of agronomic significance (Frey and Schüepp, 1993).

Legumes in Crop Rotations

As described above, when legumes or other N_2-fixing plants affect the growth of plants grown after them in sequence, the changes in growth are termed residual effects. The literature on rice tends to use this term in a slightly different way; the term direct effect is applied to the effect of green manure on the first crop of rice, while 'residual effect' is used for any apparent effect on the second subsequent crop of rice (e.g. Lauren *et al.*, 1998). In this book, to avoid confusion, the term residual effect is applied to the soil fertility benefit for the first crop, and any references to the second crop in sequence are specified. Intercropped legumes can also have residual effects, though these are likely to be reduced as the overall biomass and hence the amounts of N added to the system are smaller.

The beneficial effects on soil fertility that inclusion of legumes in cropping systems may bring are not restricted to inputs of N. Other potential benefits and how they are brought about are described in Table 5.2, and all of these must be considered when evaluating contributions from N_2-fixation.

Crop yield responses and N fertilizer equivalency

The simplest experiments conducted to demonstrate residual effects of crops are those in which the growth of a cereal is compared in plots with different cropping histories. In the first season, different legume and cereal crops are grown, or the land is left fallow; in the subsequent season, the growth of the cereal in these plots is compared. This is essentially an extension of the N difference method, which was applied for measuring N_2-fixation in Chapter 4. As indicated above, although this

Table 5.2. Benefits that may accrue from inclusion of N_2-fixing plants within agricultural fields. (After Young, 1989, with additions.)

Benefit	As a result of	Mediated by
Control of soil erosion	Root penetration	Increased infiltration
	Old root channels	Increased soil cover
	Litter fall	Reduced runoff
	Increased soil organic matter	Improved stability
Reduced water losses	Increased soil organic matter	Increased moisture retention
Reduced nutrient losses	Deep rooting	Smaller leaching losses
	Maintenance of roots during fallow	Reduced soil erosion
	Increased soil organic matter	Increased cation exchange capacity
Increased nutrient inputs	N_2-fixation	Nutrient-rich litter
	Uptake of nutrients from deep soil horizons	

method gives an indication of the benefits of including a N_2-fixing plant in the rotation, it does not confirm whether the advantages are due to sparing of soil N or an actual contribution from N_2-fixation.

A development of this approach is to include a range of fertilizer N applications as experimental treatments when a cereal crop is grown in the subsequent season. The residual benefit of the previous legume crop is then expressed in equivalent units of fertilizer N required to match the amount of N provided by the legume (Fig. 5.1). This method has a number of potential pitfalls. N fertilizers should be managed as normally recommended to gain a true comparison with the benefit of the legume, and this often means that they should be applied in split doses rather than all at sowing, otherwise losses may be high and the residual N benefits of the legume will be overestimated. For example, in India, comparisons of the N benefits from four green manure legumes showed that all gave rice yields greater than those achieved with the addition of 120 kg N ha^{-1} as urea (Beri *et al.*, 1989b). This does not mean that the green manures actually supplied 120 kg N ha^{-1}, as in most cases the amount of N in the shoots turned into the soil was less than this. Instead the results suggest that the efficiency of use of urea-N was poor in the alkaline soil in which these experiments were conducted.

^{15}N measurements of residual effects

There are a few direct measurements of the amounts of legume N recovered by the following non-legume crops using ^{15}N-labelled legume residues (Ladd, 1981). The major advantage of this method is that it is a direct measurement, as the proportion of the residue N recovered by the test crop is estimated in the same way as fertilizer recovery or fertilizer utilization efficiency, i.e.:

$$\%N \text{ from legume stover} = (E_{\text{test crop}}/E_{\text{legume stover}}) \times 100\%$$

where E = atom % ^{15}N excess.

It is essential that the legume residues are uniformly labelled with ^{15}N, otherwise the above equation is invalid. There is also a danger that ^{15}N-labelled residues of plants grown under controlled conditions may have a very different composition than the residues of plants grown in the field, which will influence the speed with which N is released. Leaves of glasshouse-grown ^{15}N-labelled soybean plants contained 3.2% N (a C : N ratio of 13) compared with 1.5% N (C : N ratio of 28) in the leaf litter of field-grown plants (Bergersen *et al.*, 1992). Stover of field-grown soybeans is lignified with a C : N ratio of 45 (Toomsan *et al.*, 1995).

There is evidence (Fox *et al.*, 1990) that the use of ^{15}N-labelled residues may underestimate the true amounts of N made available to subsequent crops if the inorganic ^{15}N mineralized during the decomposition of the labelled residues can substitute for unlabelled inorganic N that would otherwise have been immobilized – the process of pool substitution (Jenkinson *et al.*, 1985). It is also possible that addition of legume N may increase the availability of native soil N – that is, cause a real and positive priming effect or 'added nitrogen interaction' (Jenkinson *et al.*, 1985). A

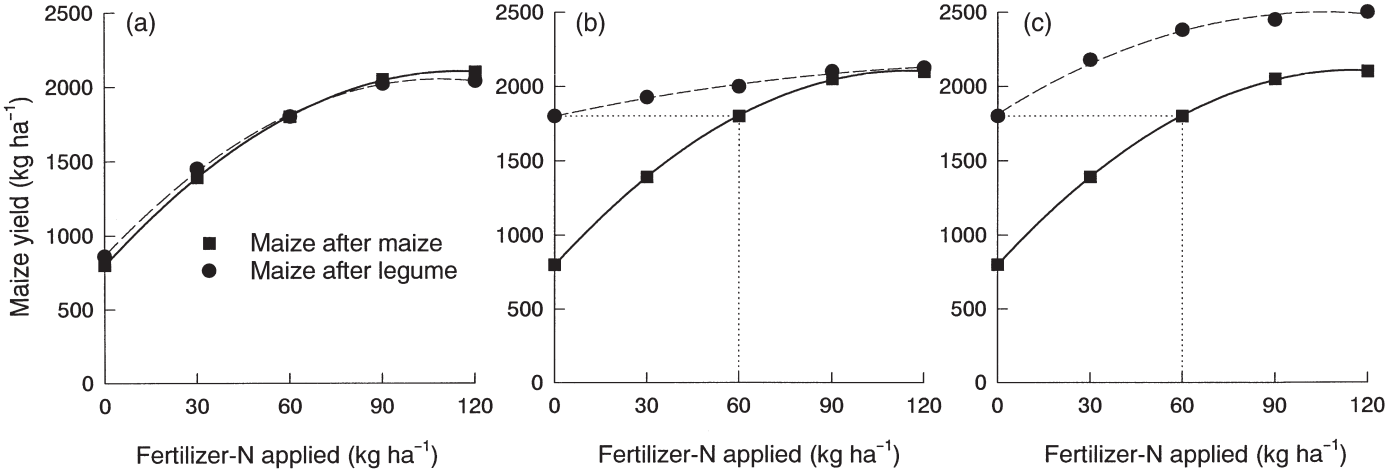

Fig. 5.1. A hypothetical example of calculation of fertilizer equivalence values by use of an N-fertilizer response curve. The dotted line shows how the residual benefit is calculated, in this case to be equivalent to 60 kg N ha^{-1} of N as urea. The response curves give examples of the types of crop response often found after growing a legume: (a) shows a case where there is no residual N benefit from growing the legume; (b) shows an example where the residual benefit can be ascribed solely to an N effect, as the legume effect is substituted by increased amounts of fertilizer N; (c) shows a case where the residual benefit cannot be explained solely on the basis of N effects, as the benefit is maintained when larger amounts of N are added.

comparison of results from several studies suggested that use of ^{15}N-labelling tends to give smaller estimates of N recovery from legume residues compared with N balance estimates, presumably due to pool substitution effects (Giller and Cadisch, 1995).

A further variation of this approach is to use an isotope dilution method, where unlabelled organic residues are added to plots previously labelled with ^{15}N and the contribution of N is calculated as described earlier (Chapter 4) by comparison of the ^{15}N-enrichments of plants grown in plots with or without addition of residues (Suwanarit *et al.*, 1986; Kumar Rao *et al.*, 1987). If this isotope dilution method and ^{15}N-labelled residues are both used separately (in what have been termed 'mirror image' plots), this should allow the relative importance of pool substitution effects to be determined (provided that all of the soil N that mineralizes is uniformly labelled). In a series of experiments using this approach to measure residual benefits from groundnut to maize or rice in northeast Thailand, variability was disappointingly large and obviated conclusions being drawn with any certainty (McDonagh *et al.*, 1993; Toomsan *et al.*, 1995). The results tended to indicate that priming effects were more important than pool substitution, although others have found little evidence for priming effects (Cadisch *et al.*, 1998). McDonagh *et al.* (1993) concluded that this isotope dilution approach was unlikely to yield useful results.

N contributed below ground in roots and nodules

Even where the above-ground biomass of the N_2-fixing plant is removed, there may be significant amounts of N added to the soil in the form of dead roots, nodules and other contributions from rhizodeposition. Recovery of roots of grain legumes has indicated that only 10% or less of the plant N is present in roots at harvest (e.g. Kipe-Nolt and Giller, 1993). Considerably more N may be present in the root systems of growing plants. Although nodules are rich in N, they often constitute such a small proportion of the below-ground biomass that the amounts of N in nodules are small, though some N_2-fixing trees such as *Erythrina* are exceptions (Nygren and Ramirez, 1995) (Chapter 11). The N in roots and nodules of annual legumes may be translocated to the shoots during senescence, but less than 30% of the N in roots is usually remobilized (Peoples and Gifford, 1997). Standing crop measurements of roots tend to underestimate the amounts of N that may be contributed to the soil as fine roots are formed and senesce continuously (e.g. Schroth and Zech, 1995). As discussed above, grazing or cutting of legume shoots causes senescence of roots and nodules and part of the N released below ground will be recovered by a companion crop through N transfer, or can later be reabsorbed by the legume when it regrows.

The methods used to determine residual benefits often include below-ground N contributions from N_2-fixation. Modifications of these methods can be applied to separate out what benefit is derived solely from roots and nodules of the legumes. For example, N yield of a subsequent crop can be assessed both in paired plots where the biomass is removed or returned, and where a non-nodulating genotype of the legume was grown (e.g. McDonagh *et al.*, 1993; Toomsan *et al.*, 1995). The residual benefit from the above-ground legume biomass can be distinguished by including additional

plots where no legume was grown previously and an equivalent amount of legume biomass to that produced is transferred.

There are only a few studies where ^{15}N-labelled roots have been added to soil and direct recovery of the N has been measured. Bergersen *et al.* (1992) found that negligible amounts of N were recovered from soybean roots using this method. The N content of roots is often so limited that it actually *increases* during decomposition as the wide C : N ratios of the roots result in immobilization of N (Lehmann and Zech, 1998; Urquiaga *et al.*, 1998).

The use of ^{15}N-labelling has been proposed for estimating amounts and turnover of N in root systems of growing plants (Janzen and Bruinsma, 1989). Labelling of the root system can be achieved by exposure of plants to ^{15}N-labelled ammonia (Janzen and Bruinsma, 1989), or to leaf application of ammonium or urea (Ledgard *et al.*, 1985a; Russell and Fillery, 1996b; McNeill *et al.*, 1997) as N is rapidly distributed throughout the plant. The total amount of root N, including the N in fine roots and that which has been lost to the soil through rhizodeposition, can be calculated from the ^{15}N-enrichment of a 'clean' root fraction (Russell and Fillery, 1996a). The main assumption is that the root system is uniformly labelled with ^{15}N, so that if the ^{15}N-enrichment measured from a sample of the rhizosphere soil is less than that of the clean root, this is due to isotope dilution by soil N. The equation that can be used for calculating root N in the soil surrounding the roots is:

$$N_{\text{fine roots and rhizodeposits}} = (E_{\text{clean root}} \times E_{\text{rhizosphere soil}})/N_{\text{clean root}}$$

where E = atom % ^{15}N excess.

The accuracy of estimates of total root N made using this method have not yet been fully verified; other potential problems are contamination of the soil during leaf labelling, and differences in ^{15}N-enrichment between nodules and roots. Results suggest that below-ground N contributions may have been underestimated substantially in the past, and may amount to 50% of the total amount of N in pasture legumes and 40% in crops at peak biomass (McNeill *et al.*, 1997, 1998; Rochester *et al.*, 1998). As indicated, some of this N may be remobilized to grain during maturation of the plants.

It is clear that all estimates of N_2-fixation, or of the residual benefits of legumes, that ignore contributions of N below ground will be underestimates, but by what magnitude remains a subject for future research.

N Release from Legume Residues

Residues of N_2-fixing plants such as legumes are particularly useful as organic manures, due to their large content of N and because this N is more likely to become readily available for uptake by other plants than N in many other crop residues. Release of N from decomposing organic material, or mineralization of N, results from the activity of microorganisms in breaking down the material. If the material contains a small proportion of N relative to the dry weight (i.e. if it has a large C : N ratio), then the amount of N available for growth of the microorganisms themselves

will be limited and any mineralized N will tend to be used immediately by the microorganisms for growth or be immobilized. Release of N into the soil (net mineralization) for use by plants is thus a balance of the processes of mineralization and immobilization. The C : N ratio (usually expressed as g C to g N) is a useful guide as to whether net release of N from organic material is likely to occur during the early stages of decomposition. The C : N ratio also provides an indication of how rapidly a plant material is likely to be decomposed (e.g. Cornforth and Davis, 1968; Frankenberger and Abdelmagid, 1985). Plant residues with a high C : N ratio (say > 20 : 1) are likely to decompose slowly, with initial net immobilization of N, whereas residues with a smaller C : N ratio are likely to decompose more rapidly, with net mineralization of N occurring right from the beginning. Legume residues commonly have C : N ratios of less than 20 : 1 and therefore tend to release N and decompose rapidly (Palm *et al.*, 2001).

This is, however, a gross simplification. Decomposition is dependent on the enzymatic cleavage of chemical bonds within the plant material. Soluble, low molecular weight substances such as glucose or amino acids are rapidly attacked by microorganisms, whereas insoluble polymeric materials tend to be cleaved primarily by slow-growing microorganisms (i.e. those with a slow basal rate of metabolism), so that breakdown of more complex substrates takes longer. Both the physical structure and the chemical composition of a plant residue thus determine whether or not it is resistant to decomposition. In particular, legume residues that contain a lot of lignins and polyphenolic compounds tend to be resistant to decay (Vallis and Jones, 1973; Palm and Sanchez, 1991; Constantinides and Fownes, 1993). The (lignin + polyphenol) : N ratio can give an excellent prediction of the N mineralization rate in legume residues, and decomposition of residues that contained less than 2% N resulted in a net immobilization of N over the first 6 weeks (Fox *et al.*, 1990). Many legumes have tissues rich in reactive polyphenols, which bind strongly to proteins and render the N resistant to microbial attack (Handayanto *et al.*, 1995). As leaves age, the N content decreases and the lignin content increases, so that older tissues decompose more slowly (Joachim and Kandiah, 1936). Older plant residues also tend to be physically harder and therefore less readily attacked by the soil fauna, which play an important role in decomposition by 'comminuting' or breaking up the residues into small fragments with a greater surface area for microbial attack.

Further discussion of the decomposition process is beyond the scope of this book and for more information on this subject readers are referred to Swift *et al.* (1979), Jenkinson (1981) and Cadisch and Giller (1997).

Managing organic residues for N release and maintenance of soil fertility

Green plant material tends to contain little lignin, which is laid down in plants as a structural component in secondary thickening of cell walls, and thus generally decomposes more rapidly than grain legume stover or woody tissues. Decomposition of shoots of forage legumes or prunings of legume trees can be rapid – several reports suggest that 40% or more of the N in legume shoot material can be released in less

than 2 weeks after addition to the soil (e.g. Cornforth and Davis, 1968; McDonagh *et al.*, 1995a). This means that the N may be released before the crop is sufficiently large to take full advantage of it, and that much of the N may be lost from the system. The management of nutrient release from organic residues to meet crop demands is a major challenge for research and a principal objective of the Tropical Soil Biology and Fertility (TSBF) programme (Woomer and Swift, 1994). Soil fertility, and N supply from soils in the longer term, is closely related to the maintenance of soil organic matter. Unfortunately there appears to be a direct trade-off: residues that release N readily for crops contribute little to build-up of soil organic matter, and recalcitrant residues that take a long time to decompose, or form stable complexes, have a poor capacity to supply nutrients (Palm *et al.*, 2000).

Legumes as feed and fodder

The quality characteristics of plant materials that determine their rates of decomposition, principally the N, lignin and polyphenol contents, are essentially the same as those that determine the utility of the plant materials as animal feed (e.g. Topps, 1992; Kumar and D'Mello, 1995; Chesson, 1997). Animals are surprisingly inefficient at utilizing N, often assimilating less than 10% of the N in the feed. Excretion of N by animals in urine is a major source of N loss from cropping systems, as hydrolysis of urea to ammonia causes development of high localized concentrations of ammonia and the high pH that favours volatilization of ammonia gas (Chapter 10). The quality of the diet fed to animals can influence the capacity of resulting manure to release N (Mafongoya *et al.*, 2000; Delve *et al.*, 2001), but the storage and handling of manure have a much stronger influence on the ability of animal manure to supply nutrients. Cattle manure collected from kraals in Africa can be as much as 90% sand (Giller *et al.*, 1997).

Conclusions

Any fixed N left in the field in the form of dead legume residues can be a source of N for other crops. The availability of the N for uptake will depend on the rate of mineralization of N in relation to the demand of growing crops. N in green manures is generally not only greater in quantity but also more available for rapid mineralization, as green legume residues have a smaller C : N ratio and lignin content than residues of mature grain legume crops. The large polyphenol contents found in some legumes have a strong influence on the capacity of their residues to release N in soil and in animal feeds.

The benefits of N_2-fixation by legumes to cereals growing in intercrops or to grasses growing in mixed swards are less clear. In many cases no benefit to the N status of cereals has been seen when they are intercropped with legumes. In cases where a benefit is found, it is mainly due to sparing of soil N rather than direct transfer from the legume. As the contact time between grasses and legumes growing

in mixed swards is generally so much longer than that of most intercrops, it would be expected that the transfer of fixed N from the legume would be a more significant process, and this generally seems to be the case. One last plea whilst on this subject is that the definition of nitrogen transfer of Henzell and Vallis (1977) should generally be adopted to include all of the possible mechanisms listed in Table 5.1 and not only that of 'excretion', which is perhaps least likely to be of agronomic significance.

Part II

Tropical Crops and Cropping Systems

Chapter 6

Cereal Crops and Grasses: Free-living, Root-associated and Endophytic N_2-fixing Bacteria

Many heterotrophic bacteria found in soil are capable of fixing N_2 (Chapter 2) but how much N_2 can they fix? Is the amount of N contributed to the soil significant for agricultural production? Do they form associations with grass roots, or inhabit grasses? And can we manipulate and enhance the rates of N_2-fixation? Or is it possible to engineer new symbioses between N_2-fixing bacteria and grasses?

N_2-fixation is an energy-expensive process but microorganisms in soil exist under near-starvation conditions for most of the time. It is thus important to consider possible energy sources that might be adequate to support N_2-fixation – for example, organic substrates such as plant litter, or carbon lost from plant roots. The rhizosphere (in its loosest definition the outer part of the root and the soil influenced by it) is known to be a relatively rich source of carbon and therefore a zone of intense microbial activity and so this will first be considered as a niche for N_2-fixing bacteria.

N_2-fixation in the Rhizosphere

The first widespread agricultural exploitation of free-living heterotrophic N_2-fixing bacteria was the sale of 'Azotobacterin' in Russia in the 1950s (Brown, 1974). Interest in this crop inoculant, which contained *Azotobacter chroococcum*, was short-lived as responses of crop growth of 10–20% were only found in some 30% of experimental trials conducted. The most encouraging results were obtained with horticultural crops.

Interest in the use of free-living N_2-fixing bacteria in agriculture gained a new lease of life with the discovery of 'associative symbioses' – the preferential occurrence of free-living N_2-fixing bacteria in the rhizosphere of cereals and grasses (Döbereiner,

1961, 1966; Döbereiner and Day, 1976). Detection of large amounts of ethylene when the new acetylene reduction assay was used with soil cores of tropical grasses (Döbereiner *et al*., 1972) led to a period of intense and exciting research activity in this field. But it was the report that inoculation of grasses, and in particular cereal crops, with *Azospirillum* could increase growth and yields (Smith *et al*., 1976) that concentrated much research effort on the goal of exploiting free-living bacteria to increase agricultural production.

It is worth noting that early research determined that the increases in plant growth observed on inoculation with *Azotobacter* were caused not by N_2-fixation but by bacterial production of plant growth hormones (Brown and Burlingham, 1968; Barea and Brown, 1974). This research was conducted in the same department at Rothamsted Experimental Station, UK, as the initial experiments to measure the ARA associated with sugarcane and *Paspalum* roots (Döbereiner *et al*., 1972). It was also soon realized that measurements of N_2-fixation rates by ARA in the laboratory bore little relation to N_2-fixation activity in the field (Witty, 1979; van Berkum, 1980). Despite this clear and long-standing evidence, reports still base evidence for associative N_2-fixation on such methods. However, the largest published estimate of 2.4 kg N ha^{-1} day^{-1} fixed in association with maize roots, which was based on ARA measurements (von Bulow and Döbereiner, 1975), perhaps helped this field of research by attracting some healthy scepticism.

The following section will review the information now available on the occurrence and activity of heterotrophic N_2-fixing bacteria in the rhizospheres of non-legume plants and attempt to draw some tentative conclusions about their importance in agriculture.

Associations between N_2-fixing bacteria and roots of grasses

The number of microorganisms is normally much greater in the rhizosphere than in soil further away from plant roots, due to the greater availability of carbon sources (Katznelson *et al*., 1956). However, the amount of N available in the rhizosphere is often limiting for microbial growth (Stotzky and Norman, 1961) and so bacteria that can fix N_2 would be expected to have a competitive advantage. In fact, most observations indicate that only 1–10% of the total bacterial population in the rhizosphere are N_2-fixing bacteria (Okon, 1982; Patriquin *et al*., 1983). An exception is found in the case of rice, where up to 85% of the rhizosphere bacteria may be N_2-fixers (Nayak *et al*., 1986). However, the accuracy of such counts is always doubtful, as they depend on the use of growth media that are selective for particular groups of organisms.

Specificity of the associations

A number of reports suggest that a degree of specificity exists between certain free-living N_2-fixing bacteria and different grasses. Numbers of *Beijerinckia* were greater in the rhizosphere of sugarcane than that of other grasses and *Beijerinckia* was preferentially enriched in the rhizosphere population compared with the surrounding

soil population (Döbereiner, 1961). *Azotobacter paspali* was found in large numbers only in the rhizosphere of tetraploid cultivars of *P. notatum* (Döbereiner, 1966, 1970). *Spirillum lipoferum* was rediscovered and found to be widespread in the rhizospheres of tropical (Döbereiner and Day, 1976) and temperate grasses (Vlassak and Reynders, 1978). These *Spirillum* isolates were then classified in the new genus *Azospirillum* with two species: *A. lipoferum* and *A. brasilense* (Tarrand *et al.*, 1978). *A. brasilense* was found in large numbers on roots of wheat that had been surface sterilized in 1% chloramine-T, implying that the bacteria were so closely associated with the root that they were protected from surface sterilization (Table 6.1). In contrast, greater numbers of *A. lipoferum* were found on the roots of a different species, maize, when subjected to the same treatment (Baldani and Döbereiner, 1980).

Umali-Garcia *et al.* (1980) showed that *A. brasilense* was attached to root hairs of millet in greater numbers than *Klebsiella* or *Azotobacter*. Specific chemotaxis of *Azospirillum* strains towards the roots of the hosts from which they had been isolated has been observed (Reinhold *et al.*, 1985). A possible link between this chemotactic response and host root exudate was suggested. Strains from roots of maize or Kallar grass (*Leptochloa fusca*) were strongly attracted to L-malate, whereas a strain isolated from wheat roots was more strongly attracted to other organic acids abundant in exudates of wheat roots. A heat-labile compound of high molecular weight found in the exudates of *L. fusca* specifically attracted the *Azospirillum* strain isolated from this grass. Study of the rhizosphere flora of this salt-tolerant grass led to the identification of the new species, *A. halopraeferens* (Reinhold *et al.*, 1987). A new genus of heterotrophic N_2-fixing bacteria, *Azoarcus* (Reinhold-Hurek *et al.*, 1993), was also first isolated from the rhizosphere of Kallar grass, in which it also occurs as an endophyte (see below).

Do the bacteria invade living cells?

Although it was postulated in 1976 that *Azospirillum* invades live cortical cells of the root (Döbereiner and Day, 1976), this has not been confirmed. Matthews *et al.* (1983) detected *A. brasilense* within pearl millet roots by immunochemistry but found no evidence for invasion of living plant cells. Most of the bacteria were present on the root surface or were found in intercellular spaces or in dead cells within the

Table 6.1. Establishment of the inoculum strain Sp 245 of *Azospirillum brasilense* in the rhizosphere and roots of field-grown wheat in Brazil. (From Baldani *et al.*, 1986b.)

	Log no. g^{-1} fresh wt		% Inoculant strain	
Site	Control	Inoc.	Control	Inoc.
Rhizosphere soil	5.30	5.71	2.0	51
Washed roots	6.40	7.12	2.0	72
Surface sterilized roots	3.16	3.74	0	84

root where the epidermis was ruptured. Similar results were obtained for *A. brasilense* infection of wheat (Levanony *et al.*, 1989). N_2-fixing bacteria were detected within the roots of Kallar grass using immuno-gold labelling techniques (Reinhold and Hurek, 1988). The bacteria appeared to enter the roots at lateral root junctions and were present between cells of the root cortex.

Is this a symbiosis?

Is there sufficient evidence of specific cooperation to allow definition of the association of *Azospirillum* (or other genera of free-living N_2-fixing bacteria) and the roots of grasses as a symbiosis? The evidence cited above certainly indicates that some species or strains of bacteria are better adapted to life in the rhizospheres of particular grasses. This is hardly surprising, as it is known that the chemical composition of root exudates varies substantially between species (see above) and the pH of the rhizosphere can differ markedly between genotypes of the same species (Brown and Bell, 1969). The evidence supports the suggestion that different strains and species of bacteria have evolved to occupy microenvironments provided in the rhizospheres of different crops. But this cannot be considered (as has been suggested in the past) to be equivalent to the legume–*Rhizobium* symbiosis. Whether the term 'associative symbiosis' is an appropriate description depends on how strict a definition is placed on the term symbiosis, but the use of the word associative is certainly accurate. The term 'diazotrophic rhizocoenoses' (*diazo* = N_2; *trophic* = nutrition; *rhizo* = root; *coeno* = common) was once proposed to describe such associations (Vose and Ruschel, 1981) but fortunately was not generally adopted.

Root carbon as an energy source for N_2-fixation

Carbon lost from living and dead roots, a process that has been called 'rhizodeposition' (Whipps and Lynch, 1985), provides an important substrate for soil microorganisms. The amount of N_2 fixed in the soil is limited by the amount of carbon available and the ability of the heterotrophic N_2-fixing bacteria to capture and use it efficiently. Various calculations of the rates of N_2-fixation that could be supported, depending on the amount of carbon available, have been made. These calculations are limited in accuracy by the difficulty of estimating how much carbon is translocated to the roots of plants, and of estimating how much of this is available for use by soil bacteria once root respiration and growth have been accounted for. However, it is possible to gain an estimate of the maximum amounts of N_2-fixation that could be sustained for a given input of carbon, assuming other factors are not limiting.

Estimates of the amount of carbon fixed from photosynthesis that is translocated below ground have been made for various temperate cereal crops and grasses. Values of up to 30% of total fixed carbon translocated for wheat, barley and maize (Barber and Martin, 1976; Helal and Sauerbeck, 1983; Swinnen *et al.*, 1994a,b) and 50% in a grassland have been obtained (Warembourg and Paul, 1977), such values being equivalent to 1–3 t carbon ha^{-1} (Whipps, 1990). No such estimates are available for

tropical cereals and grasses, and it is possible that the amounts of carbon available may be substantially greater in tropical grasses, with the more efficient C_4-photosynthetic pathway. Of the carbon that is translocated below ground, up to half is respired directly by the plant (Swinnen *et al.*, 1994a). Of the rest that does enter the soil and become available for use by microorganisms, little is present in soluble root exudates and the main addition is thought to be from death and decay of roots (Newman, 1985).

An estimate of the amount of N_2-fixation that might be supported by this carbon can be made by considering the composition and metabolic capabilities of rhizosphere microorganisms. None of the free-living N_2-fixing bacteria studied can use polysaccharides or other more complex carbon compounds for growth (Brown, 1982). The fixation of N_2 in the rhizosphere will thus depend on the ability of the N_2-fixing bacteria to capture and use either simple carbon compounds in root exudates or the breakdown products of more complex root carbohydrates provided by other microorganisms. The efficiency of carbon utilization for N_2-fixation by pure laboratory cultures of heterotrophic N_2-fixing bacteria ranges from 4 to 174 g C g^{-1} N_2-fixed in the laboratory (Giller and Day, 1985) and may be much less under field conditions. Therefore, it is assumed that the efficiency of use of root carbon in N_2-fixation is 10 g C g^{-1} N_2-fixed (a rather generous estimate), that the N_2-fixers comprise 10% of the total bacterial population in the rhizosphere and that they can acquire carbon in the rhizosphere in proportion to their numbers, then for every 100 kg C ha^{-1} that is translocated below ground, 1 kg N ha^{-1} will be fixed. The greatest flaw in such a calculation is that quantities of carbon available for N_2-fixation cannot be estimated accurately. This is because, as indicated already, most of the heterotrophic N_2-fixers cannot utilize complex carbon compounds and thus only a small proportion of the available carbon is likely to be used directly for N_2-fixation.

From such calculations it seems likely that the true amount of N_2-fixed in the rhizosphere may be closer to 1 kg N fixed for every 1000 kg C translocated below ground.

Endophytic N_2-fixing Bacteria in Cereals and Grasses

Since the realization that *Herbaspirillum* grew endophytically in grasses, a great deal of research attention has been focused on heterotrophic N_2-fixing bacteria that inhabit plants (reviews by James and Olivares, 1998; James, 2000). The various genera and many species of bacteria involved are described in Chapter 2. The impetus to search for endophytic N_2-fixers in the tissues of sugarcane actually came from indications that sugarcane gained much of its N from N_2-fixation (Boddey and Döbereiner, 1995), and the evidence for this is discussed below.

Several of the N_2-fixing bacteria that inhabit plants are said to be obligate endophytes, in that they are found in only small numbers in the soil or rhizosphere. Although many endophytic bacteria have been found in a wide range of grass species, particular interest has focused on *Acetobacter diazotrophicus* in association with sugarcane (Sevilla and Kennedy, 2000). *A. diazotrophicus* exhibits fast rates of N_2-fixation

in laboratory culture, which are not inhibited by nitrate (Stephan *et al.*, 1991); and when grown in mixed cultures, fixed N was released and supported the growth of yeast (Conjho *et al.*, 1993), suggesting that fixed N might be released within the sugarcane plant. A significant advantage of colonizing the plant tissues may be to gain access to carbon, the supply of which is restricted in the rhizosphere, but *A. diazotrophicus* is found in large numbers mainly in the xylem and the apoplast – parts of the plant where the concentrations of sucrose and other carbohydrates on which the bacteria can grow are likely to be limited.

Rather surprisingly, infection of sterile plants with *A. diazotrophicus* was only achieved with some difficulty in laboratory tests. However, when co-inoculated with arbuscular mycorrhizas, plant infection was readily achieved (Paula *et al.*, 1991), suggesting that the bacteria gained access to the plant tissues through the fungal entry points. Colonization of plants in culture also appears to be favoured by nutrient limitation in the culture medium (James *et al.*, 1994). Numbers of *A. diazotrophicus* in one variety of sugarcane grown in the field showed an inverse relationship with the N content of plants, but no effect was found in another variety (Bueno dos Reis Junior *et al.*, 2000). Colonization in laboratory tests appears to be inhibited in sugarcane that has received substantial applications of N fertilizer (Fuentes-Ramirez *et al.*, 1999). It is not clear whether the plant can regulate colonization of its tissues in some way, whether this is an effect on multiplication of the bacteria, or whether this is a result of reduced entry of the bacteria into the tissues.

As described in Chapter 2, *Azoarcus* spp. appear to be the dominant endophytes in Kallar grass, in which numbers of 7×10^7 g^{-1} dry root have been recorded (Reinhold-Hurek and Hurek, 1998). Several species of *Azoarcus* have also been found in rice, with larger numbers being found in older land races than modern varieties (Engelhard *et al.*, 2000). Endophytic bacteria were found in large numbers in rice by Barraquio *et al.* (1997) but the majority of these were not able to fix N_2.

Amounts of N_2-fixed with Cereals and Grasses

The obvious way to assess the validity of the calculations given above is by measuring the amounts of N_2 fixed in the rhizosphere, but unfortunately this is not easy to do. This subject has been thoroughly reviewed by Boddey and colleagues (Boddey, 1987; Boddey *et al.*, 1995, 1998). Most studies to date have not differentiated whether N_2 is being fixed endophytically or in the rhizosphere.

Proof of N_2-fixation using $^{15}N_2$

The first question in the study of rhizosphere N_2-fixation is simply to prove that it does take place. Uptake of $^{15}N_2$ fixed by bacteria in the soil has been demonstrated in sugarcane (Ruschel *et al.*, 1975), rice (Yoshida and Yoneyama, 1980), sorghum and millet (Giller *et al.*, 1988) and the tropical grasses *Digitaria decumbens* and *P. notatum* (De Polli *et al.*, 1977). In all of these experiments the roots and soil were incubated in

enclosed chambers for short time-periods under controlled conditions. The results conclusively demonstrated that N_2 had been fixed in the rooting medium and assimilated by the plant but did not distinguish whether the N_2 was fixed in the rhizosphere or in soil not closely associated with the roots. However, in other experiments, fixation of $^{15}N_2$ by bacteria was clearly shown to occur on or within excised roots of maize (Ela *et al.*, 1982).

In some cases rapid uptake of the fixed N by the plants was found (within 72 h of exposure to $^{15}N_2$; Giller *et al.*, 1988) but the available results are contradictory. In one set of experiments with rice, uptake of newly fixed N was slow (Eskew *et al.*, 1981) whilst other workers found 25% of the fixed ^{15}N in rice plants at the end of a 13-day incubation (Yoshida and Yoneyama, 1980). Comparison of $^{15}N_2$ incorporation with ARA estimates led Okon *et al.* (1983) to conclude that only 5% of the N_2 fixed in the rhizosphere had been taken up by *Setaria* over 21 days, but this small recovery could be due to errors in the estimates of total N_2-fixation rates, which were extrapolated from the ARA measurements.

These experiments give no evidence as to the mechanism by which N might be released from the bacteria. It is tempting to explain rapid uptake of fixed N by plants as being due to release of NH_4 by living bacteria, but the death of bacteria and turnover of microbial N can also be extremely rapid. These experiments also cannot help in determining how much N_2 is fixed under field conditions. One attempt to measure N_2-fixation associated with field-grown sugarcane using $^{15}N_2$ resulted in an increase in ^{15}N-enrichment of the soil, but not in enrichment of ^{15}N in the plant (Matsui *et al.*, 1981).

Estimates using the acetylene reduction assay

Inaccuracies in the use of ARA for measuring associative N_2-fixation that result from the natural production of ethylene in soil have already been described (Chapter 4). Unfortunately there are many other problems with this method. Early measurements were often made over long incubation periods of 24 h or more on cores of soil containing grass roots (e.g. Döbereiner *et al.*, 1972). It was found that there was a long lag phase of 12–24 h before significant activity was detected. This lag phase could be extended by the addition of fertilizer-N and only when all the N was used up were large rates of N_2-fixation observed (Fig. 6.1). Thus the lag phase is almost certainly due to exhaustion of available N supplies prior to the induction of nitrogenase. Long incubation times were also made necessary by the slow rates of diffusion of C_2H_2 and C_2H_4 through compact cores of soil, and this exacerbated the problem of induction of artificially high rates of ARA as N deficiency led to artificially high induction of nitrogenase activity.

Various different methods of incubating grass roots under acetylene have been described. These include intact soil cores in the field, soil cores taken and incubated in containers, plants grown in containers in the glasshouse and roots excised from the plant (van Berkum and Bohlool, 1980). But all of these methods suffer from various errors and, even where immediate linear rates of ethylene production are detected,

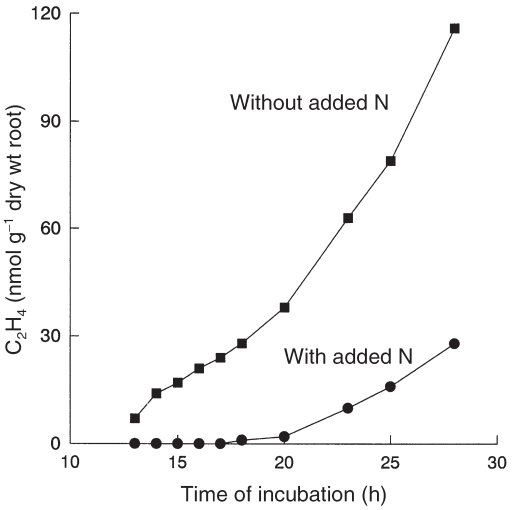

Fig. 6.1. The lag period before the onset of acetylene reduction activity associated with grass roots and the effect of added N fertilizer on the length of the lag phase. (Redrawn from van Berkum, 1978.)

much of the ethylene may come from endogenous ethylene production (Sloger and van Berkum, 1988).

If all of these problems are considered – in addition to the fact that at best the acetylene reduction assay can only provide a spot measurement of nitrogenase activity – then use of this method should be discouraged. Clark (1981) suggested that 'the ready acceptance of erroneous values is due to our enthusiasms' when considering ARA estimates of large amounts of N_2-fixation associated with grasses. Despite the detailed critique of the method given by van Berkum and Bohlool (1980) and later comments (Boddey, 1987; Giller, 1987), some papers are still published without due reference to the methodological problems.

It must be recognized that there are many reports in the literature of high rates of ARA (> 1–2 μM per plant h^{-1}) associated with the roots of cereals and grasses that are in some cases comparable to rates found with nodulated legumes. These large ARA values are often extremely variable from plant to plant and are often only obtained in very moist conditions. Several research programmes to select genotypes of cereal crops for N_2-fixation on the basis of ARA have been proposed – for instance, with rice (Ladha *et al.*, 1987) and with sorghum and millet (Wani *et al.*, 1984). With sorghum and millet, very high (yet variable) rates of ARA were only found when the growth media were watered to 60–70% of their water-holding capacity and were maximal when organic manure was added to the growth medium. This latter treatment would almost certainly override the effects of the roots in stimulating N_2-fixation by providing carbon substrates; in addition, the wet soil conditions required to promote high rates of ARA with sorghum and millet would rarely be experienced for long periods in the field.

With rice, consistent differences in root-associated ARA were found between genotypes (Ladha *et al.*, 1986) which were roughly proportional to the amount of plant biomass produced. Again, variability between plants was large (Tirol-Padre

et al., 1988) and controls to estimate endogenous ethylene produced in soil were not included. It is impossible to extrapolate from these ARA estimates to actual quantities of N_2 fixed. In another study the differences in ARA between genotypes were not correlated with variations in the natural abundance of ^{15}N ($\delta^{15}N$) in grains of rice (Watanabe *et al.*, 1987a). However, these authors point out that differences in $\delta^{15}N$ will depend on the efficiency of uptake of fixed N as well as on the rate of N_2-fixation in the rhizosphere, which is what it is supposed that ARA indicates. Neither of the research establishments at which this work on sorghum, millet (ICRISAT) and rice (IRRI) was conducted is pursuing selection programmes for enhanced ARA at present.

N balance studies

Much of the early evidence used to support the case that large amounts of N_2 are fixed in association with grass roots came from N balance studies (Moore, 1966; Dart, 1986). Several experiments were reported where different grass or cereal plots were shown to accumulate as much as 80 kg N ha^{-1} even though legumes were absent (e.g. Nye, 1958; Jones, 1971). In short-term trials where a large amount of soil N is present, gains of less than 100 kg N ha^{-1} can be difficult to measure (Dart and Day, 1975). Moreover, simple maintenance of cereal crop yields under continuous cropping cannot on its own be taken as evidence for gains from N_2-fixation. It is not at all clear from such studies where the N is coming from and there are several possiblities for gains not due to N_2-fixation. For example, although the contribution of N in rainfall may be small (< 5 kg N ha^{-1} $year^{-1}$) away from industrialized areas, uptake of N from deep soil layers may be important and might be overlooked in the N balance calculations. Gains of N could also, at least in part, be due to N_2-fixation by cyanobacteria when rainfall is sufficient. Estimates for N_2-fixation by cyanobacteria of 30 kg N ha^{-1} have been made in temperate arable agriculture (Witty *et al.*, 1979), where temperatures are considerably lower than those prevailing in most tropical soils, and cyanobacteria are abundant in many tropical upland soils (Roger and Reynaud, 1982).

^{15}N isotope dilution experiments

More recently the ^{15}N isotope-based techniques have been employed to estimate amounts of N_2 fixed in the rhizosphere. The ^{15}N natural abundance method has been judged to be insufficiently sensitive to measure N_2-fixation by free-living bacteria (Shearer and Kohl, 1988). Fried *et al.* (1983) concluded from experiments with legumes that the ^{15}N isotope dilution method was unlikely to be sensitive enough to detect rates of N_2-fixation of less than 10% of plant N. Nevertheless, it has been employed in this context.

Limitations of the methods

The main limitation in the use of isotope dilution for the measurement of associative N_2-fixation, apart from the issue of sensitivity, is the requirement for a non-fixing control plant. Comparisons of values derived from ^{15}N isotope dilution measurements with wheat genotypes grown in vermiculite, with or without inoculation with N_2-fixing bacteria, gave indications of gains from N_2-fixation (Rennie, 1980). However, given the proliferation of root hairs and stimulation of nutrient uptake often caused by inoculation with N_2-fixing bacteria, uninoculated plants are not likely to be good reference plants in such experiments. Moreover, later experiments showed that vermiculite can gradually release substantial amounts of unlabelled N (Giller *et al*., 1986) so that differences in isotope dilution between inoculated and uninoculated plants may be due to the amount of unlabelled N they were able to exploit and not due to N_2-fixation. These results highlight the danger of simply attributing N gains that are not accounted for to N_2-fixation.

Glasshouse studies

Attempts to measure N_2-fixation associated with *L. fusca* grown in sterile soil that had been amended with ^{15}N and cellulose prior to the experiment were confounded, because the inoculated plants had roots with many more branches and root hairs (Malik *et al*., 1987). Separation of the soil organic N into different acid hydrolysable fractions showed an isotopic dilution in all of the different pools of N in soils inoculated with N_2-fixing bacteria. The isotope dilution was greatest in the fraction that represented the N in the microbial biomass. In other experiments with *L. fusca*, another salt-tolerant grass (*Polypogon monospeliensis*) was found to be poorly matched as a reference plant and instead large fertilizer N additions were used to suppress any N_2-fixation and to provide a non-fixing reference treatment (Malik and Bilal, 1988). This approach may also introduce errors if the added fertilizer influences the availability of soil N in any way (Witty and Giller, 1991).

More recently the ^{15}N isotope dilution method and the natural abundance methods have been employed to study N_2-fixation with rice in flooded soils in the glasshouse. The two methods gave similar results, against a background of strong declines in ^{15}N enrichment of the available soil N, indicating that rice genotypes derived between 0 and 32% of their N from N_2-fixation (Malarvizhi and Ladha, 1996; Shrestha and Ladha, 1996).

'Field'-based estimates of N_2-fixation

Most workers have selected grass species or genotypes of cereal crops that have small amounts of ARA for use as reference plants in isotope dilution experiments. Several studies in Brazil have been conducted with different tropical grasses as test or reference plants in which the N-difference method and isotope dilution estimates have suggested similar amounts of N_2-fixation. The batatais cultivar of *P. notatum,* which has *A. paspali* in the rhizosphere, was estimated to gain 8–25% of its N from N_2-fixation, equivalent to approximately 20 kg N ha^{-1} when compared with the cultivar pensacola (Boddey *et al*., 1983). These studies were conducted in concrete cylinders filled with soil and sealed at the base with a cloth to restrict root growth but

allow drainage. Roots of the grasses did in fact penetrate into the soil below the cylinders, but the authors concluded that this was unlikely to introduce any significant error, as little N was present in the underlying soil.

Similar experiments were conducted with *Paspalum* and four other pasture grasses of the genus *Brachiaria* in which different methods of adding ^{15}N either as a soluble fertilizer or as labelled organic matter were compared (Boddey and Victoria, 1986). The different grasses were shown to exploit N from different depths in the soil profile, and the ^{15}N-labelled organic matter gave more uniform ^{15}N-enrichment than ^{15}N-labelled fertilizer and was thus chosen as the better method of labelling the soil N. The two grasses that were found to gain the largest proportion of N from N_2-fixation were *B. humidicola* (30%) and *B. decumbens* (40%) but these were planted (later than the other grasses) in cylinders where soybean had been grown in a previous experiment.

Further experiments compared ecotypes of *Panicum* for N_2-fixation using the residual labelling of ^{15}N in the same concrete cylinders that had been used for several other experiments and thus gave a stable enrichment of ^{15}N with time (Miranda *et al.*, 1990). The proportion of N from fixation in the *Panicum* was estimated to be 24–38%, equivalent to 5–10 kg N ha^{-1} $month^{-1}$ in the summer, using *Brachiaria arrecta* (syn. *B. radicans*) as a reference plant. Miranda and Boddey (1987) compared benefits to *Panicum* ecotypes from N_2-fixation in pots amended with ^{15}N-labelled compost. The ^{15}N-enrichment of the *Panicum* ecotypes decreased with time, whilst that of *B. arrecta* tended to increase (Fig. 6.2). Estimates of 16–36% of N from N_2-fixation were obtained, which correlated roughly with total N yield. There was not a particularly good agreement in the ranking of N_2-fixation between those genotypes included in both of their studies.

Much attention has focused on N_2-fixation associated with sugarcane. Two sugarcane genotypes grown in cylinders of soil amended with ^{15}N-labelled organic

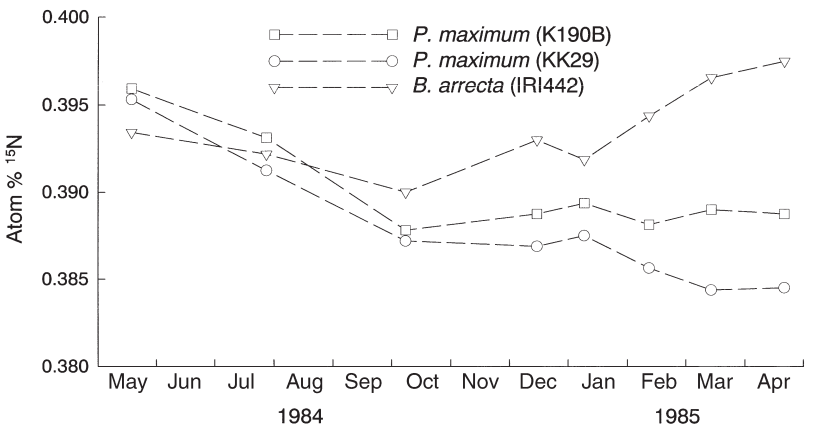

Fig. 6.2. Atom % ^{15}N of two ecotypes of *Panicum maximum* and a single genotype of *Brachiaria arrecta* grown in ^{15}N-labelled soil. (After Miranda and Boddey, 1987.)

matter were estimated to obtain 20–40% of their N from N_2-fixation, using non-nodulating soybean as the reference crop, but the sugarcane did not accumulate significantly more N than the non-nodulating soybean (de Freitas *et al.*, 1984). In a 2-year study with sugarcane grown in large pots containing 64 kg of soil labelled with ^{15}N-urea, more than 60% of the N was estimated to have come from N_2-fixation in one treatment, a conclusion supported by both ^{15}N isotope dilution and N balance methods (Lima *et al.*, 1987). The data from this experiment were extremely variable, with coefficients of variation often close to 50%, and no mention was made of the prevention of growth of cyanobacteria. Further experiments were conducted in a large concrete tank filled with soil and carefully labelled with ^{15}N fertilizer and organic matter, which was mixed throughout the soil (Urquiaga *et al.*, 1992). Initial results were confounded by a declining ^{15}N-enrichment of available soil N from around 1 atom % ^{15}N excess to 0.16 atom % ^{15}N excess over the first year. After 3 years this stabilized and estimates of 60–80% N from fixation (up to 270 kg N ha^{-1} year^{-1}) were obtained, which were supported by N balance data. The plants were watered with tap water according to the soil moisture status, but no indication was given as to the nitrate concentrations in the water. Presumably the larger plants had a greater demand for water, and would therefore have received more nitrate. When rice was later sown in these tanks, no evidence for N_2-fixation was found among 40 varieties (Boddey *et al.*, 1995).

The only true field study appears to be that of Yoneyama *et al.* (1997), who compared the natural δ^{15}N abundance of sugarcane growing on farms in Brazil, the Philippines and Japan with that of neighbouring plants. Results were highly variable, indicating that between 0 and 76% of the N in sugarcane came from N_2-fixation.

Can the results be accepted with confidence?

The attention to detail given in these experiments by Boddey and co-workers is extremely useful as it allows an evaluation of the possible flaws in the methods. There still remains a possibility that some of these differences between grasses in ^{15}N isotope dilution, δ^{15}N and N accumulation could be due to differences in their ability to access soil organic N, as Miranda and Boddey (1987) acknowledge, perhaps due to effects of the grasses on mineralization processes. However, the evidence to date supports the conclusion that the results are due to N_2-fixation. This is an important area for clarification in the future – there is still no firm field information on the contribution of N from N_2-fixation.

Responses to Inoculation with Heterotrophic N_2-fixing Bacteria

If, as discussed above, there is little evidence for a significant contribution of N from N_2-fixation with cereal crops, then how do we explain the numerous reports of responses in yield to inoculation with N_2-fixing bacteria? On careful examination of the literature it becomes apparent that crop responses to inoculation are not found consistently (Boddey and Döbereiner, 1982). Whilst the differences in results could

in part be due to use of different methods of inoculation or different inoculum strains, inconsistent results were obtained even when the same researchers carried out several experiments. Increases in grain yield were found in fewer than 50% of a large number of inoculation experiments conducted with sorghum and millet (Wani *et al.*, 1985, 1988) and responses in yield and N uptake were often greater when fertilizer-N was applied (Okon, 1982).

Production of plant growth hormones (auxins, gibberellins and cytokinins) by heterotrophic N_2-fixing bacteria has been demonstrated (Brown, 1976) and these can cause increased root branching and root hair production when cereals are inoculated (Tien *et al.*, 1979; Fig. 6.3). Inoculation with *A. brasilense* enhanced uptake of nitrate, potassium and phosphorus by *Setaria* (Lin *et al.*, 1983). Field experiments with wheat in Brazil showed that uptake of ^{15}N-labelled fertilizer, plant N content and grain yield were increased by inoculation but the ^{15}N data and ARA measurements confirmed that the yield increases were not due to N_2-fixation (Boddey *et al.*, 1986). Similar results were found with rice inoculated with *Azospirillum* where $^{15}N_2$ gas exposure, ARA and ^{15}N isotope dilution data confirmed that enhanced grain yields were due to increased N uptake and not increased N_2-fixation (Nayak *et al.*, 1986). In India, no responses to inoculation were found in a series of field trials with nine field trials with sorghum and millet, and measurements of $\delta^{15}N$ natural abundance gave no evidence for N_2-fixation (Lee *et al.*, 1994). Pot and field experiments with rice inoculated with *B. vietnamiensis* in Vietnam (Van *et al.*, 2000) or rhizobia in the Philippines (Biswas *et al.*, 2000) indicated increases in accumulation of N and other nutrients, but there was no evidence that this was due to N_2-fixation. Indeed,

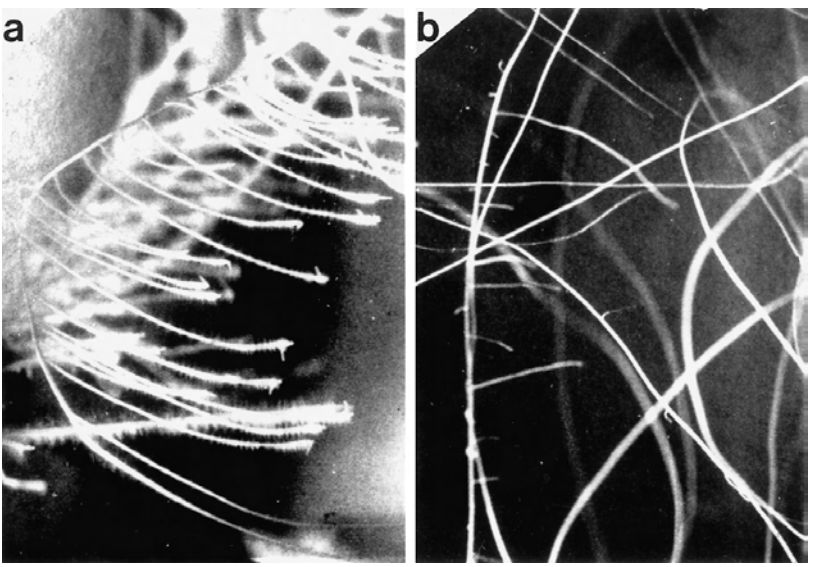

Fig. 6.3. Proliferation of hairs on roots of *Pennisetum* (a) inoculated with *Azospirillum brasilense* compared with (b) uninoculated roots. (From Tien *et al.*, 1979.)

A. diazotrophicus and *Paenibacillus polymyxa* also produce compounds which stimulate plant growth, including indole acetic acid (IAA), which may contribute to the growth benefits seen when these bacteria are inoculated on to plants (Lebuhn *et al.*, 1997; Sevilla *et al.*, 1998; Sevilla and Kennedy, 2000).

Where responses of enhanced growth and N uptake to inoculation with free-living N_2-fixing bacteria are found, they can thus often be attributed to growth stimulatory compounds produced by the bacteria. It is somewhat ironic that we have come full circle in the study of *Azospirillum* and associative symbioses to arrive at the same conclusions as those of Brown and colleagues (Barea and Brown, 1974) had from the study of *Azotobacter* in the early 1970s – the same time that grass roots were first exposed to acetylene in the same laboratory (Döbereiner *et al.*, 1972).

N_2-fixation by Free-living Bacteria in Soil

Plant residues such as straw or leaf litter provide an important input of carbon into the soil which can act as a substrate for N_2-fixation by heterotrophs. Addition of rice straw to flooded soils can cause large increases in the rate of heterotrophic N_2-fixation (Yoneyama *et al.*, 1977; Rao, 1978; Reddy and Patrick, 1979). Sugarcane litter has been shown to support a large ARA not due to endogenous ethylene production (Hill and Patriquin, 1990). A cellulolytic fungus, *Helicomyces roseus*, appears to be important in liberating carbon from the cane litter in forms that can be used for N_2-fixation by the large numbers of *A. brasilense* found in the litter. The rate of ARA was greater with warmer temperatures that stimulated microbial consumption of oxygen, thus helping to create micro-aerophilic conditions ideal for N_2-fixation by *Azospirillum* (Hill *et al.*, 1990). In many upland soils the extent to which N_2-fixation can occur on plant residues will be limited by the moisture status and the rate of decomposition of the litter. Although free-living bacteria undoubtedly fix N_2 in soil and rates of N_2-fixation are stimulated by the addition of organic matter, there are no good estimates for the amounts of N contributed in the field. It seems likely that amounts of N_2 fixed will rarely exceed 5 kg N ha^{-1} $year^{-1}$ in upland soils, although amounts may be larger under the waterlogged conditions that prevail with lowland rice cultivation (Roper and Ladha, 1995). Surprisingly little research has been targetted to N_2-fixation by anaerobic bacteria such as *Clostridium* in rice paddies, given the large amounts of rice straw available as a substrate.

Genetic Engineering of Cereals to Fix N_2

Cereal crops that can fix their own N directly from the atmosphere have long been a dream of scientists. Two approaches have been explored: to introduce nitrogenase directly into the plant so that it can fix N_2 directly; and, more recently, to manipulate the cereal plant so that it can nodulate with rhizobia.

Introducing nitrogenase into cereal crops

The principal target for introduction of nitrogenase to cereals is the chloroplast. It is now widely accepted that chloroplasts originated as symbiotic bacteria, and genes in chloroplasts are expressed in a similar way as in bacteria (Merrick and Dixon, 1984). The major problem envisaged for active N_2-fixation in chloroplasts is protection of nitrogenase from molecular oxygen produced during photosynthesis. As described in Chapter 3, the unicellular cyanobacterium *Gloeothece* synthesizes nitrogenase in the dark but is then able to continue to fix N_2 when returned to the light. By analogy it has been proposed that plants could be engineered to be nocturnal N_2-fixers, but extra challenges remain. Such a plant system must ensure the energy supply for nitrogenase throughout the night, and ensure that nitrogenase could be protected to avoid the need for daily *de novo* synthesis of the enzyme.

One of the many nitrogenase genes, *nifH*, has been inserted into the chlorophyll genome of the green alga *Chlamydomonas*, which is being used as a model system for higher plants (Dixon *et al.*, 2000). Although this gene appears to be expressed in *Chlamydomonas*, there has been surprisingly little advance in this field since the early 1980s, presumably due to a lack of targeted funding for the research. Dixon *et al.* (2000) suggested that the oxygen-tolerant nitrogenase recently described in *S. thermoautotrophicus* (Ribbe *et al.*, 1997) (Chapter 3) may be a promising alternative model for overcoming the oxygen problem in chloroplasts. Unfortunately, a set of different biochemical problems are envisaged in this case, most notably the generation of superoxide that the eukaryotic cell may not tolerate. Whichever approach is pursued, a functioning eukaryotic nitrogenase will require the additional machinery to supply reductant and electrons in adequate amounts, which may be much more problematic than engineering plants to manufacture nitrogenase.

Nodulating cereals

Substantial research on achievement of N_2-fixing nodules in cereal crops was stimulated by the observation that 'nodular structures' could be induced on roots of rice and *Brassica napus* (Al-Mallah *et al.*, 1990a,b; Cocking *et al.*, 1990; Jing *et al.*, 1990). Cocking's group induced nodules in *Brassica* and rice by treating the roots with cellulase and pectolyase in the presence of polyethylene glycol (to prevent lysing of cells when the cell wall was degraded). Surprisingly poor microscopic evidence was presented for invasion of the plant tissues by bacteria, and insignificant amounts of ethylene production were reported (see Chapter 4 for a discussion of the pitfalls of this method). A follow-up of the reports of Jing, Li and colleagues (Jing *et al.*, 1990; Li *et al.*, 1991) revealed that the 'nodules' on rice appeared to be formed as a result of fungal infections, or were short lateral root meristems, and attempts to reisolate rhizobia from these 'paranodules' failed (de Bruijn *et al.*, 1995).

Particular attention is being devoted to exploring the prospects for nodulation and N_2-fixation in rice, as the potential benefits are immense (Khush and Bennett,

1992; Ladha *et al*., 1997; Ladha and Reddy, 2000). This 'frontier project' aims to increase N_2-fixation by associated and endophytic bacteria, and to enhance the efficiency of general N metabolism in addition to exploring prospects for formation of N_2-fixing nodules in rice. Close examination reveals that 'nodular structures' reported on rice roots have a central vasculature and are, in fact, stubby lateral roots. Reddy *et al*. (1997), in a study with excellent attention to detail, examined the predisposition of rice to infection by rhizobia. Although rhizobia were able to gain entry into rice roots, the association was non-specific, with only passive involvement of the plant and no evidence for recognition between the bacteria and the plant roots. A suite of other characters in rice differ from the symbiosis between legumes and rhizobia: rice did not induce rhizobial *nod* genes; no true nodule (or induction of lateral roots) occurred; there was no root hair attachment or cellulose microfibril development; bacteria were present only in intracellular spaces or in lysing cells; and a schlerenchymatous layer of cells denied the rhizobia admission to the deeper layers of the root cortex.

On the other hand, roots of rice and most other cereal crops are infected by mycorrhizas, and this may provide some clues to establishment of closer associations with rhizobia. Homologues of nodulin genes, which are expressed specifically during nodule development in legumes, are present in rice and other grasses (Reddy *et al*., 1999). In rice, one nodulin gene appears to be expressed in the early development of vascular bundles (Kouchi *et al*., 1999). Current thinking and future approaches to achieving nodulation and N_2-fixation in rice are discussed in detail in Ladha and Reddy (2000).

Conclusions

Knowledge of the occurrence and biology of heterotrophic N_2-fixers has now increased enormously, but we still, shamefully, lack good methods for accurately quantifying the amounts of N_2 fixed. The case for an agriculturally 'significant' contribution of fixed N remains to be proved, but in natural grasslands, over long time-periods, the small inputs from free-living or associative N_2-fixation are undoubtedly valuable (Giller and Day, 1985).

Given the abundant supply of sucrose and the presence in its tissues of *A. diazotrophicus*, which can utilize sucrose to fix N_2 (Gillis *et al*., 1989), sugarcane in particular seems a very good candidate for supporting significant amounts of heterotrophic N_2-fixation (Boddey, 1995). To date there is little evidence to indicate that endophytic N_2-fixation is more important than N_2-fixation in the rhizosphere. Whilst proof of N_2-fixation by endophytes can be gained by incubating plants inoculated with N_2-fixing bacteria or *nif* mutants in the presence of $^{15}N_2$ gas, or by assessing ability of such inoculated plants to grow in N-free media (Chapter 4), this will not help in understanding what happens in the field. Long-term N balance studies, in which all sources of N are carefully monitored and controlled over periods

of 10 to 20 years, are probably the only way of understanding whether N_2-fixation with sugarcane can sustain production. Although research has been initiated to explore the transfer of the ability to fix N_2 to cereals, this still remains a fairly distant prospect.

Chapter 7

Cyanobacteria and *Azolla* as Green Manure for Wetland Rice

Rice is the staple food for almost half of the world's population and approximately 90% of the world's rice is produced in Asia (De Datta, 1981). Most rice is produced in shallow, flooded paddy fields in lowlands under rainfed or irrigated conditions, but other rice varieties can be grown in deep waters or in upland agriculture under rainfed conditions. Deepwater rice is produced in low-lying fields that are flooded to 0.5–1.0 m depth for half of the crop's growth. 'Floating' rice is grown where the floodwater is up to 6 m deep; the rice plants root in the soil but are able to elongate as the floodwater rises gradually. Over 2 million ha of rice are produced in this way in Bangladesh and flooded rice is also produced in India, Thailand and West Africa. Upland rice accounts for about 10% of the world's production and is grown in Asia, Africa and Latin America.

Yields of rice can be sustained in some regions at a moderate level of production of about 2 t ha^{-1} even where no fertilizers are used and this has caused scientists to speculate about the importance of N_2-fixation in the maintenance of soil fertility. Much research has been carried out on the role of free-living heterotrophic N_2-fixing bacteria in paddy soils. Research has also focused on the role of both the rice plant and rice straw in stimulating such N_2-fixation and this was discussed in the previous chapter. De (1939) recognized the potential importance of N_2-fixation by cyanobacteria in maintenance of soil fertility in paddy soils, and farmers have long been aware of the benefits of *Azolla*, a floating fern with symbiotic N_2-fixing cyanobacteria, which has been used to enrich the soil in parts of China and Vietnam for centuries (Lumpkin and Plucknett, 1980, 1982). The ecology and practical management of cyanobacteria and *Azolla* in rice fields are thoroughly reviewed by Roger (1995b).

Cyanobacteria

The cyanobacteria or blue-green algae are photosynthetic bacteria and some of them are able to fix N_2 (Chapter 2). They can be divided into two major groups based on growth habit: the unicellular forms and the filamentous forms. N_2-fixing species from both groups are found in paddy fields but the predominant ones are the heterocystous filamentous forms (Table 7.1). Cyanobacteria are not restricted to permanently wet habitats as they are resistant to desiccation and hot temperatures, and can be abundant in upland soils (Roger and Reynaud, 1982). However, wet paddy soils and the overlying floodwaters provide an ideal environment for them to grow and fix N_2.

Natural distribution

Free-living cyanobacteria can grow epiphytically on aquatic and emergent plants as well as in floodwater or on the soil surface. Early surveys indicated that cyanobacteria were only present in a small proportion of rice fields. Only 5% of over 900 soil samples from Asia and Africa (Watanabe, 1959; Watanabe and Yamayoto, 1971) and

Table 7.1. The main taxa of N_2-fixing cyanobacteria (indicating the Section of the Cyanobacteria to which the groups belong – see Table 2.3) found in rice soils in Southeast Asia. (From Roger *et al.*, 1987.)

Subdivision of the Cyanobacteria	Important genera and their morphology
Unicellular group Section I	Unicellular strains (*Aphanothece*, *Gloeothece*)
Anabaena group Section IV	Heterocystous strains with a thin sheath, without branching, do not form mucilaginous colonies of definite shape (*Anabaena*, *Nodularia*, *Cylindrospermum*, *Anabaenopsis*)
Nostoc group Section IV	Heterocystous strains with a thick sheath, without branching, forming mucilaginous colonies of definite shape (*Nostoc*)
Aulosira group Section IV	Heterocystous strains with a thick sheath, usually without branching, do not form diffuse colonies on agar medium (*Aulosira*)
Scytonema group Section IV	Heterocystous strains with false branching, without polarity, forming velvet-like patches on agar medium (*Scytonema*)
Calothrix group Section IV	Heterocystous strains with false branching, with polarity, forming velvet-like patches on agar medium (*Calothrix*, *Tolypothrix*, *Hassalia*)
Gloeotrichia group Section IV	Heterocystous strains, with polarity, forming mucilaginous colonies of definite shape (*Gloeotrichia*, *Rivularia*)
Fischerella group Section V	Heterocystous strains with true branching (*Fischerella*, *Westiellopsis*, *Stigonema*)

only some 33% of more than 2200 samples of Indian rice soils (Venkataraman, 1975) were found to contain cyanobacteria. This reported infrequent occurrence was almost certainly due to only small samples of soil being taken from each field and also to the use of unsuitable methods for detection. Many other studies have found cyanobacteria in all of the soils sampled (Roger and Reynaud, 1982, and references therein). Although the relative abundance may vary widely, heterocystous genera generally account for about half of the cyanobacteria in rice fields (Whitton and Roger, 1989). In fact, in deepwater rice fields studied in Bangladesh virtually all of the cyanobacteria were heterocystous forms (Whitton *et al.*, 1989).

Numbers of heterocystous cyanobacteria in rice soils expressed as colony-forming units (cfu) ranged from 10 to 10^7 cfu g^{-1} soil, with a mean value of 2.5×10^5 cfu g^{-1} soil or 8.3×10^4 cfu cm^{-2} in ten surveys in which more than 280 soils were sampled (Roger *et al.*, 1987). In most of these studies a most probable number (MPN) method for counting was used, in which serial dilutions are made and the population size is estimated on the basis of presence or absence of growth of cyanobacteria at the different dilutions. However, a modified direct-plating method using selective media gave an average value of 3.2×10^5 cfu cm^{-2} over 102 soil samples, which was roughly four times greater than the mean number found previously (Roger *et al.*, 1987). On average, heterocystous cyanobacteria formed less than 10% of the population of eukaryotic green algae and the abundance of cyanobacteria increased both with the amount of available phosphorus and with pH values over the range 4–6.5. Above pH 6.5 the numbers of cyanobacteria showed no obvious relationship with pH. These results agree with earlier observations that N_2-fixing cyanobacteria are more abundant in phosphorus-rich soils of neutral to alkaline pH (Roger and Kulasooriya, 1980).

Amounts of N_2 fixed by cyanobacteria in rice production

An average value from 38 measurements of N_2-fixation by cyanobacteria collated from the literature was 27 kg N ha^{-1} per rice crop, with a maximum of 50–80 kg N ha^{-1} (Roger and Kulasooriya, 1980). However, most of these measurements were made using the acetylene reduction assay and are unlikely to be accurate, given the problems of calibration of the assay and the well-documented diurnal fluctuations in measured rates of N_2-fixation (Chapter 4). In a detailed study of ARA due to cyanobacteria in 190 rice fields in the Philippines, the mean activity was 126 μM C_2H_2 reduced m^{-2} h^{-1}, roughly equivalent to 12 kg N ha^{-1} fixed over a cropping season (Fig. 7.1). ARA estimates of N_2-fixation indicated that greater amounts of N were fixed by cyanobacteria (7 kg N ha^{-1}) on the wet soils before flooding than in the standing waters (2 kg N ha^{-1}) of deepwater rice fields (Rother *et al.*, 1989). A bloom of cyanobacteria usually contains less than 10 kg N ha^{-1}, though a dense bloom may contain up to 25 kg N ha^{-1} (Roger *et al.*, 1986; Roger and Ladha, 1992). Such blooms may exhibit high rates of N_2-fixation and can persist for several weeks (Rother *et al.*, 1989). For a cyanobacterial bloom to be sufficiently large to

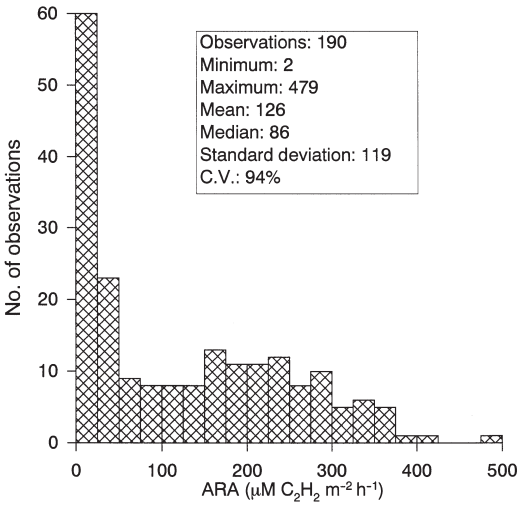

Fig. 7.1. Measurements of acetylene reduction activity (ARA) in flooded rice soils. Each value is the mean of nine to 13 measurements during a crop cycle. Over a cropping season, 10 μM C_2H_2 m^{-2} ha^{-1} is estimated to be roughly equivalent to 1 kg N_2 fixed ha^{-1}. (Unpublished data of P.A. Roger.)

make a significant input of fixed N, it would have to be readily visible in the field (Roger *et al.*, 1986).

Of course, in the short term, the important measurement is not simply the amount of N_2 fixed but the amount acquired by the rice crop. Experiments using ^{15}N-labelled cyanobacterial cells spread on the soil surface or incorporated into the soil showed that between 36 and 51% of the added N was recovered by rice in the first season (Wilson *et al.*, 1980). Similar pot and field experiments indicated that 23–28% of the N in ^{15}N-labelled cyanobacteria incorporated into the soil was recovered in the first rice crop whilst only 14–23% of the N was recovered if the cells were left on the soil surface (Tirol *et al.*, 1982). Timing is also important: N_2 fixed or released towards the end of the growing season will be too late to influence production of the current rice crop (Whitton and Roger, 1989).

Based on these data it can be assumed that one-quarter of the N_2 fixed by cyanobacteria is utilized by the next rice crop. Then if 15–25 kg N ha^{-1} are fixed during each crop, this would represent a benefit of some 4–6 kg N ha^{-1}. Thus the amounts of N_2 fixed by cyanobacteria are likely to be insufficient to sustain high yields of rice but will be important in the long-term maintenance of soil fertility in paddy fields.

Inoculation with cyanobacteria

The earlier reports of the sparse distribution of cyanobacteria in paddy fields have been used as a justification for an intensive research effort into technology for inoculation with cyanobacteria, or 'algalization', particularly in India. A method for production of algal inoculants was developed in India that was suitable for use by small-scale farmers (Venkataraman, 1981). An initial inoculum containing six species of cyanobacteria was provided to farmers by the 'All India Coordinated Project

on Algae'. The farmers then multiplied this inoculum in shallow tanks in up to 15 cm of water to which some soil, phosphorus fertilizer and insecticide were added, together with some lime where necessary. The tanks were simple in design, either consisting of a shallow pit lined with plastic sheet, or larger inoculum production units were made by mounding up soil to make shallow bunds (ridges) in the field. After a few weeks a mat of cyanobacteria and green algae developed; this was allowed to dry out and flakes of the inoculum were scraped up and stored for later use. Sufficient flakes to inoculate 1 ha (8–10 kg) were produced from a single tank in 2–3 months. The density of total cyanobacterial and algal propagules in these inocula varied from 2×10^6 to 9×10^7 cfu g^{-1} soil but only 2–32% of these were heterocystous cyanobacteria, the type most important for N_2-fixation (Roger *et al.*, 1987). It has been suggested that use of a multi-strain starter inoculum and production of inoculum using local soil in the tanks would lead to selective growth of the strains best adapted to the local soil conditions. However, this was not borne out by the results of Roger *et al.* (1987), who found that one or two strains, most commonly a *Nostoc*, were generally dominant among the N_2-fixing cyanobacteria present in the inocula produced. The results of these workers, indicating that N_2-fixing cyanobacteria are in fact abundant in rice fields, put the necessity for inoculation in question. Nevertheless, should inoculation be deemed necessary, Roger *et al.* (1987) recommended that a better multi-strain inoculum would be produced by mixing single-strain inocula at the time of inoculation. Even so, as the inoculum is produced in local soil, it is still possible that strains present in the soil may dominate and so the original inoculum strains are lost before the inoculum even reaches the field (Whitton and Roger, 1989).

Results of inoculation experiments are not encouraging, even though many of these were conducted with inocula produced in the laboratory, i.e. in which local strains had not had a chance to outcompete inoculum strains prior to inoculation. An average response of 15% in yield of rice was found in experiments where inocula of cyanobacteria were applied in the field (Roger and Kulasooriya, 1980). In many experiments the effects of inoculation and N-fertilizer application on growth of rice were often additive and were attributed to production of plant growth-stimulating compounds by the cyanobacteria and not to N_2-fixation. However, screening of 133 strains of cyanobacteria showed that 70% of the strains had an inhibitory effect on germination of rice and only 20% of the strains stimulated elongation of rice shoots (Pedurand and Reynaud, 1987). This suggests that hormonal effects of cyanobacteria are not the principal cause of improved rice growth when responses to inoculation are observed.

In many cases no benefits in yield were found after inoculation (Roger, 1995b). This is perhaps not surprising as the recommended rate of inoculum application will provide on average less than one propagule for every 130 indigenous cyanobacteria already present (Roger and Kulasooriya, 1980). In one study the inoculated strains could not be detected even immediately after inoculation, presumably as they formed such a small proportion of the total algal population (Grant *et al.*, 1985). In other experiments, inoculated strains did multiply but rarely dominated the population of cyanobacteria (Reddy and Roger, 1988). When beneficial effects on plant growth

due to inoculation have been found it is likely that establishment of large populations of cyanobacteria has been possible due to the large phosphorus content of propagules in the inocula, which will give the introduced cells a substantial growth advantage over indigenous strains (Roger *et al.*, 1986). In any case, whether the inoculum strains succeed in becoming established or not, there is little evidence indicating that they can fix N_2 more effectively than indigenous cyanobacteria.

Manipulation of indigenous populations of cyanobacteria

Given the lack of success of inoculation, an alternative strategy to improve the inputs of N_2-fixation is to enhance the growth of indigenous cyanobacteria (Roger, 1995a). Low pH, low temperatures and phosphorus deficiency are all factors that are known to limit growth, but the ecology of cyanobacteria is poorly understood (Roger and Watanabe, 1986). It is apparent that addition of phosphorus fertilizers is likely to stimulate their growth and addition of lime to floodwaters will help in acid soils.

The most important management practice is to add phosphorus fertilizers to the floodwater, combined with 'deep placement' of the N fertilizer (Roger, 1995b). When urea fertilizers are broadcast, substantial amounts of mineral N are present in the floodwater. As N is not limiting, green algae proliferate and bloom, effectively outcompeting the cyanobacteria. Dense blooms of green algae tend to cause the pH of the floodwater to rise, creating ideal conditions for loss of N through volatilization of ammonia. If N fertilizer is placed below the surface of the soil, the rice plants are able to take full advantage of the N without danger of gaseous loss of ammonia, and as N concentrations in the floodwater remain small, cyanobacteria are able to bloom and contribute N to their full potential. Unfortunately, this practice is rarely economic, due largely to the extra labour required to place the N fertilizer.

Another problem that restricts the size of populations of cyanobacteria is predation by invertebrates. Some cyanobacteria (e.g. *Aphanothece*, *Gloeotrichia* and *Nostoc*) are able to form mucilaginous colonies, which renders them more resistant to grazing by invertebrates (Grant *et al.*, 1985), but such strains generally contain little N. A standing crop of these cyanobacteria of 10 t ha^{-1} may contain as little as 3 kg N ha^{-1} (Grant *et al.*, 1985). Pesticides, including some natural products from plants such as neem (*Azadirachta indica*), can be used to reduce grazing pressure (Reddy and Roger, 1988). Research interest has been directed towards the isolation or production of pesticide-resistant strains but this work has not been extended outside the laboratory (Whitton and Roger, 1989).

Azolla as a Green Manure

The aquatic fern azolla is always found in an N_2-fixing symbiosis with a cyanobacterial species called *Anabaena azollae*, although the exact identity of the endosymbiont remains uncertain (as discussed in Chapter 2). Seven species of *Azolla* are recognized in most taxonomic schemes.

Azolla can either be grown alone in rice fields before rice planting, if sufficient water is available to flood the fields, or it can be grown among the rice plants and periodically dug into the soil. A period of 2–3 weeks' growth is required to accumulate sufficient biomass before this treatment can be carried out. In southern China, azolla is most commonly grown in the winter before transplanting of rice, whilst in the north it is either grown before rice, or intercropped with rice, or both (Lumpkin and Plucknett, 1982). In some areas rice is grown in densely planted strips with wide gaps containing azolla, which is incorporated into the soil several times during each crop. In Vietnam, a first crop of azolla is mounded up after 2–3 weeks' growth and composted, allowing further multiplication and growth of the remaining fronds for 10 days. The compost is then turned into the soil with part of the living azolla when rice is transplanted, and the remainder of the living azolla is allowed to multiply between the transplanted rice plants so that more azolla can be turned into the soil later on, giving a total of approximately 80 kg of azolla N ha^{-1} (Roger and Watanabe, 1986).

Production of 'inoculum'

As many rice paddies are dry for much of the year, azolla must be reintroduced each growing season by inoculation. Azolla can be maintained in ponds, irrigation channels or slowly moving streams. The inoculum density required to ensure good establishment is high (2–5 t ha^{-1}) to prevent the azolla from being overgrown by algae or weeds (Watanabe, 1982). The only way to achieve this practically is to multiply the initial inoculum within small areas of the fields where it is to be used (Van Hove, 1989). In Vietnam the initial inoculum, which is supplied from government farms, is multiplied in a part of the rice field until there is sufficient to be spread out over double the area. This process is then repeated, giving an exponential increase in the area covered.

Constraints to the growth of azolla

Under optimal conditions, azolla can double its mass every 2–3 days (Watanabe, 1982). In controlled environments, the growth rate of *A. mexicana*, *A. microphylla* and *A. pinnata* was greatest above 30°C but *A. caroliniana* and *A. filiculoides* grew better at temperatures below 25°C (Peters *et al*., 1980; Watanabe and Berha, 1983; Kannaiyan and Somporn, 1989). However, total biomass production was greater in all species at temperatures below 25°C and differences in tolerance to higher temperatures were observed between strains (Watanabe and Berha, 1983). *A. pinnata*, *A. microphylla* and *A. mexicana* were also the species most tolerant to transient high temperatures (> 40°C) (Uheda *et al*., 1999). Experiments in which *A. filiculoides* and *A. microphylla* were grown with reciprocal crosses of *Anabaena* isolates have demonstrated that tolerance to high temperatures requires adaptation of both symbionts (Watanabe *et al*., 1989b).

In natural conditions various constraints, such as unfavourable temperatures, phosphorus availability, lack of water and insect pests, can limit azolla production. Growth of azolla in the field is generally poor in the humid tropics when the average monthly temperature is greater than 27°C, but the highest rates of azolla production in the dry climate of Senegal (which were much greater than the rates of azolla production found for the same strains of azolla in the Philippines) occurred in months with a mean temperature above 29°C (de Waha Baillonville *et al.*, 1991).

Shading by the rice crop can help in hot periods by reducing the temperature of both the floodwater and the air close to the surface. Conversely, shading by rice can severely limit growth of azolla, as it requires a high light-intensity for growth (Lumpkin, 1987). Temperatures that are too high in summer or too low in winter for azolla to survive obviously cause problems for the use of azolla in agriculture and many different methods have been devised in China to ensure survival of an inoculum for the next growing season (Lumpkin and Plucknett, 1982).

Growth of azolla is often limited by phosphorus availability. Azolla growing in the field commonly develops a reddish-purple coloration, which has often been considered to be characteristic of phosphorus deficiency, although intense sunlight and other physiological stresses can also cause azolla fronds to produce anthocyanins (Watanabe, 1982; Van Hove, 1989). If the water is shallow (< 3 cm deep) azolla can root into the soil, which may help in uptake of phosphorus, but generally addition of phosphorus fertilizers is necessary to ensure good growth. Watanabe *et al.* (1988) suggested that phosphorus fertilizers should be applied to nursery beds in split doses. Azolla enriched in P in this way can multiply several times before it becomes P-deficient, and the better growth can increase the N benefit to rice (Singh and Singh, 1995). There appear to be differences between azolla species in their tolerance to phosphorus limitation: *A. pinnata* grew better under P-limiting conditions than *A. microphylla* or *A. mexicana* (Kushari and Watanabe, 1991). At least some of the phosphorus added to azolla will be made available to a subsequent rice crop when the green manure decomposes.

If the paddy dries out, azolla can only survive for a few days and this can severely limit use of azolla in areas with unpredictable rainfall, unless the fields are irrigated (Lumpkin, 1987). A number of insect larvae attack azolla and damage tends to be particularly severe in hot climates (Watanabe, 1982). Damage by insects appears to be one of the most important factors that lead to poor performance of azolla in the field in Asia.

Amounts of N_2 fixed

Many of the early experiments looking at amounts of N_2 fixed by azolla were carried out using the acetylene reduction assay. Calibration of ARA measurements of N_2-fixation showed that conversion ratios for $C_2H_2 : N_2$ ranged from 1.6 : 1 (theoretically impossible) to 7.9 : 1 and varied with the *Azolla* species under test, the length of the assay and the age of the culture (Eskew, 1987). Thus ARA measurements are unlikely to give reliable measurements of N_2-fixation by azolla.

Several estimates made using the ^{15}N isotope dilution method indicated that azolla derives more than 80% of its N from N_2-fixation in the field (Eskew, 1987). The proportion of N from N_2-fixation in 99 accessions of azolla ranged from 30 to 80% when grown under controlled conditions in the presence of 40 mg N l^{-1}, suggesting that there was substantial variation in the sensitivity of N_2-fixation to large N concentrations (Okoronkwo *et al.*, 1989). Isotope dilution measurements indicated that *A. pinnata* fixed 80–82% of its N in the field (Watanabe *et al.*, 1991). However, this method requires the addition of some ^{15}N-labelled fertilizer to the floodwater or the underlying soil, which can make the conditions unrealistic as the concentration of N in the water in the field rarely exceeds 1 mg N l^{-1} (Eskew, 1987). Measurements based on the natural abundance of ^{15}N using *Lemna* (a small floating aquatic plant) as a reference indicated that *A. microphylla* obtained almost all (99%) of its nitrogen from N_2-fixation when grown in the field (Yoneyama *et al.*, 1987). It therefore seems likely that azolla fixes virtually all of its N under field conditions.

Accumulation in field-grown azolla of 38–93 kg N ha^{-1} over 30–46 days has been recorded (Watanabe, 1982). With repeated harvesting, annual production rates can be as high as 500–1200 kg N ha^{-1}. Daily production rates of 0.4–3.6 kg N ha^{-1} were calculated from published values of N accumulation, with a mean rate of 2 kg N ha^{-1} day^{-1} (Kikuchi *et al.*, 1984). Azolla grown as an intercrop with rice can accumulate from 25 to 170 kg N ha^{-1}, 40 kg N ha^{-1} on average (Kikuchi *et al.*, 1984).

Recovery of fixed N by rice

Negligible amounts of ammonia are excreted into water by azolla (Watanabe and Berha, 1983; Ito and Watanabe, 1985) so uptake of azolla N by rice is dependent on the death and decomposition of the azolla. Incorporation of ^{15}N-labelled azolla fronds into the soil allows the direct measurement of recovery of N from the decomposing green manure by rice plants; using this method, recoveries of up to 28% of the azolla N were found in the field in the first crop (Ito and Watanabe, 1985). More of the azolla N was taken up by rice when the azolla was incorporated into the soil (> 25% of N recovered) than when azolla was left on the soil surface or grown in water between the rice plants (< 15% of N recovered). It is important that such experiments are carried out with fresh azolla material, as drying or freezing of azolla prior to application can reduce the recovery of N by more than a third (Kumarasinghe *et al.*, 1986). Experiments conducted using this method in Brazil, Nigeria and several Asian countries indicate that, on average, about 40% of the azolla N is recovered in the first crop of rice (Kulasooriya *et al.*, 1988; Eskew and Kovacs, 1991), and 4% in the second rice crop (Kumarasinghe and Eskew, 1993). In the Philippines, 39% of the N in *A. microphylla* turned into the soil at planting was taken up by rice but 63% of the N was recovered when a second incorporation was made among the growing crop 42 days later (Watanabe *et al.*, 1989a).

Incubation of azolla green manure in waterlogged soil in the laboratory showed that mineralization was rapid, with 60–80% of the N being released within 2 weeks

(Ito and Watanabe, 1985; Watanabe *et al.*, 1989a). As the authors indicated, the azolla used in these studies had a high N content (> 4.5%) compared with the concentrations commonly observed in field-grown azolla (*c.* 3.9%) and this could have led to more rapid decomposition than normal. This problem has been common to many of these studies, as the azolla has been labelled with ^{15}N by growing it with added fertilizer. To avoid the problem, it has been suggested that in future experiments the labelled azolla should be grown in dilute solutions of highly enriched N (Eskew and Kovacs, 1991). Although these values for recovery of N from azolla green manure may be slight overestimates, the increases in growth and yield of rice that are commonly observed support the conclusion that N release from incorporated azolla is rapid. Some of the N added in azolla also remains in the soil. In a 14-year experiment in the Philippines, the total soil N content increased by almost 20% after 27 azolla/rice cycles (Ladha *et al.*, 2000).

Losses of azolla N were found to be small (0–11%) in comparison with the loss from an equivalent amount of urea fertilizer (30%) which was probably due to direct volatilization of ammonia to the atmosphere (Watanabe *et al.*, 1989a). An interesting observation is that the presence of a mat of azolla on the surface can reduce the pH of floodwater by about 2 pH units. As a result, substantial reductions in losses of N by ammonia volatilization have been observed, which have increased recovery of fertilizer N applied as urea by up to 60%. When the azolla was then incorporated into the soil, overall fertilizer losses were reduced by 35–55% (Kumarasinghe and Eskew, 1993).

Yield responses to azolla incorporation

As discussed above, a crop of azolla produces 20–40 kg N ha^{-1} which can be turned into the soil, and in experiments in Asia this gave an increase in rice grain yield of approximately 500 kg ha^{-1} (Table 7.2). Azolla use gave a yield increase of

Table 7.2. Effects of azolla on the yield of rice. Values represent means from 12 sites in Asia. (From Watanabe, 1982.)

Treatment	Grain yield (kg ha^{-1})
Control	2.6
30 kg N fertilizer ha^{-1}	3.2
60 kg N fertilizer ha^{-1}	3.7
Azolla grown before transplanting and incorporated	3.2
Azolla grown after transplanting and incorporated	3.1
Azolla grown after transplanting	3.1
Azolla grown before transplanting and incorporated + 30 kg N ha^{-1}	3.7
Azolla grown after transplanting and incorporated + 30 kg N ha^{-1}	3.5
Azolla grown before and after transplanting and incorporated	3.6

600–750 kg ha^{-1} in a series of 1500 experiments in China (Lumpkin and Plucknett, 1982).

It is estimated that azolla is currently used on only 2% of the world's rice, although this is still some 3 million ha. Economic analysis in the Philippines, taking into account all of the associated costs of labour, phosphorus fertilizer and insecticides that may be required to use azolla effectively, indicates that fertilizer-N in the form of urea is more cost effective unless good irrigation can be ensured (Rosegrant and Roumasset, 1988). Insect resistance in azolla or effective integrated pest management techniques would substantially reduce the costs of azolla production and may offer a way of making azolla more economically feasible. Sexual hybridization between species of azolla can be achieved in the laboratory (Van Cat *et al.*, 1989) and may present opportunities for improvement of insect resistance and other desirable characters.

Other uses of azolla

If the growth of azolla is vigorous the mat produced can effectively suppress weeds, although some competition may also occur with the rice crop if seed is sown directly (Watanabe, 1982). Unfortunately, the dense mats that azolla can form in reservoirs and irrigation channels mean it can also be a problematic weed, and weevils have been identified and released in South Africa for biological control of azolla (Hill and Cilliers, 1999). The productivity and large protein content of azolla have led farmers in Asia to use it as a food for a wide variety of animals. Azolla has been used as feed for molluscs, fish, ducks, geese, chickens, pigs and rabbits but does not appear to be much appreciated by ruminants (Van Hove, 1989). The use of azolla in recipes for human consumption appears to be limited to a small body of researchers.

Conclusions

N_2-fixation by cyanobacteria is a useful input, but one that is unlikely to be manipulated easily and it is insufficient to sustain rice yields on its own. This is despite the early belief that led to moves to provide inoculum to farmers in India, supported by the FAO (Venkataraman, 1981). Deep placement of N fertilizer is the key to taking full advantage of N_2-fixation by cyanobacteria (Roger, 1995b). This allows cyanobacterial blooms to develop, and prevents proliferation of green algae that cause the floodwater to become alkaline, creating ideal conditions for loss of N by volatilization of ammonia.

Use of azolla can certainly provide a substantial amount of N for rice but requires abundant supplies of water and phosphorus. Other problems with azolla (in common with legume green manures) are that it can occupy a space in the cropping cycle that could be used for rice production in environments where several crops of rice are possible each year. Moreover, in areas where there is a single rainy season for rice cultivation, there is often insufficient time to grow azolla before the rice must be

transplanted. An advantage of using legume green manures over azolla in the period before rice transplanting is that they do not require standing water for growth and thus do not occupy the fields when rice could be grown. Legume green manures can also supply larger quantities of N to succeeding rice crops.

Despite the continued enthusiasm of some researchers (e.g. Wagner, 1997; Lejeune *et al*., 1999), the use of azolla has declined drastically since the 1950s (Roger and Watanabe, 1986). The reasons are complex (and are also discussed in Chapter 9), but ready availability of N-fertilizers in many parts of Asia has undoubtedly contributed greatly to the reduction in use of azolla. Widespread use of azolla will be limited to regions where fertilizers are unavailable and labour is abundant.

Chapter 8

Grain Legumes for Food, Fodder and Soil Fertility

The most direct way that N_2-fixation can contribute to agricultural production is by occurring in the crop itself. It is thus no surprise that improvement of N_2-fixation in grain legumes has been a major priority for research. Strictly speaking, grain legumes are those from which the seed is used directly for human consumption. Legume grain provides a protein-rich source of food and is an essential part of the diet in many parts of the tropics, particularly where meat is scarce. Grain legumes play an important nutritional role in supplying those essential amino acids (particularly lysine) that are not present in sufficient quantities in staple cereal crops, so enabling a balanced diet to be maintained even in the absence of a high intake of animal products. This is particularly important in regions (for example, parts of southern India) where religious practices ensure that a majority of the population are strictly vegetarian: grain legumes can provide up to 70% of their dietary protein intake (Smartt, 1990).

In many cases the seeds of grain legumes have additional uses, as do other parts of the plant. For example, groundnut (or peanut) and soybean are important oilseed crops; green pods of several grain legumes such as cowpea or *Phaseolus* bean are used as a fresh vegetable, as are leaves of many species (Fig. 8.1); in some cases the plant produces an edible tuber (e.g. *Pachyrhizus*); and the crop residues can be important as fodder for livestock. Many grain legumes are important as cash crops for smallholder farmers. Soybean, and to a lesser extent groundnut, are important for foreign exchange earnings, as they are exported to many of the more developed countries, where they are used for animal feeds, oil and in human food products (Smith and Huyser, 1987).

The improvement of grain legume productivity is a major objective of many national research programmes in the tropics. Several of the Consultative Group for International Agricultural Research (CGIAR) centres have undertaken

Fig. 8.1. Leaves of *Phaseolus vulgaris* collected for use as a green vegetable. In Rwanda, climbing beans are grown using stakes from tree legumes for support.

responsibility for conducting and coordinating research, including the improvement of N_2-fixation, on specific grain legume crops. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has responsibility for research on groundnut and pigeonpea and is the base of the Asian Grain Legume Improvement Programme; research on chickpea is the joint responsibility of the International Centre for Agricultural Research in the Dry Areas (ICARDA) and ICRISAT; the Centro Internacional de Agricultura Tropical (CIAT) has responsibility for *Phaseolus vulgaris* and the International Institute for Tropical Agriculture (IITA) has conducted research on cowpea and soybean.

Grain legumes are frequently grown as sole crops, but are also commonly intercropped with cereals and other staple foods, such as cassava. A further benefit of grain legumes is their contribution to improving the fertility of the soil, which largely depends on how their residues are utilized.

Tropical Grain Legumes

Most grain legumes belong to two tribes of the *Leguminosae*: the *Phaseoleae* and the *Vicieae* (Polhill and van der Maesen, 1985) (Table 8.1). Those belonging to the *Phaseoleae* are the most important crops in the tropics, together with *Arachis* (belonging to the *Aeschynomeneae*) and *Cicer* (belonging to the *Cicereae*). As might be expected for crops of such importance, grain legumes have distinct names in many languages from regions where they are grown. This can lead to confusion; for instance, the name 'field bean' is used for both *Vicia faba* (also called the 'faba bean') and for *P. vulgaris* (also known as the 'common bean'). The scientific literature is at

Table 8.1. Some characteristics of nodulation in grain legumes that are produced in the tropics. (After Sprent and Minchin, 1985.)

Species	Tribe	Common names	Centre of diversity	Nodule growth
Arachis hypogaea	*Aeschynomeneae*	Groundnut	Brazil	Determinate
Cicer arietinum	*Cicereae*	Chickpea, red gram	Turkey	Indeterminate, fan shaped
Lupinus mutabilis	*Genisteae*	Tarwi	The Andes	Indeterminate, collar shaped
Cyamopsis tetragonoloba	*Indigofereae*	Guar, cluster bean	India	Indeterminate
Cajanus cajan	*Phaseoleae*	Pigeonpea	India	Determinate when young
Glycine max	*Phaseoleae*	Soybean	China	Determinate
Phaseolus acutifolius	*Phaseoleae*	Tepary bean	C. America	Determinate
P. coccineus	*Phaseoleae*	Runner bean	C. America	Determinate
P. lunatus	*Phaseoleae*	Lima bean	C. America	Determinate
P. vulgaris	*Phaseoleae*	Common bean	C. and S. America	Determinate
Psophocarpus tetragonolobus	*Phaseoleae*	Winged bean	Africa?	Determinate when young
Vigna aconitifolia	*Phaseoleae*	Moth bean	India	Determinate
V. mungo	*Phaseoleae*	Black gram, urud dahl	India	Determinate
V. radiata	*Phaseoleae*	Green gram, mung bean	India	Determinate
V. subterranea	*Phaseoleae*	Bambara groundnut	W. Africa	Determinate
V. umbellata	*Phaseoleae*	Rice bean	SE Asia	Determinate
V. unguiculata	*Phaseoleae*	Cowpea	West Africa	Determinate
Lens culinaris	*Vicieae*	Lentil	Turkey	Indeterminate
Pisum sativum	*Vicieae*	Garden pea	Afghanistan	Indeterminate
Vicia faba	*Vicieae*	Faba bean	W. Asia?	Indeterminate

times also confusing, as the taxonomy of many important species has been revised and generic names have been changed. The taxonomic revisions have been informative in drawing together some Asiatic species within the genus *Vigna* (e.g. the green gram, *V. radiata*) that were previously placed within the genus *Phaseolus*, which is now clearly a New World family. Here commonly used Latin synonyms will be given, together with a few vernacular names. Much more information on grain legumes, and on N_2-fixation in particular species, can be found in Summerfield and Roberts (1985), Summerfield (1988) and Smartt (1990).

Arachis

The groundnut or peanut (*Arachis hypogaea*) is a tetraploid species within a predominantly diploid South American genus which has yet to be fully described (Smartt, 1990, 1994). Three main types of groundnut are recognized: the erect, bunch Spanish (with compound inflorescences; ssp. *fastigiata* var. *vulgaris*) and Valencia (with simple inflorescences; ssp. *fastigiata* var. *fastigiata*) types and the more spreading Virginia type (ssp. *hypogaea*) (Smartt, 1994). The Virginia type can be further subdivided into Virginia Bunch (var. *hypogaea*) and Virginia Runner (var. *hirsuta*). Over 90% of the world groundnut crop is produced in developing countries and roughly two-thirds of this is used for oil, making it the second most important source of vegetable oil after soybean (Freeman *et al.*, 1999). It is also important as a subsistence food crop throughout the tropics and although groundnut is principally a warm-temperature crop, varieties exist that are adapted to altitudes of 1500 m. Groundnut is generally nodulated by *Bradyrhizobium* strains (Urtz and Elkan, 1996) and has an unusual mechanism of infection (Chapter 2).

Cajanus

The pigeonpea (*Cajanus cajan*) was probably domesticated in India but spread before 2000 BC to Africa, where there is a second centre of diversity (van der Maesen, 1985). It was then carried with the slave trade from West Africa to the New World, where it is an important crop in the West Indies. The wild relatives of pigeonpea (which were placed in the genus *Atylosia* prior to the revision by van der Maesen, 1985) are spread across the Indian subcontinent, Southeast Asia and Australia and there is one wild species (*C. kerstingii*) in West Africa. Pigeonpea is a woody perennial that is grown mainly as an annual, although some genotypes can take up to 260 days to produce seed. Short-duration genotypes have been bred that can mature in 95 days in southern India (van der Maesen, 1985; Nene *et al.*, 1990). Almost 85% of the estimated 2 million t of pigeonpea produced each year is grown in India (Smartt, 1990) and pigeonpea is also grown in eastern and southern Africa – much of which is exported to India.

Pigeonpea is nodulated both by *Bradyrhizobium* strains and by fast-growing rhizobia (Bromfield and Kumar Rao, 1983). Nodules occur at depth and are difficult to recover in the field, as they are easily detached from the root system.

Cicer

Chickpea or Bengal gram (*Cicer arietinum*) is produced mainly in India, where it is the most important pulse crop in terms of production (Smithson *et al.*, 1985). The monogeneric tribe *Cicereae* contains some 40 herb and shrub species. Chickpea is thought to have been domesticated in Turkey and has its centre of diversity in western Asia (van der Maesen, 1984). There are two main seed types: the 'desi' types, with small, angular seeds, which account for more than 85% of the world's production; and the 'kabuli' type, which has larger, rounded seeds. Desi types are grown principally in India, Ethiopia, Mexico and Iran and the kabuli types in Afghanistan, North Africa, southern Europe and the Americas. Growth habits range from erect to prostrate forms. Chickpea grows best in cool conditions and is grown on residual moisture after the monsoon season in India. Yields can be high (up to 5 t ha^{-1}) in northern India, where the growing season lasts from October to June, but the yields decrease progressively further south, where the earlier increase in temperatures and moisture stress act to curtail the growing season. In Africa, chickpea is grown at altitudes between 1400 m and 2300 m.

Chickpea has a marked specificity for the strains with which it will nodulate effectively, showing a loose cross-inoculation relationship with some *Sesbania* species (Gaur and Sens, 1979). Rhizobia isolated from chickpea nodules were found to have a wide range of growth rates and were described as fast- and slow-growing rhizobia (Okon *et al.*, 1972; Bromfield and Kumar Rao, 1983; Cadahía *et al.*, 1986). Taxonomic studies later led to the description of two chickpea-nodulating rhizobial species: *Mesorhizobium ciceri* (Nour *et al.*, 1994; Jarvis *et al.*, 1997) and *M. mediterraneum* (Nour *et al.*, 1995). Chickpea nodules have indeterminate growth and can be very large (Fig. 8.2).

Glycine

The soybean (*Glycine max*) is now produced in larger quantities than any other legume crop in the world and is certainly the most important source of vegetable oil. Together the USA, China, Brazil and Argentina account for 90–95% of the world soybean production (Smith and Huyser, 1987). Soybean was domesticated in China, where its wild counterpart, *G. soja* (Sieb. & Zucc.), is found. Some taxonomists consider this to be the same species (Smartt, 1990). Other *Glycine* species are found in Southeast Asia and Australia. A wide range of photoperiod responses occur in cultivated soybeans, with some varieties being insensitive to daylength. Classifications of varieties developed in the USA are based on their growth habit and duration but are not applicable in the tropics (Shanmugasundaram *et al.*, 1980). As with many other grain legumes, in addition to the influence of photoperiod, the rate of reproductive development in soybean is inversely proportional to temperature (Hadley *et al.*, 1984). Many tropical soybean types are determinate (Hume *et al.*, 1985), but important exceptions are the 'hay' types, which tend to be more promiscuous in their nodulation ability (Mpepereki *et al.*, 2000). Soybeans are processed in a wide variety

Fig. 8.2. (a) Chickpea growing on a Vertisol in southern India and (b) the large indeterminate nodules that it can form under favourable conditions.

of ways to produce soya milk, bean curd, flour and fermented products such as the fermented soybean cake or 'tempeh' of Indonesia.

Soybean is nodulated effectively by strains of all the recognized species of *Bradyrhizobium*, including some strains isolated from other hosts, such as cowpea (Chapter 2). The fast-growing *Sinorhizobium fredii* and *S. xinjiangensis* also nodulate soybean, but *Bradyrhizobium* strains tend to occupy the majority of the nodules if the fast- and slow-growing species are inoculated together (de Chueire and Hungria, 1997). The strains commonly used as inoculants for soybean in Brazil belong to *B. elkanii*.

Phaseolus

Phaseolus is a New World genus of which the most important crop species is *P. vulgaris*, which is a major crop in Africa and Latin America. Other cultivated *Phaseolus* species are the tepary bean (*P. acutifolius*), the runner bean (*P. coccineus*) and the Lima bean or butter bean (*P. lunatus*). Several subspecies of *P. coccineus* are recognized and one of these may deserve recognition as a separate species, *P. polyanthus* (see Smartt, 1990). The archaeological study of *Phaseolus* has linked its history to that of maize and the two crops are commonly intercropped even today. Remains of *P. vulgaris* (and other *Phaseolus* species), consisting mainly of seed and pod material and dating from as long ago as 5000 BC, have been found in sites in Mexico and Peru.

It seems likely that *P. vulgaris* was domesticated separately in Central America and in the Andes, where its closest wild relative, *P. vulgaris* var. *aborigineus* is found (Kaplan, 1981; Kaplan and Kaplan, 1988).

P. vulgaris is a crop with enormously variable morphology and growth patterns ranging from determinate bush varieties that mature in 90 days (Type I) to indeterminate climbing varieties that grow for 8 months at higher altitudes (Type IV) (Laing *et al.*, 1984; van Schoonhoven and Voysest, 1991). Types II and III are bush beans, with different degrees of climbing ability and intermediate duration of the growth season. The growth habit and flowering of many varieties are strongly influenced by photoperiod and by temperature (Laing *et al.*, 1984). As the usefulness in particular cropping systems – for instance, intercropping with maize – can depend on the growth habit, transfer of promising varieties between regions requires careful evaluation. Seed types are also very diverse; seed weight can range from 50 mg per seed to 300 mg per seed, with many variations in colour and mottling (Voysest and Dessert, 1991). Local preferences for particular seed types, which are often influenced by the ease of cooking as well as the flavour, complicate the job of transferring germplasm with adapted traits.

P. acutifolius is more tolerant of hot, dry summers than *P. vulgaris*, and *P. coccineus*, which is grown in the mountains of Central America above 2000 m, is adapted to cooler climates. *P. lunatus* is grown intensively only in the USA, Peru and Madagascar but is grown as a subsistence crop in countries from Mexico to India and the Philippines and in Africa (Lyman *et al.*, 1985).

Whilst *P. lunatus* is nodulated by *Bradyrhizobium* strains, other *Phaseolus* species are nodulated by fast-growing rhizobia (Allen and Allen, 1981). The genetic diversity of rhizobial strains that can nodulate *P. vulgaris* has been the focus of considerable research and has led to the description of new *Rhizobium* species (Chapter 2). Rhizobial species that have been isolated from nodules of *P. vulgaris* include *R. leguminosarum* bv. *phaseoli*, *R. etli*, *R. tropici*, *R. giardinii*, *R. gallicum* and *Sinorhizobium* spp., as well as several other types that are sufficiently distinct that they probably represent further species. Populations isolated from nodules of *P. vulgaris*, its wild ancestor *P. aborigineus* and *P. coccineus* in Latin America appear to be dominated by *R. etli* (Souza *et al.*, 1994; Aguilar *et al.*, 1998; Straliotto *et al.*, 1999) whereas populations from Africa (Anyango *et al.*, 1995; Mhambdi *et al.*, 1999) and Europe (Geniaux *et al.*, 1993; Amarger *et al.*, 1994; Sessitsch *et al.*, 1997) are often dominated by the other species of *Rhizobium*.

Vigna

Several species of *Vigna* are widely cultivated as grain legume crops (Table 8.1). Cowpea (*V. unguiculata* syn. *V. sinensis*) is grown extensively as a subsistence crop in many countries in Africa (West Africa is the main region for production) and throughout the tropics, but is also widely used for forage or as a green manure (Tarawali *et al.*, 1997). Three different groups of cultivated *V. unguiculata* are recognized and these have been described both as subspecies and as 'cultigroups' (Ng

and Maréchal, 1985). The different cultigroups are the typical African cowpea (cultigroup Unguiculata or subspecies *unguiculata*) and two cultigroups grown in Asia: catjang (cultigroup Biflora or subspecies *cylindrica*) and the yard-long bean (cultigroup Sesquipedalis or subspecies *sesquipedalis*), which can certainly live up to its name. *V. unguiculata* was domesticated in sub-Saharan Africa and, although the precise region cannot be stated with confidence, West Africa was probably the primary centre of domestication (Ng and Maréchal, 1985). Three groups of cowpea genotypes have been recognized on the basis of photoperiod sensitivity and growth habit (Steele and Mehra, 1980). Type I genotypes are insensitive to photoperiod; they are either determinate and can mature in 60 days (Type IA), or indeterminate (Type IB) and continue to grow after flowering. The indeterminate Type II genotypes typical of the West African landraces require short days for induction of flowering and are adapted to extremely specific latitudes, such that flowering tends to coincide with the end of the rains. Part of this adaptation to specific environments is due to the influence of temperature on the rate of reproductive development (Hadley *et al*., 1983).

Bambara groundnut (*Vigna subterranea* syn. *Voandezia subterranea*) originates from West Africa and takes its English common name from a place near Timbuktu, in Mali. It is now grown as a subsistence crop in Southeast Asia and Latin America as well as in most parts of Africa (Linnemann, 1987). The deep tap root allows the Bambara groundnut to withstand severe drought but optimal growth requires evenly distributed rainfall of 900–1200 mm $year^{-1}$. Although the crop is largely unimproved, grain yields of 3–4 t ha^{-1} have been obtained on experimental plots in Zimbabwe.

Several species of *Vigna* are cultivated in Asia, of which the green gram (*V. radiata*) is the most widely cultivated. The name mungbean is used for both the green gram and the black gram or urud dal (*V. mungo*) and both are thought to be of Indian or Indo-Burmese origin (Lawn and Ahn, 1985). The adzuki bean (*V. angularis*) is cultivated in Japan and Korea and the rice bean (*V. umbellata*) is particularly important in Thailand and Indo-China (Smartt, 1990). The moth bean (*V. aconitifolia*) is reported to be the most drought-tolerant pulse crop grown in India, which is the only country in which it is produced (Anon., 1979).

Isolates from nodules of *Vigna* species are most commonly *Bradyrhizobium* strains, although a range of fast-growing rhizobia are also able to nodulate these relatively promiscuous hosts. As with most legumes, there is substantial variation in ability to form nodules and in effectiveness of N_2-fixation with rhizobial strains, both between species and among genotypes of the same plant species (e.g. Somasegaran *et al*., 1990).

Minor grain legumes

A number of other grain legumes are locally important in parts of the tropics, where they are often highly valued, suggesting that there may be potential for their wider distribution.

Drought-resistant legumes

These grain legumes manage to produce a yield under dry conditions where most other crops would fail. The grasspea (*Lathyrus sativus*) is grown as a pulse crop in India. If it forms too large a proportion of the diet it can cause lathyrism, a progressive paralysis, and this tends to occur when other crops fail (Rutter and Percy, 1984). The horse gram (*Macrotyloma uniflorum*) is another Indian pulse crop and Kersting's groundnut (*Macrotyloma geocarpum*) is grown in parts of West Africa but is said to produce consistently poor yields even when conditions are favourable (Anon., 1979). The lablab or hyacinth bean (*Lablab purpureus*) is drought-tolerant once established and is cultivated in central Africa and Asia. Guar or cluster bean (*Cyamopsis tetragonoloba*) is another legume indigenous to India and is cultivated in India and Southeast Asia. The seed provides a gum with a variety of uses, including in paper making and food products (Allen and Allen, 1981).

Minor legumes of the wet tropics

Other species are adapted to the wet tropics. The winged bean (*Psophocarpus tetragonolobus*) belongs to a genus with eight wild African species but it is only cultivated on any scale in a few parts of Asia (Newell and Hymowitz, 1979). It is remarkable in that virtually every part of the plant can be eaten, including the flowers, young shoots and root tubers (Eagleton *et al.*, 1985). Winged bean is nodulated effectively by *Bradyrhizobium* strains (Elmes, 1976). There are three cultivated species of edible yam beans: *Pachyrhizus ahipa*, *P. erosus* and *P. tuberosus*, which originate from Peru, Mexico and the Amazon basin, respectively (Sørensen, 1990). These are not strictly 'grain' legumes: although a large edible tuber is produced and the young pods can be eaten, the shoots and seeds can be toxic to humans. Yam beans are grown widely in Central and South America and the West Indies and are much valued in Southeast Asia, where their potential for soil improvement has been recognized (van der Heide and Hairiah, 1989). The jack bean (*Canavalia ensiformis*) and the sword bean (*C. gladiata*) are grown mainly as green manure crops but the immature pods and seeds can be eaten (Smartt, 1990) (Chapter 9). Two species of *Mucuna* are grown as grain legumes in parts of tropical Africa and Southeast Asia: the velvet bean (*M. pruriens* var. *utilis*) and the horse eye bean (*M. sloanei* syn. *M. urens*) (Rachie and Roberts, 1974) (Chapter 9).

Minor legumes of the cool tropics

A further group of grain legumes of minor importance in the tropics comprises crops that are grown at high altitudes. The pea (*Pisum sativum*), lentil (*Lens culinaris*) and the faba bean (*V. faba*) are far more important crops in temperate or Mediterranean regions and will not be discussed in detail here, though they are grown in mountain areas in Africa and South America. Tarwi (*Lupinus mutabilis*) is a crop of the Andean highlands of Ecuador, Chile and Peru. The seed contains bitter alkaloids, which are toxic, and the seed is usually steeped in water for several days to remove these before it can be consumed (Pate *et al.*, 1985), although Peruvian Indians use it in small quantities to remove intestinal parasites.

Amounts of N_2 Fixed

Estimates of N_2-fixation by different grain legume crops grown in the tropics are given in Table 8.2. It is essential to note that the majority of these estimates come from experiments where pests and diseases were controlled and adequate phosphorus was supplied to the crops, so that the crop yields would generally be large compared with those commonly found on farmers' fields. Even so, the amounts of N_2 fixed and the proportion of plant N derived from N_2-fixation vary enormously between grain legume crops, between different genotypes of the same crop and between different environments in which the crops are grown. So what determines the amount of N_2-fixation?

Characters required for an effective symbiosis

The most obvious requirement for a legume to form an effective N_2-fixing symbiosis is the ability to form nodules with the necessary organization and ancillary machinery for N_2-fixation. There are a number of genes for which a specific function in the formation of N_2-fixing nodules has been identified (Nap and Bisseling, 1990; Oke and Long, 1999a; Verma, 2000) and limited understanding of the genetics of legume nodulation has largely been gained by the study of plant genotypes that have lost the ability to form effective nodules, or to form nodules at all (see Table 4.7). But the simple ability to form a nodule is not enough, and many other plant characters will markedly influence the amount of N_2 fixed in the symbiosis.

Here a brief discussion on the difference between evaluating N_2-fixation on the basis of the total amount of N_2 fixed or as the proportion of the plant's N that is fixed (the % N from N_2-fixation) is warranted. The total amount of N fixed by an organism is a function of (and a contributing factor to) the total dry-matter yield of the plant. Thus virtually all plant genes involved in determining the amount of growth (which is usually measured in terms of dry-matter yield) will have an influence on the amount of N_2 fixed by that plant – both by providing the necessary photosynthate to fuel N_2-fixation and by providing a sink to use the N_2 fixed.

As all of the plant characteristics that contribute to the amount of N_2-fixation are interdependent, and are also influenced by the environment and by the rhizobial strain(s) with which the symbiosis is formed, any classification of such characters must be an oversimplification. An attempt at such a classification is presented in Table 8.3. The length of time for which an annual plant can actively fix N_2 will depend, in part, on the time taken to complete its life cycle and, even in perennial plants, the period of N_2-fixation may be strongly seasonal. This in turn can be partly controlled by the environment – for instance, if the rains end early in a seasonal climate, the maturity of annual species can be advanced. Differences in the amounts of N_2-fixation vary enormously with the length of the growing season. Genotypes that mature in 90 days have a short time to nodulate and to fix N_2 before flowering and pod-fill occur, whereas long-duration, indeterminate genotypes have a substantially longer period of N_2-fixation. The ability to form nodules and to begin fixing N_2

Table 8.2. Estimates of N_2-fixation by grain legumes grown as sole crops in the tropics. All are examples where small amounts of N-fertilizers, and generally adequate amounts of phosphorus, were applied.

Grain legume	N_2 fixed		Time period (days)	Country	Method[a]	Ref.[b]
	kg ha^{-1}	%				
Arachis hypogaea	139–206	55–64	120	Australia	NA	1
	85–131	47–53	144	Australia	NA	1
	32–120	22–49	140	Australia	NA	2
	43–72	45–67	90–106	Australia	NA	3
	68–116	54–78	110	Brazil	ID	4
	101	–	–	Ghana	Diff	5
	100–152	86–92	89	India	ID	6
	152–189	61–85	118–137	India	NA/Diff	7
	21–58	16–53	–	Indonesia	ID/NA	8
	101–130	59–64	90–110	Thailand	ID	9
	150–200[c]	72–77	106–119	Thailand	ID	10
	102[c]	68	88	Thailand	ID	11
	46[c]	62	87–97	Thailand	ID	12
Cajanus cajan	68–88	88	–	India	ID	13
	150–166	63–86	–	India	ID	14
	0–76	0–36	95–210	India	NA	15
	30–131	59–87	–	India	NA	16
	13–163	42–85	120	Malawi	NA	17
	–	52–88[c]	–	Nepal	NA	18
	1–39	64–100	–	Zimbabwe	NA	19
Cicer arietinum	60–84	60–80	160	Australia	NA	1
	67–85	63–81	170	Australia	NA	20
	0–124	0–79	–	Australia	NA	21
	0–99	0–81	–	Australia	NA	22
	35–80[c]	66–96	–	Nepal	NA	23
	<20–91	21–95	–	Pakistan	NA	24
Glycine max	85–154	70–80	110	Brazil	ID	4
	14–15	36–39	40	Congo	ID	25
	15–170	12–100	–	Nepal	NA	18
	114–188	84–87	66	Nigeria	ID/Diff	26
	42–83	46–87	36–75	Nigeria	ID	27
	149–176	69–74	70–84	Philippines	ID	28
	26–57	78–87	64–73	Thailand	NA	1
	108–152[c]	66–68	97–104	Thailand	ID	10
	68–174	38–74	72	Thailand	Ureide	29
	147	84	77	Thailand	Ureide	30
Lathyrus sativus	–	85–91[c]	–	Nepal	NA	18
Lens culinaris	19–83[c]	62–85	–	Nepal	NA	18
	16–83[c]	55–91	–	Pakistan	NA	31
	4–38	9–46	–	Pakistan	ID	32

Table 8.2. *continued*

Grain legume	N_2 fixed		Time period	Country	Method[a]	Ref.[b]
	kg ha^{-1}	%	(days)			
Phaseolus vulgaris	25–65	37–68	60–90	Brazil	ID	33
	3–32	15–72	61	Brazil	ID	34
	4–45	12–53	60–92	Brazil	ID	35
	25–115	27–60	–	Chile	ID	35
	18–36	32–47	56	Colombia	ID	36
	9–50	24–50	63–70	Colombia	ID	37
	12–125	22–73	–	Guatemala	ID	35
	74–91	43–52	74	Kenya	ID	38
	44–50	60–73	91	Mexico	ID	39
	0–108	0–58	–	Mexico	ID	35
	34–85	30–57	–	Mexico	ID	40
	7–81	13–56	86–116	Peru	ID	41
	8–26[c]	40–51	75	Tanzania	ID	42
Vigna radiata	0–55	0–100	–	Pakistan	NA	31
	64–66	89–90	57–64	Thailand	NA	1
	58–107	–	–	Thailand	NB	43
	10[c]	25	87–97	Thailand	ID	12
V. mungo	119–140	95–98	66	Thailand	NA	1
	0–55	0–100	–	Pakistan	NA	31
V. unguiculata	9–51	32–74	110	Brazil	ID	4
	201	–	–	Ghana	Diff	5
	47–105	61–76	66	Nigeria	ID/Diff	26
	66–120	54–70	57	Nigeria	ID	44
	63[c]	65	87–89	Thailand	ID	10

[a]ID = ^{15}N isotope dilution; NA = ^{15}N natural abundance; NB = N balance; Diff = N difference; Ureide = ureide method.

[b]1: Peoples *et al.*, 1991b; 2: Peoples *et al.*, 1992; 3: Bell *et al.*, 1994; 4: Boddey *et al.*, 1990; 5: Dakora *et al.*, 1987; 6: Giller *et al.*, 1987; 7: Nambiar *et al.*, 1986; Yoneyama *et al.*, 1990a; 8: Cadisch *et al.*, 2000; 9: McDonagh *et al.*, 1993; 10: Toomsan *et al.*, 1995; 11: McDonagh *et al.*, 1995b; 12: Toomsan *et al.*, 2000; 13: Kumar Rao *et al.*, 1987; 14: Tobita *et al.*, 1994; 15: Kumar Rao *et al.*, 1996b; 16: Kumar Rao *et al.*, 1996a; 17: Sakala *et al.*, 2001; 18: Maskey *et al.*, 1997; 19: Mapfumo *et al.*, 1999; 20; Herridge *et al.*, 1995; 21: Herridge *et al.*, 1998; 22: Schwenke *et al.*, 1998; 23: Ali *et al.*, 1997; 24: Aslam *et al.*, 1997; 25: Mandimba, 1996; 26: Eaglesham *et al.*, 1982; 27: Sanginga *et al.*, 1997; 28: George *et al.*, 1995; 29: Guafa *et al.*, 1993; 30: Yinbo *et al.*, 1997; 31: Shah *et al.*, 1997; 32: Hafeez *et al.*, 2000; 33: Ruschel *et al.*, 1982; 34: Duque *et al.*, 1985; 35: Hardarson *et al.*, 1993; 36: Kipe-Nolt and Giller, 1993; 37: Kipe-Nolt *et al.*, 1993; 38: Ssali and Keya, 1986; 39: Peña-Cabriales *et al.*, 1993; 40: Castellanos *et al.*, 1996; 41: Manrique *et al.*, 1993; 42: Giller *et al.*, 1998; 43: Firth *et al.*, 1973; 44: Awonaike *et al.*, 1990.

[c]Measurements made in experiments on farmers' fields, or in farmers' crops.

Table 8.3. Characteristics determined by the plant genotype which are important in determining the amount of N_2 fixed by the symbiosis.

Character	Contributing factors
Duration	Time to maturity; time to flowering; early nodulation; delay of senescence
Vigour	Plant size; photosynthetic area; plant architecture; N sinks
Nodulation	Number, size and longevity of nodules; host/rhizobial strain specificity for nodulation; nodulation in the presence of combined N
Physiology of N_2-fixation	Efficiency of carbon utilization for N_2-fixation; specific nitrogenase activity of nodules; oxygen transport mechanisms
Fertilizer use	Ability to exploit or ignore soil and fertilizer-N; rooting characteristics

quickly is particularly important for short-duration genotypes. In some grain legumes, such as soybean and *P. vulgaris*, the timing of nodule senescence often coincides with seed development, and the period of active N_2-fixation will be longer if senescence of the nodules is delayed so that N_2-fixation continues during pod-fill. By contrast, no decline in rates of N_2-fixation during pod-fill occurs in groundnut (Bell *et al.*, 1994). There is strong evidence that rates of N_2-fixation in legume nodules are limited by the supply of oxygen (Minchin *et al.*, 1996) and the strength of the sink for carbon in nodules will depend partly on the efficiency with which carbon is utilized for N_2-fixation. Factors contributing to the overall efficiency of carbon use for N_2-fixation include the provision of reductant and electrons to nitrogenase, the electron allocation to hydrogen production by nitrogenase and the costs associated with assimilation and transport of fixed N to the shoot (Neves and Hungria, 1987) (Chapter 3).

The proportion of a plant's N that is derived from N_2-fixation (sometimes referred to as the %N derived from atmosphere, or %Ndfa) is strongly influenced by the amount of combined N that is available for uptake by the plant, and the ability of the plant to capture and utilize that N. Where the amount of N available in the soil is very limited, grain yield will be directly proportional to the amount of N_2 fixed. The proportion of N derived from N_2-fixation will be smaller when large amounts of soil N are available. The sensitivity of legumes to high concentrations of N in soil can vary substantially. In soybean and *P. vulgaris*, nodulation and N_2-fixation are readily suppressed by N-fertilizer applications and, in fact, *P. vulgaris* is even more sensitive to N than soybean (George *et al.*, 1988; Abaidoo and van Kessel, 1989). Other legumes, notably *V. faba*, groundnut (Nambiar *et al.*, 1986) and perhaps *M. geocarpum* (Dakora *et al.*, 1992), are less sensitive to high concentrations of mineral N.

It must also be remembered that many of these plant characteristics can vary depending on the particular strain (or strains) of rhizobia with which the N_2-fixing symbiosis has been established.

Comparative ability of grain legumes to fix N_2

It is difficult to make useful generalizations as to the N_2-fixing ability of the different grain legumes. Across all of the grain legumes for which data is presented in Table 8.2, optimal rates of N_2-fixation are 1–2 kg N ha^{-1} day^{-1}, with all of the legumes recorded to fix more than 120 kg N ha^{-1} within a cropping season in at least one case. As discussed in Chapter 5, these fast rates should be considered as the *potential* of the grain legumes for N_2-fixation within a given environment. The wide variations in N_2-fixation within a given crop are found for a variety of reasons.

Cases where no N_2-fixation has been observed, or very small %N from N_2-fixation, are largely due to drought (*C. arietinum*), some other environmental constraint such as high temperatures (*P. vulgaris*), or perhaps nutrient limitations where the measurements were made in farmers' crops. The largest amounts of N_2-fixation have been recorded where there have been long, favourable growing seasons, generally on research stations.

Early-maturing pigeonpea varieties appear to nodulate and fix N_2 poorly compared with long-duration varieties (Kumar Rao *et al.*, 1995, 1996b). In Australia, pigeonpea exhibits highly variable nodulation even when inoculated and is often considered to fix N_2 poorly (Brockwell *et al.*, 1991), although estimates from India and Africa show that it can often fix large amounts of N (Table 8.2). The smaller amounts of fixation with *V. unguiculata* and *P. vulgaris* tend to be for shorter-season, determite types and the larger amounts are found with spreading or climbing varieties.

P. vulgaris has often been judged to be poor in N_2-fixation (e.g. Piha and Munns, 1987a); yet under optimal conditions estimates of N_2-fixation of up to 72% of N derived from fixation have been obtained (Table 8.2) and in longer growing seasons amounts up to 125 kg N ha^{-1} fixed have been recorded (Rennie and Kemp, 1983). These are comparable to estimates for soybean, which is considered to fix N_2 abundantly. Under controlled conditions in growth rooms, *P. vulgaris* nodulates well and fixes N_2 at similar high rates to other grain legume species (Eaglesham, 1989). In relatively cool, long seasons in Austria, three climbing varieties of *P. vulgaris* from the African highlands were among the best in terms of largest amounts of N accumulation and N_2-fixation (Hardarson *et al.*, 1993). The success of grain legumes in N_2-fixation in the field will be strongly influenced by the prevailing environmental conditions, and sensitivity of grain legumes to environmental stresses may be the overriding factor influencing the amount of N_2 fixed. This may partly explain why *P. vulgaris* has been classed as a poor N_2-fixer. However, whereas soybean may often be able to meet all its requirements for growth and high yields from N_2-fixation (e.g. Hungria *et al.*, 2000), *P. vulgaris* may still respond to N fertilizer even under conditions where it grows and fixes N_2 well (Redden and Herridge, 1999).

Need for Inoculation

The centres of diversity of cultivated plants are important sources of genetic variation for use in breeding programmes for crop improvement and are often the regions in

which the crops were originally domesticated. Lie (1981; Lie *et al.*, 1987) highlighted the importance of these centres as a source of genes involved in symbiotic N_2-fixation in both host plants and rhizobial strains. Wild legumes that are capable of forming effective N_2-fixing symbioses are invariably nodulated in the field in their centres of diversity, even when they fail to nodulate when imported into other regions. This is presumably due to coevolution of adapted host plant genotypes and compatible rhizobial strains (Lie *et al.*, 1987). Inoculation is therefore most likely to be necessary when legumes are introduced into new regions, although the need for inoculation will of course be conditioned by the requirements of the introduced legume for its own specific strains of rhizobia. If the introduced legume crop can nodulate effectively with rhizobia that are present in the soil in sufficient numbers, then inoculation may not be necessary. The contrasting requirements of grain legumes for inoculation with rhizobia can best be examined by comparing the examples of soybean, which often requires inoculation, and cowpea, which has rarely been found to respond to inoculation.

The soybean story

Soybean was domesticated in China and has been grown traditionally in many parts of Southeast Asia. The crop was first grown in North America in the 18th century but production has increased since the early part of the 20th century to the extent that soybean is now the most important grain legume crop in North America (Smith and Huyser, 1987). Soybean is a relatively specific host and does not nodulate when grown in the field for the first time in many parts of Africa, the Americas, Europe and some parts of Asia. Yields of soybean in North America were poor until soil containing compatible rhizobia was introduced from Japan (Allen and Allen, 1981). Thus soybean crops routinely respond well to inoculation and a substantial soybean inoculum production industry has grown up, particularly in Brazil and the USA (Eaglesham, 1989).

However, the story is by no means so simple. In the centre of diversity of soybean and in countries of Southeast Asia, where the crop has been grown for centuries, soybean usually nodulates without inoculation. Strains of *Bradyrhizobium* isolated from nodules of other legume hosts, such as cowpea, grown in the same soils, can also nodulate the local soybean genotypes effectively. In some Chinese soils, fast-growing *Sinorhizobium* species dominate the indigenous populations of soybean rhizobia.

Promiscuous varieties in southern Africa

Soybean cultivation in East and southern Africa appears to be first documented in the early 20th century (see review by Mpepereki *et al.*, 2000) but it is likely that the crop was introduced much earlier through the extensive trade around the Indian ocean. Corby (1965, 1967) first described nodulation of soybean by rhizobia indigenous to African soils. He found that one variety, 'Hernon 147', nodulated effectively at five out of six sites in Zimbabwe and Zambia and did not respond to rhizobial inoculation. At the other site, in a more arid area of Zimbabwe, plants were ineffectively

nodulated and inoculation resulted in strong increases in crop yields. In earlier inoculation trials in the 1950s with a different variety, 'Hernon 237', crop yield responses to inoculation had been observed (Davis, 1986). Corby concluded that inoculation was unnecessary, as long as varieties able to nodulate with indigenous rhizobia were grown. The 'Hernon' varieties are 'hay' types, with luxuriant growth but relatively low yield potential, which were grown largely for fodder.

Nodulation of soybean varieties with indigenous rhizobia was also reported in South Africa (Van Rensburg *et al.*, 1976) and Tanzania (Chowdhury, 1977). Indeed, all varieties evaluated in Tanzania formed some nodules, but only those bred locally from 'Hernon' varieties or an Asian variety, 'Malayan', formed many nodules. More recent investigations have revealed a similar pattern in Zimbabwe, where even highly specific North American genotypes such as 'Bragg' nodulated in at least one of the soils tested, while 'Hernon 147' nodulated in most of the soils (Mpepereki *et al.*, 2000).

Observations by a farmer in 1977 of good nodulation and yields of uninoculated soybean (again 'Hernon 147') in a field in southern Zambia with no history of rhizobial inoculation led to further detailed investigations (described in Javaheri and Joshi, 1986; Javaheri, 1996). Out of 400 varieties evaluated, more than 40 formed nodules and, without exception, the varieties that nodulated shared common parentage with either 'Hernon' varieties or 'Gilbert', an introduction from Australia. The link between promiscuous nodulation and the variety 'Gilbert' was surprising, until it was realized that both parental lines of 'Gilbert' were largely unimproved selections taken to Australia from Africa (Mpepereki *et al.*, 2000). Further screening led to the identification of one exceptionally promiscuous variety, which was named 'Magoye' and released in Zambia in 1981. 'Magoye' was selected from a cross between 'Gilbert' and 'K53', which themselves were both selections with the same parents. These parental lines were 'Avoyelles' (a selection from a farmer's field in Taiwan) and Mamloxi (a cross between selections from China and Japan), initially introduced to Africa from the USA (Mpepereki *et al.*, 2000). So although these varieties had almost circled the earth by the time Javaheri evaluated them in Zambia, they had been through a very limited breeding programme since their initial collection in East Asia. 'Magoye' nodulates readily in virtually all soils in southern Africa where it has been tested, and rarely shows any response to inoculation in Zambia and Zimbabwe, whereas 'Hernon 147' responds to inoculation more often (Mpepereki *et al.*, 2000). The lack of response to inoculation, together with good and consistent yields of 'Magoye', have led to its widespread promotion in southern Africa (Chapter 14).

Breeding for promiscuity in soybean

Asian varieties also nodulated well in the field in Nigeria, whilst American varieties formed very few nodules (Nangju, 1980). As in southern Africa, Asian varieties – in this case 'Orba' and 'Malayan' – had been introduced around the turn of the century and were grown sporadically by smallholder farmers. The American varieties probably show restricted nodulation for two reasons: firstly, the genetic base from which they have been bred is limited (Hartwig, 1973; Gizlice *et al.*, 1994); and secondly, only a limited range of inoculant strains of *B. japonicum* were introduced to North America, leading to increased cultivar-strain specificity.

Inoculation of the varieties of Asian origin seldom increased yields in the field in Nigeria although yields of American varieties were more than doubled in most cases (Pulver *et al.*, 1982). On the basis of these results, a breeding programme was initiated at IITA, Nigeria, in 1978 to reintroduce the ability to nodulate with indigenous strains of rhizobia into the American varieties, as they had far greater yield potential and better resistance to diseases (Kueneman *et al.*, 1984; Pulver *et al.*, 1985). The programme was based on selection of progeny from crosses between Asian and American varieties with good nodulation in local soils (using visual scores for nodule mass) and was partially successful. As a result a number of varieties were released that nodulate without inoculation in soils not previously cropped with soybeans, but few nodules are produced and the nodules are widely distributed over the root system rather than clustered around the tap root – the situation characteristic of small ($< 10^2$ cells g^{-1} soil) populations of compatible rhizobia. Moreover, these 'promiscuously nodulating' soybeans are not in fact nodulated by the same range of rhizobia as cowpea when grown in the same soil (Bromfield and Roughley, 1980; Eaglesham, 1985; Abaidoo *et al.*, 1999). Hence their nodulation can be very poor even in soils where cowpeas of the same age are abundantly nodulated (Eaglesham, 1989). When the promiscuously nodulating soybean varieties were inoculated, yields were sometimes increased, at least in the first season, indicating again that the initial nodulation with indigenous bacteria is poor in comparison with the plant's capacity for nodulation (Ranga Rao *et al.*, 1985; Okereke and Eaglesham, 1993; Sanginga *et al.*, 1996a). It is likely that over a few seasons the number of compatible bacteria may increase in response to the presence of the host, at least where soil conditions are favourable. On the basis of these results, Eaglesham (1989) concluded that it may be safer to rely on effective inoculant strains than to breed for the ability to nodulate with indigenous strains of unknown potential – a case of 'better the devil you know'.

Despite the problems with initial nodulation in some soils, promiscuous soybean varieties from the IITA breeding programme have been widely adopted by farmers in parts of Nigeria (Manyong *et al.*, 1998; Sanginga *et al.*, 1999). The breeding programme at IITA has continued and recent materials have substantially improved ability to nodulate and fix N_2 in farmers' fields without inoculation (Sanginga *et al.*, 1997, 2000), as well as having higher yield potential (Sanginga *et al.*, 2001).

Breeding for symbiotic specificity in soybean

In the USA, by contrast, exactly the opposite approach has been adopted, namely that of breeding for highly specific nodulation. In North America, strains of *B. japonicum* serogroup USDA 123 are prevalent in many soils, and are highly competitive in nodulation with the improved American soybeans (Moawad *et al.*, 1984; Zdor and Pueppke, 1988). Unfortunately, serogroup USDA 123 is less effective in symbiosis with most American soybean cultivars than other *B. japonicum* strains now present in the soils or used as inoculants (Caldwell and Vest, 1970). To enable establishment of more effective soybean symbioses, therefore, attempts have been made to breed cultivars that specifically exclude the less effective serogroup USDA 123 from nodulation (Cregan and Keyser, 1986; Keyser and Li, 1992). Restricted nodulation ability of soybean genotypes with other strains has been

identified but whether this is a wise strategy remains a matter of debate. Kipe-Nolt *et al.* (1992) identified accessions of *P. vulgaris* that had been collected in the wild and were resistant to nodulation with *R. tropici*, which indicates that it may be possible to breed for nodulation specificity in *P. vulgaris*. As Herridge and Rose (2000) suggested, success of this approach will depend on the continual development of effective and compatible host genotype/strain combinations as infective but ineffective populations may build up in the soil.

'Promiscuous' grain legumes

Other grain legumes are less specific in their nodulation with rhizobia than soybean. Cowpea is a very promiscuous legume host (Ahmad *et al.*, 1981; Ranga Rao *et al.*, 1985; Lewin *et al.*, 1987) and *Bradyrhizobium* strains with which it can form effective nodules are normally present. Thus cowpea, and some other tropical legumes, have rarely been found to respond to inoculation unless they are grown in soils where the conditions are not conducive to the survival of rhizobia (Lewin *et al.*, 1987). For example, a response to inoculation was found in cowpea and black gram in only one of three experiments conducted in the field (Bushby *et al.*, 1983) and inoculation of pigeonpea resulted in a yield increase in only two of 12 field experiments conducted at the ICRISAT centre in India (Thompson *et al.*, 1980). Chickpea is more exacting in the specificity of its requirements for rhizobia and both nodulation and plant growth can commonly be increased by inoculation where it is introduced for the first time, but not in traditional growing areas (Smithson *et al.*, 1985). Problems for the survival of rhizobia in soil, inoculant technology and screening of rhizobial strains for use as inoculants are considered further in Chapters 13 and 14.

Phaseolus – a special case?

One of the most perplexing problems in research on improvement of N_2-fixation in grain legumes has been the generally poor nodulation of *P. vulgaris* in the field (Graham, 1981). Frustratingly, although poor nodulation is frequently observed, *P. vulgaris* rarely responds to inoculation. For example, in Cameroon inoculation did improve nodulation in trials over 3 years but improved grain yield in only one season (Salez and Saint Macary, 1987).

It has been shown in Colombia and in eastern Africa that, even in soils where nodulation of *P. vulgaris* with indigenous rhizobia is sparse, these soils often contain large numbers ($> 10^3$ g^{-1} soil) of compatible and effective rhizobia. This poor nodulation is not due to an intrinsic inability of *P. vulgaris* to nodulate, as profuse nodulation can occur in controlled conditions in the glasshouse. Therefore, it indicates either that some environmental constraint is limiting nodulation in the field, or that some factor other than N is limiting crop growth, or a combination of the two (Giller, 1990). In eastern Africa, although the nodule mass of *Phaseolus* plants was often increased by inoculation alone, application of phosphorus was the

only treatment that gave consistent responses in N_2-fixation and grain yield (Ssali, 1988; Amijee and Giller, 1998; Giller *et al.*, 1998). In Tanzania, the improvement in growth of *Phaseolus* when phosphorus was added revealed that the ability of the soil to supply potassium was chronically deficient, and small potassium additions doubled grain yields in farmers' fields (Smithson *et al.*, 1993).

Where *P. vulgaris* has responded to inoculation this is where effective, compatible rhizobia were absent, or present only in small numbers (Amijee and Giller, 1998). Absence of compatible rhizobia is particularly unlikely in the case of *P. vulgaris*, due to its promiscuity of nodulation. Although for a long time it was presumed that *P. vulgaris* nodulated only with *R. leguminosarum* bv. *phaseoli* strains, it is now known that it can nodulate with many different species of rhizobia (see above). The wide range of rhizobia able to infect *P. vulgaris* increases the likelihood that nodulation may occur with strains that are ineffective or poorly effective in N_2-fixation. Further research is required to clarify the particular reasons why nodulation is poor in different environments, but there is no clear evidence to suggest that *P. vulgaris* is particularly unusual in its response to inoculation.

Potential for strain selection for grain legumes

The vast majority of rhizobial strains that are recommended for use as inoculants have not been subjected to rigorous selection in soil in competition with native strains. Thus inoculation is rarely beneficial if populations of effective, compatible rhizobia are already present in the soil (Singleton and Tavares, 1986) but, despite the poor record of success, there remains considerable scope for strain improvement in the future (Chapter 14). A large proportion of rhizobia indigenous to Kenyan soils were more effective in N_2-fixation with *P. vulgaris* than the widely recommended strain CIAT899 (Anyango *et al.*, 1995). Selection of adapted strains for *P. vulgaris* sown directly in pots of soils containing large populations of indigenous, compatible rhizobia has resulted in many cases in yield increases when these strains were tested in the field (Pineda and Kipe-Nolt, 1990; Sylvester-Bradley and Kipe-Nolt, 1990).

In some cases particular strains have shown promising results with grain legumes considered to be highly promiscuous in nodulation with rhizobia. An interesting example is found with groundnut, where a particular cultivar (Robut 33–1) gave increased pod yields on inoculation with a specific strain, *Bradyrhizobium* sp. (*Arachis*) strain NC92 (Nambiar *et al.*, 1984). Several experiments at a range of locations in India indicated the advantage of this host/strain combination but later work failed to achieve significant increases in yield and interest in this combination appears to have waned.

Breeding Grain Legumes for Enhanced N_2-fixation

There are numerous examples of experiments with a wide range of grain legumes where different genotypes of the same legume have been shown to have differing

capacity to nodulate and fix N_2. For instance, large differences in nodulation in the field have been shown between genotypes of cowpea (Zary *et al*., 1978), *P. vulgaris* (Graham, 1981), groundnut (Nambiar *et al*., 1982), chickpea (Rupela and Dart, 1980), pigeonpea (Kumar Rao and Dart, 1987; Rupela and Johansen, 1995) and soybean (Neuhausen *et al*., 1988). But apart from the case of breeding for promiscuity of nodulation in soybeans discussed above, there have been few concerted efforts to enhance the potential for N_2-fixation in grain legumes through plant breeding.

In groundnut, the Virginia types were shown to have greater capacity to nodulate and fix N_2 than the Spanish or Valencia types (Arunachalam, 1984; Giller *et al*., 1987). Recombination of the ability to form a large mass of nodules was demonstrated in a series of crossing experiments at ICRISAT in India (Nigam *et al*., 1985) but this work has not been pursued. In Australia, the rates of N_2-fixation in Virginia and Spanish types were similar and greatest amounts of N_2 were fixed by long-duration genotypes (Bell *et al*., 1994). Greater N_2-fixation in groundnut varieties was found to be due mainly to better light interception, suggesting that alterations of row spacing to optimize light interception would give more immediate benefits of increased N_2-fixation than breeding (Williams *et al*., 1990). A breeding programme to combine the high N_2-fixation ability of some cowpea genotypes into well-adapted, high-yielding varieties was established (Miller and Fernandez, 1985; Miller *et al*., 1986) but appears not to have been pursued.

Breeding for enhanced N_2-fixation in *P. vulgaris*

At CIAT a programme of crossing and recurrent selection was begun to increase the contribution from N_2-fixation in *P. vulgaris* (Graham, 1981; Graham and Temple, 1984). The effort was concentrated on improving N_2-fixation in small-seeded bush beans of a similar maturity group and materials were screened for nodulation, ARA, total N and grain yield under glasshouse conditions before promising materials were advanced for screening in the field. An evaluation of materials produced from this programme (designated RIZ lines) in the field in Colombia indicated that the RIZ lines generally nodulated better and fixed more N_2 than the early parents used in the breeding programme (Kipe-Nolt and Giller, 1993). However, when compared with other *P. vulgaris* genotypes from CIAT breeding programmes, the RIZ lines were no better in N_2-fixation than several of the other promising lines that had not been selected for N_2-fixation potential. In this comparison the same genotypes were grown at two different sites in the Cauca valley in Colombia in two seasons and the ranking of genotypes for N_2-fixation was remarkably consistent, although the absolute amounts of N_2 fixed varied enormously between the separate experiments (Kipe-Nolt *et al*., 1993). The soils in which this bean breeding programme was carried out in Colombia are rich in N and one can only speculate as to whether it would have been more successful with a more severe selection pressure for N_2-fixation. The same recurrent selection method gave marked improvements in N accumulation over three generations in N-limited soils (Barron *et al*., 1999). A programme to select varieties for adaptation to infertile soils in Africa (Wortmann *et al*., 1995) has been

remarkably successful in improving yields in farmers' fields, at least in part due to enhanced N_2-fixation.

A different approach to the improvement of N_2-fixation in *P. vulgaris* was adopted with the aim of improving N_2-fixation in 'Sanilac', a poorly nodulating, white-seeded, determinate (Type I) variety that is widely grown in the USA. A back-cross method was used to combine the characters of 'Sanilac' with 'Puebla 152', an abundantly nodulating, black-seeded, indeterminate bush bean (Type IIIB) from Mexico. Selection criteria used were visual nodule scores and ARA. The lines selected were of intermediate character: all formed more nodules and showed greater capacity to fix N_2 than the poorly nodulating parent 'Sanilac', but none nodulated or fixed N as well as 'Puebla 152' (Dubois and Burris, 1986; Rosas and Bliss, 1986; Pereira *et al.*, 1989). Thus N_2-fixation was increased by crossing two genotypes with widely differing abilities to nodulate and fix N_2 but, as might be expected, when the two parental genotypes were only slightly different in their ability to fix N_2, success in improvement of N_2-fixation was limited (St Clair *et al.*, 1988). The back-cross method is, however, a useful method to improve N_2-fixation in genotypes that nodulate poorly but otherwise have good agronomic characteristics (Bliss, 1993).

After 15 years of screening in Queensland, Australia, in which a total of 1500 genotypes of *P. vulgaris* were evaluated, two were identified that fixed roughly 30% more N than commercial check cultivars (Redden and Herridge, 1999). These genotypes, however, lacked disease resistance and responded strongly to N fertilizer, which indicated that the supply of N from N_2-fixation was inadequate. On the basis of these results, Redden and Herridge (1999) concluded that prospects for reliance on N_2-fixation in *P. vulgaris* were limited in the short term and that recommendations for use of N fertilizer should remain.

Breeding for enhanced N_2-fixation in soybean

A programme designed to improve the ability of soybean to fix N_2 in fertile Australian soils rich in nitrate was established in 1980 (Herridge and Rose, 2000). Of some 500 soybean varieties that were screened in glasshouse and field experiments, a group of varieties from Korea was able to form many more nodules and fix N_2 when grown in soils with an abundant nitrate supply (Betts and Herridge, 1987; Herridge and Betts, 1988). Screening of progeny from crosses between the nitrate-tolerant genotypes and other high-yielding varieties, using a non-destructive ureide assay on part of the plant shoots, led to the identification of lines with clearly enhanced nitrate tolerance (Herridge and Rose, 1994). The effectiveness of this approach in improving the efficiency of N use in cropping systems depends on any nitrate that is not used by the legume crop being retained in the soil. It can be argued that a legume that is able to utilize mineral N when it is available, but rely on N_2-fixation when soil N is limiting, is ideal, and this questions the rationale for selecting legume varieties that are able to fix N_2 in the presence of large concentrations of nitrate (Chapter 14).

Selection criteria

Obviously, to conduct a breeding programme, clear selection criteria are required. The acetylene reduction assay was frequently used as a selection criterion in breeding for improved N_2-fixation in the past, but the method is now known to be inaccurate. The extent of nodulation, assessed as nodule number, nodule mass or simple nodule scores, has also been used as a criterion and is sometimes correlated with total N_2 fixed, at least in soils poor in N. Total N accumulation, which is a good indication of the total amount of N_2 fixed, at least in soils with a poor capacity to supply combined N (Chapter 4), is probably the best broad criterion for selection programmes in the tropics. In soybean, ability to fix N_2 appears to be closely related to early formation of nodules, a simple criterion which could be used in breeding (Pazdernik *et al*., 1996, 1997a,b).

A large number of plant characters contribute to N_2-fixation and it is thus important to clarify whether the plant selection can discriminate between plants with a real ability to nodulate and fix N_2 better consistently in the field and plants that are simply more vigorous, and thus nodulate better, under a given environment. Genetic adaptation to specific environments can complicate the selection of genotypes with enhanced N_2-fixation. This is particularly apparent in *P. vulgaris*. Rio Tibagi, a genotype considered to have a poor capacity to nodulate and fix N_2 in Brazil (Duque *et al*., 1985), nodulates and fixes N_2 as well as some of the genotypes specifically selected for N_2-fixation in Colombia (Kipe-Nolt and Giller, 1993). The genotype Puebla 152, which nodulates profusely in Colombia and has been used as a parent line with good nodulation in breeding for enhanced N_2-fixation (Rosas and Bliss, 1986), was found to be among the poorest nodulators when many genotypes of *P. vulgaris* were compared in Queensland, Australia (Redden *et al*., 1990). This is perhaps not surprising given the enormous breadth of environmental adaptation within *P. vulgaris*, but it emphasizes the need either to breed specifically for local environments, or to screen genotypes across the wide range of environmental conditions that they are likely to encounter in the field. This is especially true given the unpredictability of the climate in many parts of the tropics.

Intercrops of Grain Legumes and Cereals

Intercropping of grain legumes with cereal crops is common in the tropics. The crop combinations and planting arrangements are infinitely variable and range from mixed cropping, in which many species are sown randomly in a field, to more strict row or strip intercropping (Francis, 1986). Often climbing varieties of legumes such as *P. vulgaris* may be intercropped with a cereal that provides physical support (Fig. 8.1).

Effects of intercropping on N_2-fixation

Often the overall benefit of growing two crops in a mixture will be a net benefit in which the increase in growth of one crop exceeds a small competitive reduction in the growth of the other (Willey, 1979) and this is often seen where a low-growing legume is intercropped with a tall cereal. For example, nodulation and N_2-fixation of groundnut were greatly reduced when it was intercropped with maize, sorghum or millet (Nambiar *et al.*, 1983a). Similarly, growth and N_2-fixation of soybean were reduced by a tall sorghum intercrop, whereas N_2-fixation per plant was enhanced by a dwarf sorghum (Wahua and Miller, 1978), indicating that the reduction in yield and N_2-fixation was partly caused by shading. Studies using the ^{15}N isotope dilution method indicated that, although the total amount of N_2 fixed was substantially reduced from 97 to 62 kg N ha^{-1} in ricebean (*V. umbellata*) intercropped with maize, the proportion of N derived from N_2-fixation in the ricebean was increased from 72% to 90% (Rerkasem and Rerkasem, 1988). This increase in the proportion of N from N_2-fixation in the legume was due to efficient depletion of mineral N from the soil by the cereal crop, thus reducing nitrate-induced suppression of nodulation (Chapter 3). However, experiments using similar methods with maize/cowpea intercrops found no such effects on N_2-fixation (Ofori *et al.*, 1987; Van Kessel and Roskoski, 1988). Some caution must be exercised in drawing conclusions from isotope dilution experiments as the differences in ^{15}N-enrichment used to calculate N_2-fixation may be due either to competition for soil N in the intercrops or to different ^{15}N uptake patterns between crops (Abaidoo and van Kessel, 1989) (Chapter 4).

Competition for soil N between cereal crops and legumes often results in the legume deriving a greater proportion of its N from N_2-fixation, as demonstrated with pigeonpea/cereal intercrops (Tobita *et al.*, 1994; Sakala *et al.*, 2001). The extent to which growth and the total amount of N_2 fixed by the legume crop is decreased in the intercrop depends on the degree of complementarity between the crops. A much quoted example of the benefits of intercropping legumes and cereals is that of pigeonpea intercropped with maize or sorghum (Dalal, 1974; Ong *et al.*, 1996). The early growth of pigeonpea is very slow so that it affords little competition and yields of the cereal crop are unaffected (Sakala *et al.*, 2001). When intercropped with maize or short-duration varieties of sorghum, pigeonpea continues to grow on residual soil moisture long after the cereal crop has been harvested, and the amounts of N_2 fixed by pigeonpea are the same when grown in mixture or as sole crops (Sakala *et al.*, 2001).

As discussed in Chapter 5, there is little evidence for direct transfer of significant amounts of N between roots of legumes and cereals in mixtures, and this conclusion is supported by measurements of natural ^{15}N abundance in intercrops of pigeonpea and sorghum (Tobita *et al.*, 1994). Although pigeonpea loses large amounts of N in leaves that fall during crop growth, these cause an initial immobilization of soil N when they decompose and so little of the N is available for use by intercropped cereals (Sakala *et al.*, 2000). The available evidence indicates that inputs of fixed N are more likely to benefit subsequent crops (see below).

Although intercrops can produce greater yields, they generally do so by extracting more nutrients from the soil than sole crops (Dalal, 1974; Mason *et al.*, 1986) and may therefore cause more rapid decline in soil fertility. Similarly, intercrops use more water for growth: when rainfall was adequate a cowpea/maize intercrop gave superior crop yields, but competition for moisture in a drought year caused drastic reductions in yields of intercropped maize (Shumba *et al.*, 1990).

Net N benefits and residual effects of grain legumes

Substantial quantitative information on the amount of N available to crops succeeding legumes in a rotation – that is, on the residual effect of the legume – is available (Table 8.4). The beneficial effects of legumes on succeeding crops can often arise due to a variety of other effects, such as reduction of disease incidence (e.g. Marcellos *et al.*, 1997), or by reducing attack by striga, a parasitic weed that can often devastate yields of cereal crops (Reddy *et al.*, 1994), as well as other changes in soil fertility (see Table 5.2). These other 'rotation effects' must also be considered when assessing the benefits from N_2-fixation in crop rotations.

Net N benefits of grain legumes

For grain legumes to play an important role in the maintenance of soil fertility for other crops in the rotation, they must obviously leave behind more N from N_2-fixation than the amount of soil N that is removed in the crop. Clearly the two purposes served by the crop – one to provide grain yield and the other to leave residual N – are somewhat contradictory. The role of grain legumes in contributing N to cropping systems is bound, therefore, to be compromised by the breeding priority of optimizing the efficiency of conversion of N into the grain removed (Henzell and Vallis, 1977). The amounts of N added to the cropping system that have been measured are very variable for all of the main species on which there is substantial information (Table 8.4). In many cases there is no net benefit from including a grain legume in the crop rotation, even when the legume stover is returned to the field. The largest net benefits tend to be found with groundnut and cowpea, as at least some varieties of these crops generally have a smaller N harvest index (NHI). However, some newer varieties of groundnut have higher yield potential and larger NHI as a result (Bell *et al.*, 1994). The 'hay' or fodder varieties of cowpea and soybean produce large amounts of stover. The promiscuously nodulating variety 'Magoye' yielded over 10 t of stover ha^{-1} when grown on a fertile soil in Zimbabwe, containing almost 140 kg N ha^{-1} (Kasasa *et al.*, 1999). Similarly, lablab (*L. purpureus*), a relatively 'unimproved' legume with vigorous vegetative growth and poor grain yield, generally left more residual N than groundnut, soybean or pigeonpea (MacColl, 1989). If the legume stover is removed from the field, the net effect of growing a legume crop on the N balance of the cropping system is always negative unless contributions from fallen leaves, roots and nodules is considered. Based solely on above-ground plant parts, the net loss of N from the system from growing grain legumes can easily be 100 kg N ha^{-1} or more.

Table 8.4. Amounts of N_2 fixed and contributions to soil fertility by grain legumes in the tropics grown as sole crops, if only above-ground plant parts are considered and legume stover is returned to the soil.

Grain legume	Duration (days)	Grain yield (t ha^{-1})	Stover yield (t ha^{-1})	Harvest index (%)	% N from N_2-fixation (%)	Amount of N_2 fixed (kg N ha^{-1})	N in stover (kg N ha^{-1})	N harvest index (%)	Net input from N_2-fixation (kg N ha^{-1})	Recovery of stover N (%)	Residual effect in fertilizer equivalents (kg N ha^{-1})	Refs[a]
Arachis hypogaea	90–140	0.3–3.1	1.4–6.7	25–47	16–92	21–206	52–166	30–70	–37 to 100	12–26	0–97	1
Cajanus cajan	90–241	0.2–1.4	1.8–13.8	8–54	0–88	0–166	12–50	21–68	–32 to 41	9–15	0–67	2
Cicer arietinum	140–175	0.6–2.9	5.9–7.5	23–78	0–96	0–124	–	43–66	–47 to 46	–	?	3
Glycine max	96–104	0.8–3.0	1.0–10.4	16–57	12–100	26–188	30–170	37–88	–37 to 59	14–23	0–22	4
Phaseolus vulgaris	72–114	0.1–4.0	0.1–7.5	21–64	0–73	2–125	3–38	44–93	–	–	–	5
Vigna radiata	70–84	0.7–1.7	1.3–3.9	28–46	0–100	61–107	30–88	54–67	–20 to 10	4–58	68–94	6
Vigna unguiculata	69–115	0.2–2.7	0–8.4	9–42	32–76	9–201	20–94	29–66	–11 to 136	12–24	38–205	7

[a](References in addition to those cited in Table 8.2) 1: Bandyopadhyay and De, 1986; MacColl, 1989; Anuar *et al.*, 1995; 2: Dalal, 1974; Jones and Wild, 1975; MacColl, 1989; Cobbina, 1995; Mandimba, 1995; 3: None; 4: Wetselaar and Ganry, 1982; Suwanarit *et al.*, 1986; Ofori and Stern, 1987; MacColl, 1989; Sisworo *et al.*, 1990; Bergersen *et al.*, 1992; Ying *et al.*, 1992; Kasasa *et al.*, 1998, 1999; 5: Jones and Wild, 1975; Davis and Garcia, 1983; Davis *et al.*, 1984; Ssali and Keya, 1984b; 6: Bandyopadhyay and De, 1986; Senaratne and Ratnasinghe, 1995; Sharma *et al.*, 1996; 7: Agboola and Fayemi, 1971; Balasubramanian and Nnadi, 1980; Ssali and Keya, 1984a; Bandyopadhyay and De, 1986; Ofori *et al.*, 1987; Van Kessel and Roskoski, 1988; Ntare *et al.*, 1989; Sisworo *et al.*, 1990; Bationo *et al.*, 1991; Franzluebbers *et al.*, 1994; Klaij *et al.*, 1994; Reddy *et al.*, 1994; Senaratne and Ratnasinghe, 1995.

Residual effects on cereal crops

In northern Nigeria, maize grain yields were found to be greater following a groundnut crop than after crops of cowpea, cotton or sorghum. The yield increase was related to an increased availability of mineral N in the soil after groundnuts (Jones, 1974). The fact that no such beneficial effect was found after growth of cowpea in the same experiment indicates that residual effects do not always occur even with legume crops (Table 8.4). In Zimbabwe, the yield of maize was greater after bambara groundnut (7.6 t ha^{-1}) than after groundnut (6.2 t ha^{-1}), unplanted fallow (4.3 t ha^{-1}) or maize (3.9 t ha^{-1}) (Mukurumbira, 1985). Groundnut and cowpea were found to have roughly equal residual effects on the growth of a subsequent maize crop in northern Ghana, equivalent to the addition of 60 kg fertilizer-N ha^{-1}. This was despite the fact that 68 kg N ha^{-1} was left behind in above-ground residues after groundnut and 150 kg N ha^{-1} after cowpea (Dakora *et al.*, 1987). The residual benefit of the groundnut was additive with the application of fertilizer-N, giving an increase in yield over that in maize grown after maize even when 60 kg fertilizer-N ha^{-1} was applied, whilst that of cowpea was replaced by the application of N-fertilizer. This suggests that the organic residues of groundnut had additional benefits that were either not due solely to their provision of N or were due to a more efficient use of the N by maize. Direct evidence of the benefits from N_2-fixation was obtained where yields of sorghum grown after nodulating varieties of chickpea were better than yields after non-nodulating chickpeas (Kumar Rao and Rupela, 1998).

As most of the above-ground parts of grain legumes are removed at harvest, residual effects must come from the below-ground parts and any leaves that fall to the soil during growth of the crop. In India, pigeonpea was found to give a residual benefit to a subsequent maize crop of 38–49 kg N ha^{-1} (Kumar Rao *et al.*, 1983), which was partially attributed to a contribution of N from pigeonpea leaf fall of 30–40 kg N ha^{-1}. The amount of N in leaves that fall during growth of long-duration pigeonpea may be as much as 68–84 kg N ha^{-1} (Kumar Rao *et al.*, 1996b; Sakala *et al.*, 2001). Over 12 years, yields of sorghum were consistently higher following a sorghum/pigeonpea intercrop or than after an oilseed crop, safflower (*Carthamnus tinctorius*), and the soil N content had increased significantly where pigeonpea had been grown (Rego and Rao, 2000). Other legumes may also contribute substantial amounts of N during crop growth; inputs of 81 kg N ha^{-1} were measured in leaf fall from soybean in Australia (Bergersen *et al.*, 1992). A pigeonpea/sorghum intercrop gave no residual N benefit (Kumar Rao *et al.*, 1987) but a reduced but significant benefit (< 10 kg N ha^{-1}) of soybean/maize and groundnut/maize intercrops was found elsewhere (Searle *et al.*, 1981) – much less than the residual benefit following the sole legume crops (soybean, 46 kg N ha^{-1}; groundnut, 54 kg N ha^{-1}). All above-ground plant parts of the legumes were removed when they were harvested and so the residual effect must have been due to leaves lost during the growth of the legume crop, or to decomposition of roots and nodules.

Yields of maize grown after soybean on an Alfisol were increased to 2.5 to 4 t ha^{-1}, compared with only 1.8 t ha^{-1} in continuous maize cropping where all the legume stover had been removed (Kasasa *et al.*, 1999), but this may be partly due to other rotational effects than simply N_2-fixation. The below-ground contribution that

specifically came from N_2-fixation in groundnut was estimated by comparing N uptake by maize following nodulating and non-nodulating varieties (McDonagh, 1993; Toomsan *et al.*, 1995). The amounts were small, ranging from 0 to 10 kg N ha^{-1} and included inputs from both roots and fallen leaves.

Measurements of residual benefits using ^{15}N

As stated earlier, the residual benefit does not necessarily demonstrate a contribution of N from the legume N_2-fixation but could simply be due to sparing effects of soil N. There are still relatively few studies in which the sources of N for the second crop have been separated. Measurements using ^{15}N-labelled residues of grain legumes indicate that, with a few exceptions, some 10–20% of the legume N is recovered in the first subsequent crop (Giller and Cadisch, 1995) (Table 8.4). More than 70% of the N in shoot residues of cowpea was recovered by six successive crops in cowpea/rice/soybean or cowpea/rice/maize rotations in Indonesia and up to 27% of this was found in the first crop of rice (Sisworo *et al.*, 1990). In experiments using the isotope dilution approach to study residual effects of pigeonpea, Kumar Rao *et al.* (1987) found evidence that decomposing roots as well as the fallen leaves were supplying N to the subsequent crop. Recovery of N from ^{15}N-labelled roots of soybean was negligible, presumably due to their poor content of N (Bergersen *et al.*, 1992).

As indicated, groundnut has a particularly large potential to contribute N to cropping systems if the stover is returned to the soil, for two reasons: it produces large amounts of stover; and because the crop is harvested when the tops are still green, the stover is rich in N. Up to 166 kg N ha^{-1} in the stover of a groundnut crop grown on a farmer's field in northeast Thailand have been recorded (Toomsan *et al.*, 1995). Using ^{15}N-labelled residues the fate of the N applied in groundnut stover was followed in an upland groundnut/maize/maize cropping sequence (Fig. 8.3). In this experiment the groundnut grain contained 75 kg N ha^{-1}, and the groundnut residue that was returned to the soil contained 120 kg N ha^{-1}. Using ^{15}N-labelled residues, 20 kg N ha^{-1} was recovered in the first crop; of the remaining 100 kg N ha^{-1}, half was recovered in the surface 30 cm of the soil and roughly half was missing. The N in groundnut stover is released rapidly (McDonagh, 1993) and the N that was unaccounted for was presumed to have been lost from the cropping system, either to the atmosphere through denitrification and/or volatilization or by leaching. Of the 50 kg N ha^{-1} that remained in the soil, 5 kg N ha^{-1} was recovered in the second crop of maize. Groundnut yielded roughly 2 t grain ha^{-1} in the field experiments on which this N balance was based – the average yield on smallholder farms around the world is estimated to be only 0.7 t ha^{-1} (Freeman *et al.*, 1999) and is often much less.

Residual benefits on farmers' fields

Smallholder farmers invariably carry the whole shoots of grain legumes from the fields for threshing or shelling, or leave them in the fields to be grazed by livestock. In on-station trials on sandy granitic soils in Zimbabwe, the yield of maize was almost doubled, from 2.5 to 4.6 t ha^{-1}, after a groundnut crop that yielded only 0.4 t grain ha^{-1}, even though most of the stover was removed by cattle (Waddington and Karigwindi, 2001). Benefits were much smaller and generally insignificant on farms

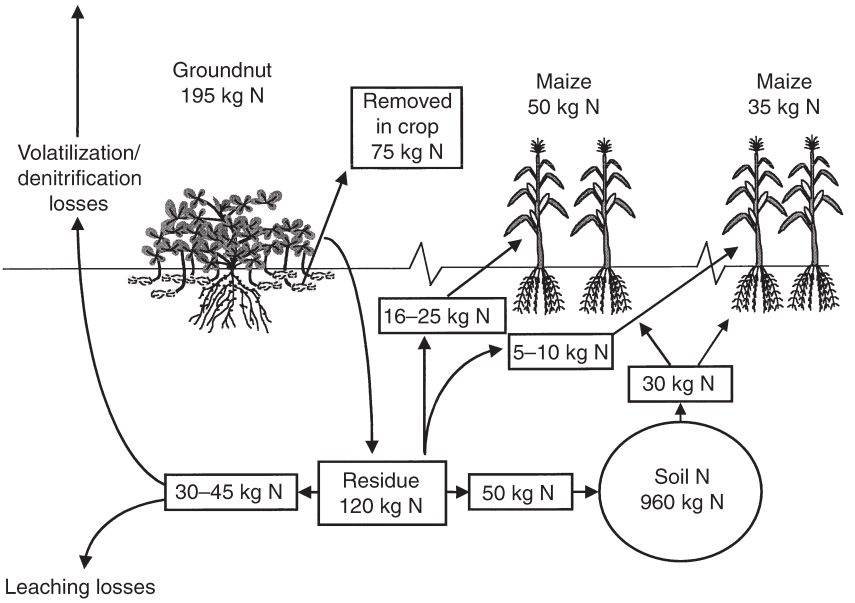

Fig. 8.3. Fate of the N in a crop of groundnut (*Arachis hypogaea*) grown on a sandy soil in northeast Thailand traced using ^{15}N-labelled groundnut residues. (From McDonagh, 1993.)

of five smallholders on similar soils where yields of maize were less than 0.8 t ha^{-1}. Researcher-managed experiments in farmers' fields in northeast Thailand, also on very sandy soils, showed that groundnut and soybean yields of 2 t ha^{-1} and major impacts on yields of subsequent rice were readily achievable (Toomsan *et al.*, 1995), but with much greater inputs than normally feasible for many smallholders in terms of basal fertilizers and labour for weeding.

The major role of crop residues as dry-season fodder, either for feeding to animals in stalls or by free-grazing of animals, and the extra labour involved to incorporate grain legume stover are probably the greatest limitations to residual effects in smallholder agriculture. This must, of course, be weighed against the benefits to the farmer in terms of both animal production and the cattle manure, which is a major source of nutrients for crop production in many tropical farming systems (Giller *et al.*, 1997).

Conclusions

Although large differences in N_2-fixation potential among genotypes have been demonstrated for many grain legumes, only a few serious attempts have been made to enhance N_2-fixation in grain legumes. Many legume breeders have adopted the approach of adding large amounts of N-fertilizers to help to reduce variability in their

selection plots, rather than ensuring that the plants are effectively nodulated. This approach may have resulted in selection against N_2-fixation whilst selecting for other desired traits. The need of American soybean varieties grown in regions outside North America for inoculation with specific rhizobia to ensure effective nodulation can be ascribed partly to the activities of plant breeders in selecting the plant genotypes in the absence of the symbiont, but it must also be recognized that soybean is far more selective than other legumes such as cowpea in its requirements for rhizobia. Once it is possible to make inoculants of selected strains freely available for use in the tropics, it may be considered that a highly specific host is preferable. However, given the lack of inputs commonly available to smallholders in the tropics (even inputs as inexpensive as rhizobial inoculum), legumes that fix N_2 and grow well without the need for inoculation are the best solution for the immediate future. Guidelines on how best to assess the requirements of legumes for inoculation are discussed in Chapter 14.

Grain legumes can contribute large amounts of N to the soil in fallen leaves and stover that can provide N for subsequent crops, sometimes resulting in spectacular yield increases on sandy soils. If the legume stover is removed, however, there is often no observable benefit to the next crop and there is usually a net removal of N from the cropping system in the legume grain. Increases in the amount of legume N contributed through residual effects is generally possible only if grain yield of the legume is decreased. This can rarely be justified in economic terms (Schwenke *et al.*, 1998), but might be worthwhile for smallholder farmers in remote areas who are unable to participate in a cash economy.

Chapter 9

Legumes as Green Manures and Cover Crops

In contrast to the role of grain legumes in cropping systems, a green manure legume is one that is grown specifically for use as an organic manure and this obviously maximizes the amount of N from the legume that is available for a subsequent crop. As, by definition, the green manure is returned to the soil whilst the plant material is still green, it will have a higher moisture content and higher N content than grain legume residues – both factors that favour rapid mineralization of the N.

Although often used interchangeably, the terms green manure and cover crop have very different meanings. As indicated, a green manure is an organic fertilizer, whereas the principal use of a cover crop is to protect the soil from erosion or to suppress weeds by maintaining a dense canopy close to the surface of the soil. Cover crops are therefore especially useful on steeply sloping lands and for control of pernicious weeds, and are widely used in plantations (Chapter 11).

There are many reports of increases in the growth and yield of crops sown after incorporation of green manures. Spectacular increases in yields of maize have been reported in some experiments. For example, maize yields were more than doubled by incorporation of a 3-month-old green manure of *Mucuna pruriens* var. *utilis* or *Crotalaria juncea* grown in an alluvial soil on the island of Java, Indonesia (Hairiah and van Noordwijk, 1989). The N uptake of the maize grown after the green manures was, in fact, increased by more than the total amount of N incorporated. This serves to reinforce the point made earlier (Chapter 5) that the advantages of growing legumes are not solely due to the provision of N, but are often due to other beneficial effects of organic matter additions and root penetration on the soil structure and water-holding capacity (Hulugalle *et al.*, 1986), or on incidence of pests or diseases (e.g. Abawi and Thurston, 1994). Green manures were widely used to maintain the fertility of the soil in the southern states of the USA (Tracey and Coe,

1918) and in tropical countries such as Zimbabwe (Rattray and Ellis, 1952) in the earlier part of the 20th century.

There has been a resurgence of interest in green manures and cover crops, for use both in uplands to improve the growth of crops such as maize and upland rice and stabilize the soil, and in the tropical lowlands to improve the productivity of paddy rice.

The Main Green Manure and Cover Crop Legumes

Trials to compare growth of different legumes for use as soil cover and for soil fertility improvement have been conducted in many parts of the tropics since the early 1900s. Thus there is a wealth of information on the relative growth potential and uses of different legume species, although much of this resides in rather obscure sources and in 'grey' literature. To date, selection of cover crops has largely relied on hard-earned judgement and knowledge of researchers and on empirical testing, often of a rather limited range of species. Attempts to collate this information into databases are still at a fairly early stage of development, but have the potential to become powerful tools for preliminary selection of potential cover crops and green manures in future (COMBS, 1994; Kiff *et al*., 1996; Weber *et al*., 1997). Recent attention to effects of temperatures and photoperiod on development of legume cover crops (Keatinge *et al*., 1996, 1998; Qi *et al*., 2000) will undoubtedly help in future selection of appropriate species for testing in different agroecologies. Many of the legumes that are useful as green manure crops are described elsewhere as grain legumes (Chapter 8), as pasture legumes (see *Aeschynomene*, *Centrosema* and *Pueraria*, Chapter 10), or as agroforestry legumes (see *Sesbania* and *Tephrosia*, Chapter 12).

Canavalia

The jack bean (*C. ensiformis*), which originates from the New World, and the sword bean (*C. gladiata*), an Old World species, are grown mainly as green manure crops but the immature pods and seeds can be eaten (Smartt, 1990). Both species are well adapted to acid and infertile soils, and can grow in a range of climates, from very wet to very arid. Bush and climbing types of *C. ensiformis* have a wide range of maturity: from 59 to 109 days to flowering and 161 to 225 days for seed harvest (Kessler, 1990). Grain of *C. ensiformis* is a useful feed for pigs and chickens but care must be taken to remove the non-protein amino acid canavanine, an anti-nutritional factor which is present in the seeds (Udedibie, 1990; Belmar and Morris, 1994). *C. brasiliensis* has also been tested as a green manure. Little is known of the rhizobia that nodulate species of *Canavalia*, but they are said to be nodulated by slow-growing rhizobia.

Crotalaria

There are more than 600 species in the genus *Crotalaria*, at least 500 of which are found in Africa (Allen and Allen, 1981; Polhill, 1982). Several species are useful green manures but most are not good fodder plants due to their high content of toxic alkaloids. Although some species such as *C. retusa* are highly toxic, the leaves of others such as *C. ochroleuca* are used as vegetables (Wortmann *et al*., 1994; Raussen and Kawimbe, 1997). The most widely used green manure species is *C. juncea*, the 'sunnhemp', which is a useful source of fibre (Duke, 1981). It is grown on the Indian subcontinent for making ropes and sacks, and the low ash content makes it ideal for high-quality paper for cigarettes and tissues (Anon., 1979). *C. juncea* has a shrubby, branched growth habit, but grown in dense stands it has a straight stem, which can reach 3 m in height. Collections in Madagascar identified a shrubby species with palmate leaves, *C. grahamiana*, which is being tested by farmers and researchers for improved fallows in western Kenya (Chapter 12).

Crotalaria spp. are reported to be fairly promiscuous hosts, and are said to be nodulated by *Bradyrhizobium*, although early reports indicated a range of growth rates among isolates from their nodules (Allen and Allen, 1981). A study of rhizobia from nodules of *Crotalaria* species in Senegal provided an explanation for this confusion: some six species were nodulated only by *Bradyrhizobium*, but three species, *C. glaucoides*, *C. perrottetii* and *C. podocarpa*, were nodulated only by fast-growing strains (Samba *et al*., 1999). These fast-growing strains are unusual and appear to belong to the genus *Methylobacterium* (Sy *et al*., 2000).

Mucuna

The taxonomy of the pan-tropical genus *Mucuna* is confusing and there are many different species, but the non-stinging varieties used in agricultural experimentation invariably belong to the species *M. pruriens* var. *utilis* (R. Polhill and B. Verdcourt, 1997, personal communication; Wulijarni-Soetjipto and Maligalig, 1997). Thus the different accessions of mucuna, which are often referred to as *M. deeringiana*, *M. cochinchinensis*, *M. aterrima* and so on, should strictly be named as *M. pruriens* var. *utilis* cv. deeringiana, cv. cochinchinensis and cv. aterrima. Here the velvet bean, *M. pruriens* var. *utilis*, will simply be referred to as 'mucuna' unless another species is discussed. Part of the taxonomic confusion has undoubtedly arisen from the enormous variability in colour and size of the seeds and wide range in growth duration (100–290 days to maturity) between these cultivars.

A historical account of the spread and utilization of mucuna in agriculture is given by Buckles (1995b). In screening programmes of potential green manures in a wide range of tropical and subtropical environments, mucuna is routinely found to be one of the most successful green manure species. Its large seed gives relatively rapid early growth which, together with the spreading or trailing habit, means that mucuna gives rapid establishment of soil cover (Carsky *et al*., 1998). Both *M. pruriens* var. *utilis* and the horse eye bean (*M. sloanei*) are used for food (Rachie and Roberts,

1974). Mucuna seed contains significant amounts of L-dopa, an amino acid used in the treatment of Parkinson's disease, and the seed must be boiled for hours with several changes of water to remove this toxic compound. In Mozambique an outbreak of acute psychosis was attributed to consumption of poorly cooked mucuna in time of drought (Infante *et al*., 1990). Considerable variability in L-dopa concentrations between mucuna accessions indicates that efforts to select varieties with lower seed contents may be worthwhile (Lorenzetti *et al*., 1998). Mucuna also provides an excellent hay for livestock, and its seeds are used as a feed supplement after processing (Maasdorp *et al*., 2001).

Rhizobia isolated from nodules of mucuna are slow-growing (Allen and Allen, 1981; Sanginga *et al*., 1996c); some of them are able to nodulate cowpea and soybean effectively. *Bradyrhizobium* isolates from *C. juncea*, *Mimosa pudica*, *Stylosanthes guianensis* and *Phaseolus vulgaris* all nodulated mucuna effectively (Mandimba, 1998).

Green manures for cool climates

In the cool climates of the tropical highlands, species of *Lupinus*, *Trifolium* and *Vicia* are better adapted for use as green manures. In the mountains of Burundi and Rwanda, *Lupinus luteus*, *Vicia sativa* and, despite the high altitude, mucuna and *Canavalia* were among the species that gave largest biomass (Potts *et al*., 1989; Yamoah and Mayfield, 1990). In Nepal, *L. mutabilis*, *Vicia benghalensis*, *Trifolium repens* and *T. resupinatum* were among the best adapted species (Schulz *et al*., 1999, 2000). A number of species of vetch grow well in the tropical highlands, including *V. benghalensis*, *V. sativa*, *V. villosa* ssp. *villosa* and *V. villosa* ssp. *varia* (syn. *V. dasycarpa*).

Legumes as Cover Crops and Green Manures in Upland Soils

The usefulness of legume green manures in maintaining or building up soil fertility has long been recognized. Legumes have been traditionally used for this purpose in some regions (Sturdy, 1939). There was substantial research on legume green manures from the 1920s in various parts of the tropics – for example, India (Singh, 1963), East Africa (Gethin Jones, 1942), West Africa (Doyne, 1937) and southern Africa (Davy, 1925). Much of the early interest focused on use of mucuna and *C. juncea*, both of which still appear to be the most widely used throughout the tropics.

The characteristics that are desirable in a green manure or cover crop legume depend on the use to which it will be put. If weed suppression or erosion control is a major goal, then rapid development of a dense soil cover is essential, but if the major aim is to intercrop with a cereal, species that grow slowly initially or that have a more erect growth habit are more suitable. In either case, the ability to produce sufficient seed is usually an important selection criterion, and the legumes should not be important secondary hosts for diseases of legume crops grown in the cropping

system, nor should they be susceptible to damage by pests or diseases. A major reason that legumes are used as green manures is their ability to fix N_2 in abundance, and that is the focus of the discussion here.

N_2-fixation in legume green manures

Need for inoculation

As testing of legumes as cover crops and green manures has invariably been done without inoculation, promiscuity of nodulation with indigenous rhizobia has been a hidden selection criterion. However, sufficiently large populations of compatible rhizobia to give rapid nodulation are not always present, even for promiscuously nodulating legumes. When 11 green manure legumes, which are all thought to be promiscuous hosts, were grown in five Nigerian soils most of the legumes nodulated poorly at 4 weeks, but all nodulated in all of the soils after 8 weeks (Sanginga *et al.*, 1996b). This indicates that the soils contained only small populations of compatible rhizobia.

In farmers' fields in Benin in which mucuna had not been grown, compatible rhizobia were absent, or were present in very small numbers (<10 cells g^{-1} soil) in the majority of the soils (Sanginga *et al.*, 1996c). Indeed, mucuna formed nodules in only 20% of the soils in glasshouse tests. Inoculation with *Bradyrhizobium* increased yields of mucuna in farmers' fields by over 40% on average, with growth responses at the majority of sites in two villages but at fewer than half the fields in another village (Hougnandan *et al.*, 2000a). Marked increases in mucuna growth were mainly found in fields where numbers of rhizobia were fewer than 5 cells g^{-1} soil. Plants inoculated with *Bradyrhizobium* also had higher rates of infection with mycorrhiza (Hougnandan *et al.*, 2000a). This was probably due in part to better growth of the inoculated plants, and in turn the enhanced mycorrhizal infection may have contributed to their better growth.

These observations explain much of the variability in growth of mucuna that had been observed in Benin, but they also raise a dilemma. Should inoculation with rhizobia be recommended? And does this justify establishment of an inoculation production facility? It appears that this may be a more widespread problem, as mucuna was also found to benefit from inoculation in Ghana (Dogbe *et al.*, 2000). Given the other constraints to green manure use, transfer of rhizobia in soil from fields where mucuna is well nodulated would seem the most straightforward option. There are significant dangers in this approach, however, as seeds of parasitic weeds such as *Alectra* and *Striga* as well as soil-borne pests and diseases may be spread around.

Contributions from N_2-fixation

Surprisingly little research has been conducted specifically on N_2-fixation by legume green manures and cover crops, although there are many estimates of the amounts of N they can accumulate. The available estimates of N_2-fixation by different legume green manures in the tropics (Table 9.1) confirm their ability to fix large amounts of N. The amounts of N_2 fixed commonly exceed 100 kg N ha^{-1} within 6 months, with

Table 9.1. Amounts of N_2-fixation by green manure legumes and N accumulated and incorporated into the soil as above-ground plant residues in the field.

Species	Duration (days)	N accumulated (kg ha^{-1})	N_2 fixed (kg ha^{-1})	N_2 fixed %	Country	Method[a]	Ref.[b]
Aeschynomene	56	205	143	70	Philippines	ID	1
afraspera	87–89	71	59	85–87	Thailand	ID	2
A. histrix	180	27–159	9–137	35–86	Côte d'Ivoire	NA	3
	70	–	–	93	Philippines	NA	4
A. indica	–	–	–	75–94	Thailand	NA	5
Cajanus cajan	180	45–221	10–157	55–71	Côte d'Ivoire	NA	3
	190–195	154–235	111–167	71–72	Philippines	NA/Diff	6
Calopogonium mucunoides	180	4–108	1–76	35–79	Côte d'Ivoire	NA	3
Canavalia ensiformis	180	77–256	45–174	57–69	Côte d'Ivoire	NA	3
C. rosea	180	41–150	22–139	54–78	Côte d'Ivoire	NA	3
Centrosema pubescens	180	6–132	3–104	48–79	Côte d'Ivoire	NA	3
Clitoria ternatea	180	14–86	6–52	45–69	Côte d'Ivoire	NA	3
Crotalaria anagyroides	180	10–270	4–181	36–67	Côte d'Ivoire	NA	3
C. juncea	180	48–79	29–142	59–86	Côte d'Ivoire	NA	3
	74–80	33–88	16–43	48–49	Malawi	NA	7
	190–195	271–279[c]	199–223	72–80	Philippines	NA/Diff	6
C. retusa	180	15–194	5–93	31–48	Côte d'Ivoire	NA	3
Desmanthus virgatus	190–195	251–283[c]	196–226	78–80	Philippines	NA/Diff	6
Lablab purpureus	180	4–96	7–70	35–76	Côte d'Ivoire	NA	3
	132–154	7	3	45	Malawi	NA	7
Macroptilium atropurpureum	190–195	132–178[c]	91–132	69–74	Philippines	NA/Diff	6
M. lathyroides	180	32–80	19–66	57–83	Côte d'Ivoire	NA	3
Mucuna pruriens	180	30–257	18–213	60–83	Côte d'Ivoire	NA	3
var. *utilis*	84	23–163	18–38	36–49	Benin	Ureide	8
	140	22–193	41–76	49–57	Benin	Ureide	8
	92–108	71–126	42–73	58–78	Malawi	NA	7
	84	302–305	139–224	64–86	Nigeria	ID	9
cv. cochinchinensis	180	49–210	32–172	66–82	Côte d'Ivoire	NA	3
Pueraria phaseoloides	180	13–87	6–53	44–61	Côte d'Ivoire	NA	3
	–	225	172	76	Nigeria	Diff	10
Stylosanthes guianensis	180	40–201	25–159	63–79	Côte d'Ivoire	NA	3
Sesbania cannabina	45–55	127–199	119–188	93	Philippines	ID	11
S. rostrata	45–55	157–312	140–286	88–91	Philippines	ID	11
	50	–	–	78	Philippines	NA	4
	60–75	193–222	68–125	35–56	Philippines	ID	12
	50	23	13	55	Sri Lanka	ID	13
	87–89	83	64	77–81	Thailand	ID	2
	88–99	105–143	82–126	79–89	Thailand	ID	14
S. speciosa	50	31	19	62	Sri Lanka	ID	13
Tephrosia villosa	180	27–119	18–82	57–80	Côte d'Ivoire	NA	3
T. vogelii	184–220	32–88	12–45	37–78	Malawi	NA	7
Vigna unguiculata	180	11–39	4–34	38–86	Côte d'Ivoire	NA	3

Footnotes on *opposite* page.

Table 9.1. *continued*

[a]ID = ^{15}N isotope dilution; NA = ^{15}N natural abundance; NB = N balance; Diff = N difference; Ureide = ureide method.
[b]1. Becker *et al.*, 1990; 2. McDonagh *et al.*, 1995b; 3. Becker and Johnson, 1998; 4. Yoneyama *et al.*, 1991; 5. Yoneyama *et al.*, 1990b; 6. Ladha *et al.*, 1996; 7. Giller *et al.*, 2000; 8. Houngnandan *et al.*, 2000; 9. Sanginga *et al.*, 1996b; 10. Tian *et al.*, 1999; 11. Pareek *et al.*, 1990; 12. George *et al.*, 1998; 13. Seneviratne *et al.*, 1992; 14. Toomsan *et al.*, 2000.
[c]Total from repeated clippings.

the maximum amounts of N_2-fixation recorded in upland soils of 220–230 kg N ha^{-1}. Although this is probably as much N as that required to be added to the soil for any crop, the rates of N_2-fixation are not as large as observed in wetland rice systems (see below). Using the N balance method, Schulz *et al.* (1999) estimated that *L. mutabilis* fixed more than 460 kg N ha^{-1} in small plots in Nepal, and contributions from a range of other green manure legumes ranged from 79 to 260 kg N ha^{-1}.

Many of the species listed in Table 9.1 fixed 80% or more of their N when growing well. In Côte d'Ivoire the mean %N from N_2-fixation in 21 legumes was 68–73% in two sites with bimodal rainfall but only 52–54% where there was a single rainy season (Becker and Johnson, 1998). The %N from N_2-fixation in the legumes showed a loose positive relationship with shoot N accumulation, indicating that poor nodulation and N_2-fixation were linked to poor growth in general. Performance of green manures on farmers' fields can be very variable even when the plots are managed by researchers. In Benin the amount of N accumulated by mucuna in 140 days ranged from 30 to 193 kg N ha^{-1} between different fields in three villages (Houngnandan *et al.*, 2000). On average, 55% of the mucuna N came from N_2-fixation, amounting to 60 kg N ha^{-1}. When seed of mucuna was harvested from the fields, the N balance from growing the green manure ranged from loss of 37 to gain of 30 kg N ha^{-1} (Houngnandan *et al.*, 2000).

In all of the above estimates it is likely that inputs of N from the legume green manures have been underestimated. When a dense canopy develops the plant becomes self-shading, so that leaves lower in the canopy senesce. The fallen leaves may contribute substantial amounts of N; mucuna leaf fall over 6 months contained 40 kg N ha^{-1} and was almost equivalent in mass to the live shoots of the plant (van Noordwijk and Purnomisidi, 1992).

Managing legume green manures

The most useful way to manage a legume green manure or cover crop depends on its major intended use and on local conditions. When sown as cover crops, the legumes are normally sown alone to develop as uniform stands that can smother weeds. As different species are better adapted under the varied conditions that may prevail within the same region, the sowing of legume mixtures has been recommended (Schulz *et al.*, 1999), in a similar way that legume mixtures are used for soil cover in plantations (Chapter 11).

Green manures can also be intercropped or relay-planted by undersowing within a crop. Mucuna sown into maize tends to climb up the stalks and can cause significant losses in grain yields of the cereal. Delaying the planting of mucuna among the maize plants can reduce its competitiveness, but may also reduce its productivity drastically (Gilbert, 1998). Intercrops of maize and mucuna were harvested together for animal feed in the southern states of the USA in the early 20th century (Tracey and Coe, 1918), a practice still used today on some commercial farms in Zimbabwe. Slower-growing legumes, such as *Pueraria phaseoloides*, can be undersown at the same time as maize without reducing the crop yield, and then be allowed to grow through the following season (Tian *et al*., 1999).

Green manure legumes that are more erect, such as some species of *Crotalaria*, are less competitive when intercropped with cereals. *C. juncea* sown 28 days after maize gave no significant reduction in crop yield (Jeranyama *et al*., 2000), whereas another erect species, *C. ochroleuca*, reduced maize yields when planted at the same time as maize in Uganda (Fischler *et al*., 1999). If relay-planting of *C. juncea* or *C. ochroleuca* into maize was delayed by more than 2 weeks, production of the green manure was drastically reduced (Gilbert, 1998; Fischler *et al*., 1999). In fact, in Uganda the optimal management for *C. ochroleuca* appeared to be intercropping with *P. vulgaris* (Fischler *et al*., 1999).

In climates with a long dry season there is often little choice but to grow the legume as a sole crop. The rapid growth of *C. juncea* in Zimbabwe allowed it to be sown after the busy time for planting of other crops (Rattray and Ellis, 1952). The benefits to production of the maize crop grown in the following year were greatest when *C. juncea* was sown late, as the legume could be turned into the soil at the end of the rainy season when it was still green. If incorporated earlier in the season, the N may be released from the residues and easily leached. As the name 'sunnhemp' suggests, if left to grow for too long *C. juncea* becomes very fibrous. The mean nitrogen concentration in the plant tissues drops from around 3% at flowering to close to 1% in the mature plant, and the lignin increases from 6 to 18% (Fischler, 1997; Maasdorp and Titterton, 1997). This substantially reduces its ability to supply N for crop growth. In experiments conducted between 1935 and 1960, sugarcane production was just as good where sunnhemp had been harvested for fibre as where the stems were left in the field (Singh, 1963). By the time the plant is mature, most of the leaves have fallen to the soil and this, together with the long period between incorporation and planting of the cane, probably explains why removal of the sunnhemp plants did not decrease its benefit to the crop.

Of course, the green manure legumes cannot fix large amounts of N_2 if other nutrients are not available in the soil. In Zimbabwe, rehabilitation of the most exhausted sandy soils by mucuna was possible only if phosphorus fertilizer was added, and in some cases phosphorus alone was not enough to stimulate good growth (Hikwa *et al*., 1998). The ability of mucuna to grow luxuriantly in abandoned acid Ultisols dominated by *Imperata* in Southeast Asia is wholly dependent on additions of phosphorus fertilizers (von Uexküll and Bosshart, 1989; von Uexküll and Mutert, 1994). If large amounts (1 t ha^{-1}) of rock phosphate are used together with mucuna to 'kick start' the productivity of the system, these degraded lands can readily be

restored for arable cropping (Sri Adiningsih and Fairhurst, 1998). Although mucuna is somewhat tolerant of aluminium toxicity (Hairiah *et al.*, 1995) (Chapter 13), the rock phosphate will also help to ameliorate problems of soil acidity.

Managing the mulch

When ready, the legume can be left on the soil surface as a mulch or turned into the soil. In Nigeria, burning was compared with ploughing in because of the huge effort needed to incorporate the mucuna vines into the soil. The ash produced gave a more immediate liming effect (Doyne, 1937), and although there was more nitrate in the topsoil when the mucuna mulch was ploughed in, maize yields were equal with both methods of management (Vine, 1953). Similarly, there was no difference in yields of upland rice if mucuna was burned, removed or turned into the soil (Becker and Johnson, 1999). Recent measurements in Malawi indicated that it takes around 60 man days to turn the mulch of a luxuriant stand of mucuna into the soil on 1 ha of land (R. Gilbert, 2000, unpublished results), suggesting that methods for managing the mulch could be the key to successful green manuring for smallholder farmers.

Direct planting into the mulch without tilling the soil reduces the risk of soil erosion and requires much less labour (Kannegieter, 1968). As many green manure legumes grow very vigorously, it can be difficult to control their growth when a subsequent crop is sown. The legume can be slashed back, but often it will regrow under the next crop. This can be an advantage, as it maintains soil cover and re-establishes the green manure for a further season, although the green manure will generally compete with the crop to some extent. In fact, the legume can be used as a 'live mulch' in which crops are sown directly into a ground cover of a creeping legume. In some experiments weedkillers and growth retardants have been used to reduce competition from the legume (Mulongoy, 1986a; Mulongoy and Akobundu, 1990), which may prohibit the use of this technology by smallholders.

Effects on growth of subsequent crops

How large the benefit a green manure gives for growth of the following crop depends mainly on two things: firstly, the initial fertility of the soil, as benefits of green manures are likely to be smaller on fertile soils; and secondly, the amount of biomass (principally the amount of N) that the green manure contributes.

Yields of upland rice increased linearly with the amount of N added by a wide range of green manures across four sites in Côte d'Ivoire (Becker and Johnson, 1998) (Fig. 9.1). Vine (1953) found that yields of maize were generally higher after mucuna if the soil had been cropped continuously for several years. The fertilizer value of mucuna in farmers' fields in southern Benin was equivalent to 38–66 kg N ha^{-1} as urea, and this was increased to 74–76 kg N ha^{-1} where the mucuna had been inoculated and more N was added to the soil (Houngnandan *et al.*, 2001a). Overall maize yields were nearly doubled where mucuna had been grown and there is also some

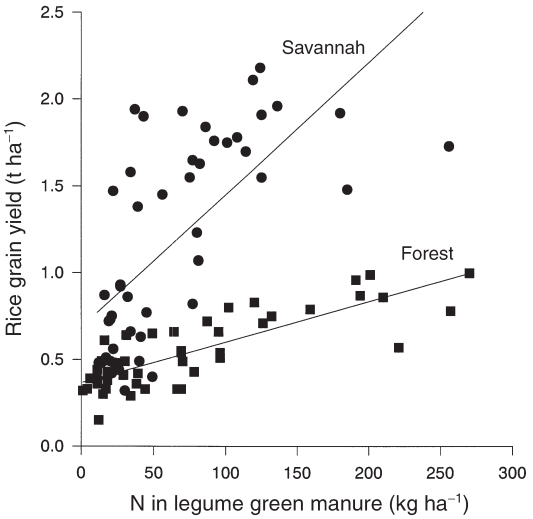

Fig. 9.1. Yields of upland rice in relation to the amount of N applied in 21 different species of green manure in Côte d'Ivoire. Mulch was incorporated in the savannah environments but was slashed and burnt in the forest environments. (Redrawn from Becker and Johnson, 1998.)

evidence that fertilizer is used more efficiently when combined with the mucuna mulch (Houngnandan *et al.*, 2001b). At least part of the benefit from mucuna to growth of the subsequent maize crop may have been due to increased colonization of the maize roots with arbuscular mycorrhiza. In Uganda, yields of the first maize/bean intercrop were substantially increased in trials on a research station and on farmers' fields, with no significant benefit to subsequent crops (Fischler and Wortmann, 1999; Fischler *et al.*, 1999). Pure stands of *C. ochroleuca* gave almost double the yield increase than where the green manure had been intercropped with maize.

In regions with less rainfall, the effects of legume green manures tend to be attenuated. Although N was shown to be strongly limiting at trial sites in the northern Guinea savannah of Nigeria, mucuna, *C. juncea* or cowpea green manures gave yield increases in maize that were equivalent to 6–14 kg N ha^{-1} (Carsky *et al.*, 1999).

Green manures tend not to have long-term effects on crop yields or on soil organic matter, as they decompose so readily. In long-term trials in Zimbabwe, yields of the second crop following *C. juncea* were only 60% of the yields found in the first maize crop (Rattray and Ellis, 1952). Even in trials where green manures had been used in rotation with crops for 12 years, there was no readily detectable effect on the build-up of organic matter in the soil (Vine, 1953; Singh, 1963). A trend of increasing soil organic matter and N contents with years of mucuna use was found in Honduras (Buckles *et al.*, 1998), but as these results were not corrected for any changes in bulk density, which tends to become less under a green manure for a variety of reasons (Chapter 5), firm evidence for such an effect is still lacking. In Bolivia, although mucuna and *L. purpureus* were the best legumes at competing with weeds, grass cover crops proved to be better for increasing the soil organic matter (Barber and Navarro, 1994). Roots of legume green manures are more lignified (Tian and Kang, 1998) and are therefore more likely to contribute to soil organic matter than the plant shoots.

Farmers' experimentation and use of cover crops in upland soils

Mucuna has been used in rotation with maize by smallholder farmers in Guatemala, where it was introduced for forage in the 1920s. From Guatemala, the use of mucuna spread spontaneously to farmers in neighbouring countries. Mucuna was introduced in Honduras in the early 1970s, where it was used by a small proportion of farmers over the first 10 years, but then the rate of use rose dramatically. Known as the 'abonera' or 'fertilized field' system, mucuna green manuring was adopted by almost 70% of farmers in northern Honduras by 1992 (Buckles *et al.*, 1992). This is a live mulch system in which the farmers control growth of the mucuna by heavy slashing two or three times during each maize crop (Bunch, 1990). Labour inputs were actually less than when no mulch was used, due to the reduced requirements for weeding (Buckles, 1995a). The 'fertilizer effect' was highest on the list of reasons that farmers gave for using mucuna, but ease of land preparation, moisture conservation and reduction of weeds and soil erosion were also important (Buckles *et al.*, 1998). Farmers also identified a number of problems that they associated with using mucuna, including a build-up of rat populations and risks of small landslides, perhaps due to the mulch assisting in water retention. In particular, a pernicious grass weed (*Rottboellia cochinchinensis*) invades the mucuna green manure, as gaps are often present when it regenerates. Control of this weed may require some change to the management system, such as regular planting of the mucuna rather than reliance on spontaneous reseeding (Buckles and Triomphe, 1999).

In Benin, mucuna was introduced in the late 1980s specifically in response to farmers' problems in controlling *Imperata cylindrica* (Versteeg and Koudokpon, 1990). Although *Imperata* can be effectively controlled by mucuna, some slashing back of the *Imperata* regrowth or use of herbicides is generally necessary as the green manure establishes (Sri Adiningsih and Fairhurst, 1998). Prolific growth of mucuna in most cases gave good weed control and the number of farmers using mucuna rose rapidly from 20 in 1988 to more than 10,000 in 1996 (Versteeg *et al.*, 1998; Manyong *et al.*, 1999). Land tenure was an important factor in determining whether or not farmers invested their time in growing mucuna, and farmers recognized the important contribution of the mulch in improving soil fertility.

Joint experimentation with farmers on green manures in Uganda gave good increases in crop yields, although over a three-crop cycle the green manure did not compensate for the time it occupied the field (Fischler and Wortmann, 1999). An economic evaluation of different green manure legumes indicated that only those species that could control weeds effectively, and reduce labour costs, gave increased savings over continuous cropping. In consultation with farmers, a simple guide was developed to assist in deciding which of the green manure species was best for a given niche in the cropping system (Fischler and Wortmann, 1999). An interesting farmers' observation was that *Tephrosia* benefited production by deterring mole rats from digging up their fields.

As discussed earlier, it has been suggested that development of toxin-free varieties of mucuna increases the acceptability and reduces the cooking time required. In Malawi, where mucuna is well known as a 'hunger food', it can produce

seed yields of 2 t ha^{-1} without any fertilizer inputs on soils where maize yields less than 1 t grain ha^{-1} (Gilbert, 1998). Utilization of mucuna as food is a focus for current research, but whether it will prove more acceptable to farmers than existing grain legumes remains to be seen.

Legume Green Manures in Lowland Rice Production

A wide range of legumes have traditionally been used as green manures for rice production in many parts of Asia, including some legumes that are usually grown as grain crops, such as cowpea (*V. unguiculata*) or mungbean (*V. radiata*). Much research interest has focused on the use of the stem-nodulating species *Sesbania rostrata* and *Aeschynomene* spp., which are tolerant of waterlogging and can thus be grown in soils susceptible to flooding. This subject is also reviewed in detail by Becker *et al.* (1995a), Ladha and Kundu (1997), Lauren *et al.* (1998) and Kundu and Ladha (1999).

In many rice production systems where there is abundant water throughout the year, there is little opening for use of legume green manures when the field is not occupied, as three, or exceptionally four, crops of rice can be grown in a year. In seasonal climates the green manure can be grown either before rice is transplanted or after rice harvest, depending on the local climate. Early rains, at the beginning of the wet season, are often insufficient to flood the fields for rice and this moisture can be used for green manure production. Residual soil moisture remaining after rice harvest can also be used.

N accumulation and N_2-fixation in legume green manures in rice systems

Experiments over 4 years in the Philippines showed that *V. radiata* and *V. unguiculata* grown between the wet and the dry seasons can accumulate 75–100 kg N ha^{-1} in 40 days (Morris *et al.*, 1986b). Incorporation of this green manure gave a mean increase in rice yield of 2 t ha^{-1}, double that found with N-fertilizer applied at a rate of 80 kg ha^{-1} (Morris *et al.*, 1986a). The use of grain legumes provides the possibility for the pods to be harvested whilst leaving the residues to be turned into the soil. For example, *V. radiata* produced 0.9 t grain ha^{-1}, and crop residues that gave an increase in rice yield equivalent to 25 kg N-fertilizer ha^{-1} (Meelu and Morris, 1988). Multi-purpose varieties of cowpea, from which the green pods can be used as vegetables or left to mature for grain, had residues containing 26–41 kg N ha^{-1} in northeast Thailand (McDonagh *et al.*, 1995b) whereas *V. radiata* produced and fixed N very poorly in this environment (Toomsan *et al.*, 2000).

Stem-nodulating species, such as *S. rostrata* and *A. afraspera*, are very fast growing (Rinaudo *et al.*, 1983; Alazard and Becker, 1987) and have the advantage that they can continue to fix N_2 under waterlogged conditions when root nodulation is poor (Saint Macary *et al.*, 1985; Becker and George, 1995). Thus they are excellent candidates for use as green manures in paddy fields. In small plots in Senegal,

A. afraspera and *A. nilotica* accumulated 42 and 53 g N m^{-2} in only 7 weeks of growth (i.e. 420–530 kg N ha^{-1}), equivalent to almost 10 kg ha^{-1} day^{-1} (Alazard and Becker, 1987). In a series of experiments in the Philippines, *A. afraspera* and *S. rostrata* yielded between 100 and 250 kg N ha^{-1} in 8 weeks (Becker *et al.*, 1990). *A. afraspera* is insensitive to the small variations in photoperiod over the year and gave consistent yields of N, whilst *S. rostrata* flowers early under short days and thus produced much less biomass (Rinaudo *et al.*, 1988), although it grew more rapidly than *A. afraspera* under long days (Becker *et al.*, 1990).

When *S. rostrata* was intercropped with alternating rows of multi-purpose cowpea, the amount of stover N (98 and 135 kg N ha^{-1}) and the amount of N_2 fixed (97–140 kg N ha^{-1}) were both equal to or greater than that of the sole green manure (McDonagh *et al.*, 1995b; Toomsan *et al.*, 2000). Yield of pods from the cowpea was slightly greater when it was intercropped with *A. afraspera* than with *S. rostrata*, but growth of *A. afraspera* was strongly reduced, indicating that it is less suitable for intercropping with a low-growing legume. Of the other green manure legumes that can be grown in lowland rice paddies, only *Sesbania cannabina* and *C. juncea* can match the rapid rates of accumulation in the stem-nodulated species (Lauren *et al.*, 1998). Within 45 days, accumulation of 225 kg N ha^{-1} has been recorded in *S. cannabina* and up to 169 kg N ha^{-1} in *C. juncea* (Meelu *et al.*, 1992b). An analysis of 222 measurements in rice-based cropping systems showed that legume green manures accumulated from 2 to 324 kg N ha^{-1}, with a mean value of 102 kg N ha^{-1} (Becker *et al.*, 1995a).

Estimates made using various methods indicate that a large proportion (80–95%) of the N in field-grown *Sesbania* or *Aeschynomene* green manures comes from N_2-fixation (Table 9.1). Exceptions are when the green manure does not grow very well, or where a large amount of nitrate is available in the soil when the green manure is planted (George *et al.*, 1998). The amounts of N_2 fixed within a short space of time can be very large – up to 286 kg N ha^{-1} in 55 days, representing a rate of N_2-fixation above 5 kg N ha^{-1} day^{-1}. The optimal conditions for N_2-fixation in rice paddies, with unlimited water and other nutrients supplied, are the underlying reasons for this amazing productivity.

Release of N and recovery by the rice crop

Release of N from *Sesbania* green manure in rice paddy soils can be very rapid. Over half of the N from *S. speciosa* was released in just 4 days in one experiment (Palm *et al.*, 1988). N release from *S. cannabina* was so rapid that yield of rice was less if the green manure was turned into the soil a week before transplanting compared with rice transplanted the day after incorporation of the green manure (Beri *et al.*, 1989a). More than 80% of the N in the green manure was released within 10 days of incorporation into soil in this case. N release from *S. rostrata* and *Indigofera tinctoria* is also rapid, whereas green manure from *Sesbania emerus*, which has more lignified tissues, releases N more slowly (Becker *et al.*, 1994b; Clément *et al.*, 1995). In fact, the leaves of *S. rostrata* have such little lignin that they disappear within 2 days,

whereas the stems take longer to decompose (McDonagh *et al.*, 1995a). Tissues of *A. afraspera* are more lignified than those of *Sesbania* and release N more slowly (McDonagh *et al.*, 1995a; Clément *et al.*, 1998).

These rapid rates of N release mean that much of the N is in mineral form, and therefore susceptible to being lost before it can be absorbed by the rice plants. Synchronization of the rate of N released with the N demands of the rice crop can be achieved by mixing *S. rostrata* green manure with rice straw (Becker *et al.*, 1994a; Becker and Ladha, 1997) and this has the potential to cut down N losses from the system completely. Despite the rapid release of N from legume green manures in flooded soils, recovery of the legume N by rice can be equivalent to or greater than those observed in upland soils. Several estimates have been made of N recovery from ^{15}N-labelled residues by rice under flooded conditions. Using this method, rice was found to recover 33% of the N supplied in an *S. cannabina* green manure but only 21% of the equivalent amount of N added in green manure of *C. juncea* (Rao and Shinde, 1991). Similar recoveries by rice of 20–29% of N were measured from green manure of *S. rostrata* or *A. afraspera* (McDonagh *et al.*, 1995b; George *et al.*, 1998; Toomsan *et al.*, 2000), whereas only 11–17% of the N from an *S. speciosa* green manure was recovered by rice (Seneviratne *et al.*, 1992). The efficiency with which N from *A. afraspera* green manure was utilized by the rice crop was found to be greater than that from *S. rostrata*, due to the slower rate of N release from the green manure (McDonagh *et al.*, 1995a).

Incorporation of green manures such as *A. afraspera* and *S. rostrata* increased rice yields in proportion to the amount of N added (Becker *et al.*, 1990), with yield increases of up to 2 t ha^{-1} to 100 kg N ha^{-1} in green manure. Fertilizer equivalency values for legume green manures range from 34 to 200 kg N ha^{-1} as urea, with the majority of values between 70 and 120 kg N ha^{-1} (Lauren *et al.*, 1998). In fact, the N from legume green manures supplied at rates up to 75 kg N ha^{-1} is used more efficiently by rice than N fertilizer supplied as urea (Becker *et al.*, 1995b; Clément *et al.*, 1998) (Fig. 9.2). If more than 100 kg N ha^{-1} is added in legume green manure, the efficiency with which the N is utilized for crop growth decreases rapidly compared with urea. The urea fertilizer is used more efficiently when supplied at high rates because the N is supplied in split doses, a method of management that is not feasible with a legume green manure. As up to 32% of the N in the green manure can be lost when larger amounts of N are added (George *et al.*, 1998), it is probably more sensible to add no more than 100 kg N ha^{-1} as green manure and to top-dress the crop with fertilizer. When large amounts of green manure N from *Sesbania* were incorporated, N benefits were found to be sufficient to increase the yield of a second crop of rice by 600 kg ha^{-1} in the Philippines (Morris *et al.*, 1989; Meelu *et al.*, 1992a) and in Senegal (Ndoye *et al.*, 1996). In general, however, little benefit from green manures is carried over into the second rice crop (Lauren *et al.*, 1998).

Yield responses of rice to legume green manures in northeast Thailand were much less striking, but the green manures still gave 1 t ha^{-1} greater yields than the unfertilized control, with large increases in production of rice straw (McDonagh *et al.*, 1995b). The main rice varieties grown in this region are glutinous or 'sticky' rice. These varieties are photoperiod sensitive and do not have the same capacity to

respond to N as the high-yielding varieties that are widely grown. If too much N is supplied in the green manure, these rice varieties are susceptible to lodging (Toomsan *et al.*, 2000).

Apart from the N contributed directly for the first crop of rice, a substantial amount of the legume N may remain in the soil in undecomposed roots and in the soil organic matter (George *et al.*, 1998). Over a 14-year period, during which two cycles of *S. rostrata* green manure followed by rice were grown each year, the soil N increased significantly while the soil N content remained constant in control plots cropped with unfertilized rice (Ladha *et al.*, 2000). In a series of long-term experiments in wet season rice followed by dry season wheat in India, green manuring with addition of mineral fertilizers sustained yields, whereas fertilizer alone could not prevent declines in yields of the wheat crop (Yadav *et al.*, 2000). These examples show that legume green manuring can be important to assure the long-term sustainability of crop production in intensive agriculture.

Legumes as nitrate catch crops

During the dry season, a large amount of mineral N can accumulate in the surface soil horizons in uncropped paddy fields due to mineralization of organic N in the soil. This can amount to more than 150 kg N ha^{-1} and is largely present in the form of nitrate (Buresh *et al.*, 1989; George *et al.*, 1993). This nitrate can easily be lost by leaching or denitrification if heavy pre-season rainstorms occur or when the soil is flooded for rice cultivation. More than 95% of ^{15}N-labelled nitrate fertilizer was lost from the soil by denitrification after only 9 days of flooding (Buresh *et al.*, 1989). Green manure crops of *S. rostrata* or *V. radiata* grown before the soil is flooded are able to absorb much of this mineral N and help to reduce N losses from the soil

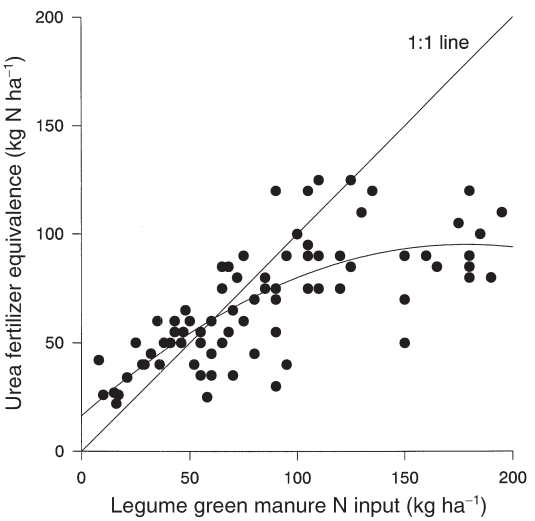

Fig. 9.2. The equivalent fertilizer value of N from green manures for production of lowland flooded rice in terms of N fertilizer applied as urea in two split-doses. (Redrawn from Becker *et al.*, 1995a.)

(Buresh *et al.*, 1993; George *et al.*, 1993). Weeds are also quite effective in reducing losses of nitrate from the soil but do not provide edible pods, or as much N for rice (George *et al.*, 1994).

In intensive vegetable gardens in the Philippines up to 700 kg N ha^{-1} of mineral N has been measured (Shrestha and Ladha, 1998). Not surprisingly, under such conditions *Indigofera tinctoria* fixed only 20% of its N, but it proved to be very effective at capturing nitrate, due to its deep root system.

Current use and problems of green manures

Much of the above evidence indicates that use of green manures could be highly beneficial. A survey of green manure use by rice farmers indicated that green manures are currently used by a small proportion of farmers in India, China and the Philippines (Garrity and Flinn, 1988). Most green manures are used in irrigated rice production; the most widely used species in China was *Astragalus sinicus*, whilst *Sesbania* spp. were used in parts of India and Bangladesh. In the Philippines, *I. tinctoria* is grown after rainfed rice throughout the dry season (often being sown initially as an intercrop with food or cash crops) and is then incorporated into the soil at the beginning of the wet season, prior to rice cultivation (Garrity and Flinn, 1988; Garrity *et al.*, 1994). Other species, such as *C. juncea* and *Vicia* spp., are also still used to some extent.

Where there is sufficient water for green manures to be grown at a time when they are not directly preventing growth of crops, the principal limitations to the adoption of green manuring in rice production derive from the inputs of labour required and also difficulties with propagation of the legumes. Legume green manures can be relay-planted into rice when time between crops is too short for pre-rice green manure (Torres *et al.*, 1995). Seed availability is often a problem with *S. rostrata* because, although plants produce large numbers of flowers and seeds, a large proportion can be damaged by insects. The use of stem cuttings has been suggested as an alternative to seed propagation (Becker *et al.*, 1988) but is likely to be too labour intensive to be practical. Biomass production can be stimulated by ratooning (cutting back) the plants after they have established and this is an additional way to reduce the amount of seed required initially (Becker *et al.*, 1988). Whatever method was used for establishment of the green manure, Becker *et al.* (1995b) concluded that mineral N-fertilizers were more economically attractive.

It should also be noted that in many experiments which demonstrate the benefits of green manures, phosphate fertilizers had been added to support the luxuriant growth of the green manure and in some cases the green manure had been irrigated. Without added phosphorus and potassium, growth of *S. rostrata* can be very poor (Engels *et al.*, 1995). Growth and N_2-fixation in an intercrop of *S. rostrata* and multi-purpose cowpea on a typical sandy soil in a farmer's field in northeast Thailand were poor unless lime, phosphorus and potassium were added (McDonagh *et al.*, 1995b) (Table 9.2). The green manure gave a substantial boost to yields of the subsequent rice crop only when it had been fertilized and a large amount of N had

Table 9.2. Amounts of N_2-fixation by *Sesbania rostrata* green manure intercropped with multi-purpose cowpea as affected by different fertilizer treatments, and the residual effect of these treatments on yield and N accumulation in rice. (From McDonagh *et al.*, 1995b.)

Pre-rice treatment	Fertilizer added	%N from N_2-fixation		Total legume N fixed (kg ha^{-1})	Stover N added (kg ha^{-1})	Grain yield of rice (t ha^{-1})	N uptake by rice (kg ha^{-1})
		S. rostrata	Multi-purpose cowpea				
Sesbania rostrata + multi-purpose cowpea	Lime + PK	88	61	84	84	2155	45
Sesbania rostrata + multi-purpose cowpea	PK	53	53	29	29	1685	30
Sesbania rostrata + multi-purpose cowpea	Lime	65	32	17	17	1610	30
Sesbania rostrata + multi-purpose cowpea	None	57	47	20	20	1450	26
Rice	PK	–	–	–	–	1335	25
Fallow	None	–	–	–	–	1245	21

been turned into the soil, clearly showing that N was the nutrient most limiting rice yields.

Economic analysis using partial budgets showed that the net benefits of using green manures in rice production were small or negative, whereas only small yields of grain legumes were necessary to give economic benefits (Ali, 1999). The major limitation of green manures is the large amount of labour required, and the disappointing conclusions of the economic analysis were reinforced by the opinions of Indian farmers. They considered green manures to be uneconomic, and stated problems with water, seed supply and incorporation of the green manure biomass as the major obstacles. As Becker *et al.* (1995b) indicated, the profitability of green manures depends on the relative prices (and availability) of fertilizer and labour.

Farmers in northeast Thailand showed considerable interest in the combination of *S. rostrata* and multi-purpose cowpea or stakeless variety of the yard-long bean as pre-rice intercrops (McDonagh *et al.*, 1995b). Pods of these legumes are highly prized as vegetables and the intercrop produces a similar green biomass, and benefit to rice yields, as the pure green manure. Although economic analysis indicates that this system is highly profitable (Whitmore *et al.*, 2000), there has been no spontaneous adoption by farmers in the villages where this grain legume/green manure combination was tested over the past 10 years.

Conclusions

One of principal reasons for the success of legumes as green manures and cover crops is their ability to grow in poor soils, which is of course in no small part due to their

ability to fix N_2 abundantly. It is unusual for green manures to be adopted solely for their beneficial effects on soil fertility, but where other benefits are also found – for instance, suppression of weeds, reduction of incidence of pests or control of soil erosion – then farmers may choose to use them (van der Heide and Hairiah, 1989). The spontaneous dissemination of mucuna through Central America, and its widespread adoption in Benin, were due to effective weed control and the labour that was saved as a result. A major problem for adoption is that the green manure legume must often occupy land at a time when other crops could be grown and therefore a 'cut and carry' system has been proposed in which the green manure is grown on land that cannot be readily used for agriculture and is subsequently brought to the fields (Wade and Sanchez, 1983). However, this practice adds still further to the other main problem restricting adoption of green manures: the extra investment of labour often required. For several decades, green manuring with mucuna or *C. juncea* formed the basis of intensive commercial agriculture in the southern USA and in Zimbabwe (Rattray and Ellis, 1952; Buckles, 1995b). Two significant factors coincided with the move away from growing green manures: the promotion and ready availability of mineral fertilizers, and new varieties of soybean that became widely grown as a highly profitable oilseed crop.

The area of land sown to green manures in Asian rice production has declined since the 1950s. Various factors are involved, including the demands on labour and the preference of farmers for growing food crops rather than devoting their land to production of green manures (Garrity and Flinn, 1988). The ready availability of N-fertilizers is certainly a major factor that has contributed to the reduction in the area sown to green manure. Fertilizer use allows the farmer time for other tasks (or to earn income elsewhere) and can lead to production of cereal yields greater than those possible with green manures alone. However, the arguments concerning adoption of green manures (also discussed in Chapters 7 and 15) may be quite different in other regions of the tropics where fertilizers are beyond the reach of most small-scale farmers.

A possible compromise, in both upland and lowland cropping systems, is the use of legumes that can provide leaves, young pods as vegetables, or grain (e.g. cowpea or *Canavalia*) and still give sufficient green leafy material to be useful as a green manure.

Chapter 10

Forage Legumes in Pastures and Leys

Savannah – that is, vegetation with a large component of grasses – covers a huge area of the tropics. Savannahs used for animal production are commonly restricted to the more acid and infertile soils, or to regions where there is insufficient rainfall for crop production. The productivity of tropical pastures is often limited by the infertility of the soils; in particular, the lack of N in the fodder leads to protein deficiency in the livestock. Native savannahs can maintain a stable but low productivity and two main approaches are available for increasing output: an intensive approach, in which fast-growing grasses such as elephant grass (*Pennisetum*) are grown with heavy dressings of N and other fertilizers and are fed to cattle together with food concentrates; and an extensive, low-input approach, which relies on the selection of grasses and legumes that can grow in infertile soils with little addition of fertilizers. It is in the second, extensive approach that legumes play a crucial role by contributing N to the grazing system by N_2-fixation and this approach will be discussed here. Given the costs and lack of availability of fertilizers and the inaccessibility and scale of the savannahs, if pasture productivity is to be improved inclusion of legumes would seem to be the only feasible option, but as yet they have not been widely adopted.

Research into the use of legumes in tropical pastures was initiated in Australia, where it has a long tradition, as it does also at a few research centres in Africa. Research by the Centro Internacional de Agricultura Tropical (CIAT) in Latin America and the International Livestock Research Institute (ILRI) in various African countries has been focused on improving animal production in the tropics. The main role of the legume is to improve fodder quality, as forage legumes are rich in N and provide an extra source of protein for the grazing animals, particularly during the dry season when the grasses are poorly nutritious. At the same time the legume can contribute to the overall N economy of the pasture through N_2-fixation, giving rise to an increased N content of the associated grasses. Tropical pasture can be managed

in rotation with cropping in ley systems. Forage legumes can also be grown in pure or mixed stands as 'fodder banks', which the animals are allowed to graze periodically, or from which the forage can be cut and fed to animals.

Detailed background texts on tropical pastures and forages include Crowder and Chheda (1982) and Humphreys (1991, 1994, 1997). Bogdan (1977) and Skerman *et al.* (1988) are mines of information on tropical pasture legumes and grasses. Legumes more commonly recognized as agroforestry trees (Chapter 12), green manures (Chapter 9) and grain legumes (Chapter 8) can also provide fodder and feed supplements for animals and these uses are discussed in the relevant chapters.

Tropical Pastures

The vegetation on land used for grazing varies from open grassland savannah, with almost no trees, to densely wooded savannah. It is estimated that savannahs cover 65% of Africa, 60% of Australia, 45% of South America and roughly 10% of the Indian subcontinent and 10% of Southeast Asia; some 23 million km^2 in all (Cole, 1986). In most savannah areas there is at least one pronounced dry season during the year. Here the term 'pasture' is used loosely to encompass all grassland and rangeland used for grazing.

Distribution of tropical savannah

Africa

Wooded savannah covers much of the wet-and-dry to the dry regions of Africa. There are many regions of 'derived' savannahs with sparse tree cover as a result of forest clearance – for instance, in the Guinea zone of West Africa. Thorny trees, especially *Acacia* species, characterize the sparse tree and shrub savannahs of the drier regions. In the highlands of eastern Africa, from Ethiopia through Kenya to parts of Tanzania and Uganda, lie large tracts of temperate grasslands, which are the centre of diversity for the African clovers (*Trifolium* spp.) and for many of the grasses used in tropical pasture improvement (see below). Much of the natural vegetation of southern Africa is open savannah woodland dominated by the non-nodulating legume species of the genera *Brachystegia* and *Julbernardia* (Campbell, 1996). Overall more than half of the land area of Africa is covered by vegetation in which grasses are a major component. However, the susceptibility of cattle to sleeping sickness restricts the use of large areas of African savannahs for grazing where the tsetse fly (*Glossina*) is present.

Central and South America

The largest areas of grasslands in this region are the cerrados of Brazil and the llanos of eastern Colombia and Venezuela. The Brazilian cerrados consist of densely wooded savannah, which covers much of central Brazil, and of grasslands with fewer trees and shrubs to the east and south of the country. The Llanos del Orinoco in

Colombia and Venezuela, and the campos of northern Brazil and southern Guyana, are largely treeless, apart from the gallery forests that stretch along the many rivers that dissect the savannah. Five main agroecological zones have been identified within the central lowlands of tropical South America that could be developed for tropical pasture use: poorly drained savannah; isohyperthermic savannah; isothermic savannah; semi-evergreen seasonal forest; and tropical rainforest (Cochrane *et al.*, 1985). Dry savannah with sparse shrubs stretches from the north of Mexico into the southern parts of North America.

Asia

Much of the uncultivated land of the Indian subcontinent is covered by sparse grasslands with few shrubs, largely resulting from earlier clearance of woodland and forest. In Southeast Asia there are some natural savannahs but the majority of the grassland is marginal land that has become dominated by star grass (*Cynodon* spp.) or alang-alang (*I. cylindrica*) as a result of forest clearing and burning. These *Imperata* grasslands are very important for animal production in Southeast Asia (Falvey, 1981). The understorey vegetation in coconut, oil palm and rubber plantations is commonly used as pasture in Asia and the Pacific islands (Chapter 11).

Australia

An enormous area of open and wooded grasslands covers the north and northeastern parts of Australia. The naturally occurring grasses will not support heavy grazing with livestock and large areas have been replaced by sown pastures.

Types of grazing systems

In many parts of the tropics most livestock is kept within agricultural systems based on arable farming in which animal densities can be very high (Ruthenberg, 1980). Animals in small-scale arable farming systems may be kept inside buildings and fed on crop residues and on harvested forage in 'cut-and-carry' systems, or may graze communal lands and fallowed fields (Humphreys, 1991). Extensive grazing systems occupy large areas of land, often where rainfall is insufficient for intensive arable agriculture, and are known as 'rangelands' (Anon., 1990). Where rainfall is very sparse, only nomadic stock-keeping is feasible and the herders have no permanent place of residence (Table 10.1). With more rainfall, semi-nomadism is practised where some cultivation is carried out around settlements that may be kept for several years, but the animal keepers travel with their herds for long periods to find grazing. Partial nomadism is a term used to describe arable farming in permanent settlements where cattle graze on communal lands, whilst ranching is distinguished by the land being under the ownership of a single stock-keeper and the land is normally fenced. Fodder banks have been adopted mainly in areas of partial nomadism. In essence, ranching is a commercial alternative to nomadism (Ruthenberg, 1980). Ranches vary enormously in size. In the Colombian llanos or the cerrados of Brazil, ranches are commonly 500–20,000 ha, whilst in Central America the typical ranch size is only

Table 10.1. Types of livestock production systems in the tropics. (Adapted from Ruthenberg, 1980.)

Rainfall (mm year^{-1})	Predominant type of grazing system	Main animal kept
Under 50	Occasional nomadic stock keeping	Camels
50–200	Nomadism with long migrations	Camels
200–400	All types of nomadism, transhumance and supplementary animal farming	Cattle, goats, sheep
400–600	Semi-nomadism, transhumance and partial nomadism with stronger emphasis on arable farming	Cattle, goats, sheep
600–1000	Transhumance and partial nomadism	Cattle
Above 1000	Partial nomadism and permanent stock-keeping (ranching)	Cattle

20 ha (CIAT, 1989). Most ranching in the tropics is for meat production, due to the large distances between grazing lands and major markets, but in some areas, such as the llanos of Venezuela and the Brazilian cerrados, milk may become an important part of the produce (Toledo, 1985).

In ranching, several different systems of grazing can be adopted. Grazing regimes can vary from continuous stocking of unimproved savannah or improved pastures, to alternating or rotational systems where the animals are moved between one or more small areas of improved pasture and larger areas of unimproved savannah. Current estimates of the area of improved pastures are hard to find. In the late 1980s some 20% of the Brazilian cerrados were sown to improved pastures, whilst in the eastern llanos of Colombia 90% of farms had improved pasture (CIAT, 1990). This amounted to 90,000 ha, or 13% of the land area, although only 8% of this area was planted with legume/grass mixtures. Pastures are also now part of zero-tillage systems that occupy over 10 million ha in Brazil.

Approaches to improvement of pastures and rangeland

The productivity of pastures is measured both in terms of the output (e.g. gain in liveweight) of each animal and as the output per unit area of land (Fig. 10.1). In unimproved savannah in Colombia the liveweight gain was less than 20 kg ha^{-1} year^{-1}, whilst on improved pastures that had been sown with the grass *Andropogon gayanus* the liveweight gain was increased to 236 kg ha^{-1} year^{-1}. Animal productivity of pastures sown with legume/grass mixtures was even greater at 280–290 kg ha^{-1} year^{-1} (Fig. 10.1) and milk production was also greater in animals grazed on legume/grass mixtures than on pure grass swards (Fig. 10.2). On unimproved pastures in Colombia the stocking density is typically only one animal for every 2–5 ha (0.2–0.5 animal units (AU) ha^{-1}) which can, on improved pastures, be increased to 0.4–1.0 AU ha^{-1} in the dry season or to 1–3 AU ha^{-1} in the wet season (Paladines and Leal, 1979).

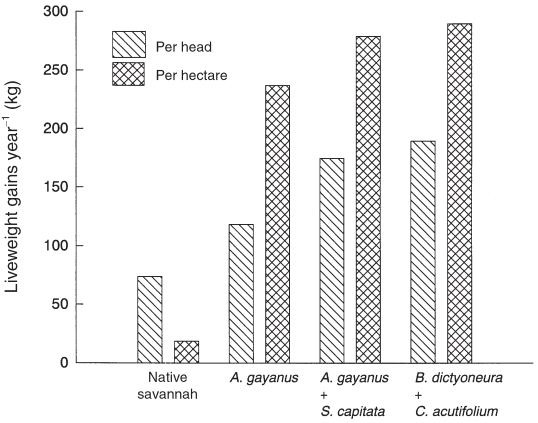

Fig. 10.1. Productivity of cattle grazed on native savannah and improved pastures of the grasses *Andropogon gayanus* or *Brachiaria dictyoneura* in a pure grass sward or in mixtures with the legumes *Stylosanthes capitata* or *Centrosema acutifolium*, at Carimagua in the Colombian llanos. (Redrawn from CIAT, 1990.)

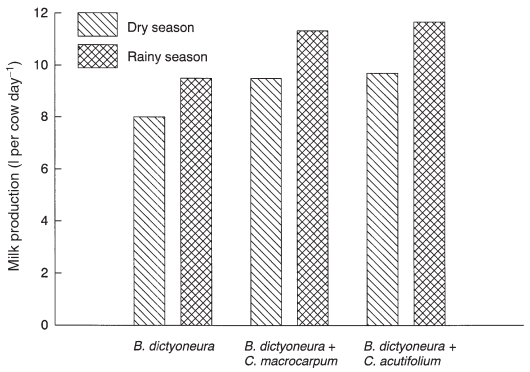

Fig. 10.2. Milk production from different pastures at Quilichao, Colombia, consisting of an improved grass species (*Brachiaria dictyoneura*) alone or in mixtures with species of *Centrosema*. (Redrawn from CIAT, 1990.)

Three low-input approaches to pasture improvement using legumes have been recognized ('t Mannetje, 1986): (i) oversowing of a legume into existing pasture; (ii) replacement of the existing vegetation with a mixture of an improved grass and a legume; and (iii) establishment of a legume monoculture (or protein bank) in association with existing pasture or with pasture that has been improved as in (i) or (ii). Thus, in each case the improvement of pastures is dependent on the introduction of new species of legumes and it often requires the introduction of new grasses as well. Africa has proved to be a major source of grasses for tropical pasture improvement. *Brachiaria*, *Cenchrus*, *Chloris*, *Cynodon*, *Panicum*, *Setaria* and *Urochloa* have been widely distributed from their centres of diversity in East Africa, *Andropogon* from West Africa and *Digitaria* and other species of *Urochloa* from southern Africa. *Hyparrhenia rufa* occurs widely in Africa and is also found in South America, where it is not certain whether it is a native species or whether it results from an early accidental introduction from Africa (Bogdan, 1977). Many of the improved grass species of the genera *Axonopus*, *Bromus*, *Paspalum*, *Sorghum* and *Tripsacum* originate from subtropical South America.

The main source of legumes for tropical pasture improvement has been Central and South America, although some useful species have been identified in Africa and

Southeast Asia. Within each species there is often considerable variability between ecotypes, which can be exploited in selection of types with favourable characteristics for pasture improvement. Given the enormous variability in environments and pasture production systems, a decentralized approach has been recommended for selection of well-adapted plants for specific ecosystems (Toledo, 1985). There is still much unexploited variability in the genetic resources of pasture legumes both in new species not yet identified or tested and in different ecotypes of species already recognized as having potential.

Tropical Pasture Legumes

Legume evaluation

What criteria are used to select a legume for pasture improvement? The primary concern is that the legume should give a large yield of good quality fodder that is rich in protein, but it is equally important that the legume should be able to establish and persist in the sward. Thus the legume must not only be well adapted to the environment in a given region, but must also be compatible with the dominant grasses in the pasture under grazing, whether they are constituents of the native savannah or selected species sown at the same time as the legume. Survival of the legume is dependent not only on competition for resources such as light, moisture and nutrients, but also on its palatability and on the selectivity of the grazing animal for different species within the sward. It is important that the legume is not too palatable – otherwise it will be quickly eliminated by grazing. The ability to regrow rapidly following defoliation will also strongly influence persistence, and tolerance to fire is important in areas managed by burning. In general, climbing or shrubby legumes (e.g. *Centrosema* spp., *Pueraria*) are most suitable for introduction with tall grasses such as *Panicum*, and low-growing stoloniferous or rhizomatous legumes (e.g. *Arachis pintoi*, *Stylosanthes* spp.) are most suitable for mixing with spreading grasses such as species of *Brachiaria*.

As a low-input approach is the only feasible strategy for pasture improvement in most of the tropics (Toledo, 1985), legumes selected must be able to yield well and persist with minimal fertilizer additions, or preferably without their use. An added advantage of some species is that they can extend the period of grazing into the dry season, either by remaining green or simply by the dry leaves providing better quality feed than the grasses. It is essential that seed production of the legume is not a problem, so that it is easy to propagate and distribute, although, once established, many biennial and perennial legumes persist by rooted stolons.

The philosophy of scientists at CIAT towards screening of legumes for introduction into tropical pastures is to select germplasm that is adapted to low soil fertility and is resistant to the major pests and diseases (Thomas *et al.*, 1995; Vera, 1998). This is achieved by screening accessions or breeding lines with minimal use of fertilizers and with no disease control in multi-locational trials using standard cultivars or commercial materials as checks. Further selection is then conducted under grazing of

the grass/legume mixture, as early work in which grasses and legumes were selected for biomass production under clipping (meant to simulate grazing) led to the selection of species that could not persist, or that yielded poorly, under actual grazing. A flexible grazing system has been proposed for the evaluation of grasses and legumes in which the stocking rates and rest periods of the pasture are adjusted in response to the production and legume content of the pasture, thus ensuring persistence of the legume in the pasture (Spain *et al.*, 1985; Fisher *et al.*, 1996). The length of time spent grazing the animal on the improved pasture is determined by the liveweight gain of the animal and the proportion of the legume forage in the mixed sward (Spain *et al.*, 1985).

At CIAT, substantial emphasis was placed on selection of well-adapted rhizobia for the legumes (see below, e.g. Sylvester-Bradley and Kipe-Nolt, 1990), and a large collection of more than 4000 rhizobial strains for tropical forage legumes is held at CIAT, Colombia (Franco *et al.*, 1993). By contrast, legume screening for West Africa, conducted mainly in Nigeria by ILRI, concentrated on identification of legumes that would nodulate readily with indigenous rhizobia, to avoid the need for inoculants (S.A. Tarawali, 1999, personal communication). These alternative strategies are discussed further in Chapter 14. Although rhizobia isolated from nodules of many of these forage legumes are generally strains of *Bradyrhizobium*, promiscuous fast-growing rhizobia can form effective nodules with many of them (e.g. Pueppke and Broughton, 1999). In addition the rhizobial species *R. hainanense* was recently proposed to include fast-growing strains isolated from nodules of *Centrosema*, *Desmodium*, *Stylosanthes* and other legumes in Hainan, China (Chen *et al.*, 1997).

Some important legumes for pasture improvement

Many legumes are present in savannahs – in fact, most of the legumes selected for introduction to improve pasture productivity occur naturally in savannah vegetation and only a few have been specifically bred. However, most of the herbaceous legumes found in savannahs are small, have low productivity and are of poor quality for grazing, many being relatively unpalatable.

Given the lack of available soil N in tropical pastures, the ability to fix large amounts of N_2 is an essential requirement for production of protein-rich fodder and it is perhaps not surprising that all of the legume species selected for pasture improvement are able to nodulate. Almost all of the species with potential are members of the *Papilionoideae*, with the exception of species such as *Leucaena* and *Desmanthus*, which belong to the *Mimosoideae*, and *Chamaecrista* (syn. *Cassia*) *rotundifolia*, a nodulated species of the *Caesalpinioideae*. Here some of the legumes that show potential for improvement of tropical pastures are described, but it is important to realize that only a fraction of the possible pasture legume species have been evaluated. The genera considered are ones for which there is at least some information on rhizobia, nodulation and N_2-fixation.

Aeschynomene

Several species of this pan-tropical genus – *Aeschynomene americana*, *A. falcata*, *A. histrix* and *A. indica* – produce useful forage (Bishop *et al*., 1988). *A. histrix* was among the most prolific biomass producers of legumes tested in subhumid Nigeria (Peters *et al*., 1994; Tarawali, 1994). *Aeschynomene* spp. are generally well adapted to wet conditions and survive periods of waterlogging. *A. afraspera* forms stem nodules (Chapter 2) and is a useful green manure for rice (Chapter 9). *Aeschynomene* spp. are nodulated by rhizobial species that have been found to fall into three cross-inoculation groups with respect to host range (Alazard, 1985). When originally examined these rhizobia appeared to be intermediate between fast- and slow-growers, having generation times of 5–8 h (Alazard, 1985), but those for which 16S rRNA sequences have been looked at have proved to be *Bradyrhizobium* strains, including the photosynthetic strain that nodulates *A. indica*, BTAi1 (Young *et al*., 1991).

Arachis

This genus, best known for the groundnut (*A. hypogaea*), is native to Brazil (see Chapter 8). Of particular interest for use in tropical pastures is *A. pintoi*, which is a relatively new discovery compared with other forage groundnuts such as *A. glabrata* and *A. repens* (Kerridge and Hardy, 1993). *A. pintoi* is a perennial native to the central and coastal regions of Brazil and is nodulated by *Bradyrhizobium* (Sylvester-Bradley *et al*., 1988a; Thomas, 1993). It combines well with *Brachiaria* grasses and is resistant to heavy grazing, part of its success in persistence being due to the underground production of seeds (Grof, 1985; Ibrahim and 't Mannetje, 1998).

Calopogonium

Of the 12 tall, climbing species of *Calopogonium* that occur throughout tropical America and the West Indies, *C. mucunoides*, *C. orthocarpum* and *C. caeruleum* have been used in agriculture, mainly as cover crops in plantations (Chapter 11). *C. mucunoides* shows promise for use as a forage legume in *Brachiaria* pastures of central Brazil (Seiffert *et al*., 1985), whereas *C. caeruleum* is not palatable. As *C. mucunoides* is not particularly palatable, it is largely ignored by animals until the dry season, when it becomes an important part of the diet. *Calopogonium* species are nodulated by *Bradyrhizobium* strains (Allen and Allen, 1981).

Centrosema

Several of the 33 species of *Centrosema* have proved to be very useful pasture legumes (Williams and Clements, 1990). The genus has a wide distribution in tropical and subtropical America and *C. pubescens* was the first species to be widely used for pasture improvement, due to its productive growth, good herbage quality and ability to combine well with grasses (Clements *et al*., 1983). Evaluation of collections of *Centrosema* in the Colombian llanos has led to identification of three species well adapted to the acid soils: *C. brasilianum*, *C. macrocarpum* and *C. acutifolium* (Grof, 1986). *C. arenarium* is poorly palatable and has proved to be unsuitable for grazing (Grof, 1991). *C. macrocarpum* produces high yields under cutting but does not persist under grazing, due to high palatability and poor seed set, whilst prolific seed

production in *C. brasilianum* ensures better survival under grazing. *C. acutifolium* has a more stoloniferous growth habit that enables it to persist under heavy grazing; it is more resistant to pests and diseases, and consistently yielded more forage under grazing trials than *C. brasilianum* and *C. macrocarpum* (Thomas and Grof, 1986b). *C. pascuorum* is useful for short-term pastures in West Africa and Australia (Tarawali and Peters, 1996), and a number of *C. brasilianum* accessions are well adapted in subhumid West Africa, remaining green into the dry season (Peters *et al*., 1998).

Rhizobial strains isolated from nodules of *Centrosema* species are invariably *Bradyrhizobium* strains (Sylvester-Bradley *et al*., 1990) but ineffective nodules can be formed when *Centrosema* is inoculated in the laboratory with fast-growing isolates (Trinick, 1980). There are marked differences in cross-compatibility between *Bradyrhizobium* strains and *Centrosema* genotypes, with some strains being highly effective on one species but completely ineffective on another (Miranda *et al*., 1985; Sylvester-Bradley *et al*., 1990).

Chamaecrista

Only one member of this large, pantropical genus has been widely used for forage. *Chamaecrista rotundifolia* from Brazil was well known as *Cassia rotundifolia* until Irwin and Barneby (1981) revised the taxonomy of this tribe, such that all nodulating species now fall into the genus *Chamaecrista* (Chapter 2). Accessions of this species range from prostrate, creeping forms to large bushes up to 1.5 m tall and are successful in a wide range of environments. Little seems to be known about rhizobia for *Chamaecrista*, but it is able to nodulate with fast- and slow-growing strains.

Desmodium

Members of the genus *Desmodium* are widespread throughout the warmer climates of the New and Old Worlds. Two species with good potential in South American savannahs, *D. ovalifolium* and *D. heterophyllum*, are from Southeast Asia. In Australia, *D. intortum*, *D. uncinatum* (which both have relatively high phosphorus requirements) and *D. heterophyllum* have been the most widely used species. These species are creeping herbaceous perennials that are resistant to heavy grazing, but some of the approximately 80 members of the genus are woody perennials (Imrie *et al*., 1983). *D. ovalifolium* is well adapted to acid soils, and to regions with very high rainfall (> 2000 mm $year^{-1}$) and short dry seasons, and it can withstand periodic flooding (Thomas and Grof, 1986b). The stoloniferous, perennial habit of *D. ovalifolium* allows it to combine well with aggressive short grasses such as *Brachiaria* spp. as well as with vigorous tufted species such as *A. gayanus*. A disadvantage of *D. ovalifolium* is its high content of tannins, such that cattle will not eat it on their first encounter (Thomas and Grof, 1986b). *Desmodium* species are nodulated by *Bradyrhizobium* but require specific strains for effective nodulation (Imrie *et al*., 1983; CIAT, 1989).

Lotononis

Lotononis bainesii is a southern African species initially collected in Zimbabwe which has been widely used in Australia. It is highly specific in its rhizobial requirement and

requires inoculation when sown outside its natural range; perhaps for this reason *L. bainesii* is said to perform rather erratically. Rhizobial isolates from nodules of *Lotononis* are said to be very slow-growing, but the full diversity of rhizobia that may nodulate this host has not been explored.

Macroptilium

Siratro, *M. atropurpureum* (syn. *Phaseolus atropurpureus*), was bred in Australia in the early 1960s and has since been grown throughout the tropics, as it is adapted to a wide range of soils and combines well with many grasses (Whiteman, 1980). It is best suited to the warm tropics in areas receiving more than 750 mm annual rainfall, but is susceptible to fungal pathogens in very wet climates. Siratro is commonly used as a trap host for isolating or counting *Bradyrhizobium* strains in soil (Vincent, 1970), though it can be nodulated by both slow-growing and fast-growing rhizobia, and is in fact more selective in its strain compatibility than widely believed (Lewin *et al.*, 1987). Other useful species such as *M. gracile*, *M. lathyroides* and *M. bracteatum* also nodulate freely.

Macrotyloma

Members of this genus are indigenous to Africa, Asia and South America, and include two grain legumes: Kersting's groundnut (*M. geocarpum*) and the horse gram (*M. uniflorum*) (Chapter 8). *M. axillare*, *M. lathyroides* and *M. uniflorum* have been used widely in Australian pastures and elsewhere, as they are relatively free of diseases. They nodulate freely with *Bradyrhizobium*.

Pueraria

Tropical kudzu (*P. phaseoloides*) is a vigorous species indigenous to Southeast Asia, from where it has been widely distributed. It is adapted to the wet tropics, with poor tolerance of drought (Grof, 1991), and is useful for pasture improvement but its high palatability can lead to its rapid disappearance due to selective grazing. It is nodulated by *Bradyrhizobium* strains from many different legumes but the most effective strains were isolated from kudzu itself (Sylvester-Bradley *et al.*, 1991).

Stylosanthes

The genus *Stylosanthes*, which contains about 30 species that occur throughout the tropics, has been an important source of plants for pasture improvement across a wide range of climates (de Leeuw *et al.*, 1994). Townsville stylo (*S. humilis*) is an annual species which has become one of the most widely used legumes in Australia, where it was first found in 1913, apparently as a chance introduction (Bogdan, 1977). It establishes easily and grows well on sandy, infertile soils and provides a high-quality fodder. Several different forms of *S. guianensis* are recognized that represent adaptations within the species to a wide variety of climatic conditions (Thomas and Grof, 1986a). *S. guianensis* is now used throughout the tropics. *S. hamata* and *S. scabra* are species that are well adapted to drier regions. *S. capitata*, *S. macrocephala* and the 'tardío' type of *S. guianensis* have been found to be good species for pasture improvement in South American savannahs (Thomas and de

Andrade, 1984; Thomas and Grof, 1986a). *S. guianensis* and *S. hamata* are the legumes most widely used in fodder banks in West Africa (Elbasha *et al.*, 1999). A number of useful accessions of *S. hamata* have been identified which stay greener in the dry season in West Africa than the commonly used cultivar 'Verano' (Tarawali, 1995).

Whilst all species of *Stylosanthes* are nodulated by *Bradyrhizobium* spp. the degree of specificity varies enormously, both between species and between cultivars of the same species in the case of *S. guianensis* and *S. humilis* ('t Mannetje, 1969; Edye *et al.*, 1974). The specificity found depends partly on the strains against which the plants are tested. For example, accessions of a fine-stemmed variety of *S. guianensis* only nodulated with a small proportion of strains tested in one experiment ('t Mannetje, 1969), but were effective with a wide range of strains isolated from soils where the plants were collected (Date and Norris, 1979). This observation fits well with the idea that centres of diversity for legume species are also centres of diversity of compatible rhizobial strains (Chapters 2 and 8) (Lie *et al.*, 1987).

Variation in symbiotic effectiveness with different strains was found to be particularly strong between accessions of *S. guianensis* and *S. hamata* (Date and Norris, 1979) and *S. capitata* (Date, 1984). An attempt to classify rhizobial strains by their ability to fix N_2 with 336 different *Stylosanthes* genotypes gave rise to six groups within the 22 strains tested (Date and Norris, 1979), but different results might be expected if a wider range of strains isolated from *Stylosanthes* was examined. Some accessions of *Stylosanthes* from specific environments – for instance, from alkaline soils in dry regions – were nodulated only by *Bradyrhizobium* strains isolated from the same environment (Date *et al.*, 1979). Other strains could only be isolated from *S. capitata* and *S. guianensis* growing on acid Oxisols when an acidified culture medium was used (Date, 1984).

Trifolium

The clovers deserve mention here as they are useful pasture species for higher altitudes in the tropics. Eastern Africa is an important centre of diversity for the genus *Trifolium*, many species of which have a very specific requirement for *Rhizobium* and only nodulate with strains isolated from the same species (Norris and 't Mannetje, 1964; Lupwayi *et al.*, 1997). Kenya white clover (*T. semipilosum*) and the temperate species white clover (*T. repens*) are the two clovers most important as pasture species (Wheeler and Jones, 1977).

Zornia

Some perennial species of *Zornia*, a genus of roughly 75 species found in the tropical savannahs of America and Africa, show promise for pasture improvement. *Zornia* species are well adapted to acid soils high in aluminium and manganese and give good seed yields (Thomas and Grof, 1986b). There is interest in use of three species in Latin America, namely *Z. glabra*, *Z. latifolia* and *Z. brasiliensis*, although disease problems and poor acceptability for grazing may limit their use. *Zornia* species are nodulated by *Bradyrhizobium* strains.

Other legumes

A wide range of other herbaceous legumes have been used in pastures or as forage, including grain and green manures. These include *Alysicarpus* spp. (Gramshaw *et al.*, 1987), *Atylosia* spp., *Clitoria ternatea*, *Galactia* spp., *Lablab purpureus*, *Mucuna* spp., *Neonotonia wightii* (syn. *Glycine wightii*), *Teramnus* spp. (Pengelly and Eagles, 1996) and many species of *Vigna*. In addition to cowpea (*V. unguiculata*), the lower-growing *V. decipiens*, *V. oblongifolia*, *V. trilobata* and *V. vexillata* seem to have promise as forage (Hacker *et al.*, 1996).

Requirements for inoculation

The commonly used pasture legumes have been classified according to their responses to inoculation with standard strains of rhizobia from culture collections (Date, 1977; Bushby *et al.*, 1986) in order to give guidance about the need to inoculate. The legumes were divided first on whether they nodulated freely with a range of strains (i.e. were promiscuous) or did not (i.e. were specific). If they did nodulate freely they were further divided into those that normally formed effective nodules and those that often formed ineffective nodules. Most, but not all, of the promiscuous, effective (PE) group were nodulated mainly by *Bradyrhizobium* strains whilst species in the promiscuous, ineffective (PI) group were nodulated either by *Bradyrhizobium* or by fast-growing rhizobia. Inoculation was thus recommended for the PI group and was considered essential for members of the specific (S) group.

Whilst this provides a useful framework for giving advice to farmers on the necessity of inoculation, there are a number of shortcomings to the approach. A major one is that classification of legumes as specific or promiscuous can depend as much on the particular range of rhizobial strains tested as anything, and attaching a label can fix a particular point of view in people's minds. For example, as mentioned above, siratro is not as promiscuous in its nodulation capacity as commonly believed. Moreover, as Date (1977) indicates, even legumes from the PE group respond to inoculation if the compatible strains of rhizobia present in the soil are less effective (and competitive) than the strain(s) used in the inoculum.

In agreement with these points, selection of adapted, competitive strains for *P. phaseoloides* and *C. macrocarpum* – pasture legumes that were said to belong to the PE group – has led to substantial responses to inoculation in the field which persisted into the second year (Fig. 10.3) (Chapter 14) (Sylvester-Bradley, 1984). Similar results have been obtained with *A. pintoi* (Sylvester-Bradley *et al.*, 1988a) and consistent responses were found when strains of *Bradyrhizobium* selected in this way at CIAT were evaluated in the field in Brazil, Colombia, Mexico, Panama, Peru and Venezuela (Sylvester-Bradley and Kipe-Nolt, 1990). The benefit of inoculation is the stimulation of more rapid and vigorous early growth, which is a critical phase to ensure successful establishment of the legume. The strain CB756, which has long been a recommended strain for legumes of the PE group (Date, 1977), was found to be of only moderate effectiveness in comparisons of a large range of strains selected for *Pueraria*, and gave no significant increase in yield in pasture soil above the

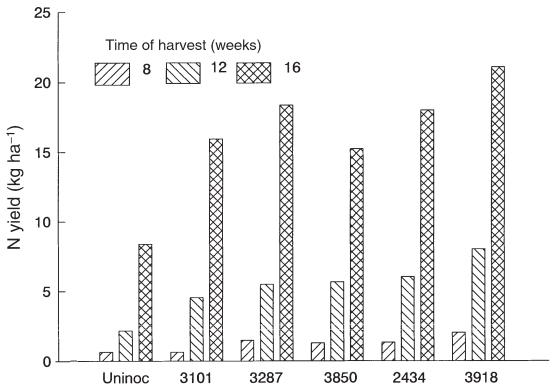

Fig. 10.3. Nitrogen yield of *Pueraria phaseoloides* without inoculation, or inoculated with strains of *Bradyrhizobium* (CIAT strains 3101, 3287, 3850, 2434, 3918) in the field in the Colombian llanos. (Sylvester-Bradley *et al.*, 1991.)

uninoculated control. In fact, it appears that there are few published reports of field trials with strain CB756 in which responses to inoculation were found (Date, 1991; Sylvester-Bradley *et al.*, 1991). If populations of compatible and effective rhizobia are small the legume may respond to inoculation with CB756, as was the case with *D. intortum* reported by Date (1991). However, the numbers of rhizobia in the uninoculated plots gradually build up so that differences in plant growth due to inoculation gradually disappear (Date, 1991, 2000).

To date, experiments with *Stylosanthes* have generally failed to identify strains that consistently give inoculation responses. With *S. capitata*, growth of the plants was stimulated by addition of N-fertilizers, but *S. guianensis* failed to respond to added N, indicating that N was not the limiting growth factor (Sylvester-Bradley, 1984). In Thailand, the inoculant strains used only formed a small proportion (10–33%) of the nodules on *S. humilis* and *S. hamata* (Homchan *et al.*, 1989b), indicating that selection of more competitive and effective strains is warranted. The strains that are often recommended for use with legumes have rarely been subjected to rigorous comparison with other strains under conditions that reflect those likely to be encountered in the field. As such strains have been used for drawing up the classification of tropical pasture legumes, it is hardly surprising that exceptions to the predicted responses to inoculation are readily encountered when selection of adapted, competitive and effective strains has been carried out. Given current knowledge of the potential for strain selection for pasture legumes, it may be better to abandon this classification altogether.

But with what should it be replaced? It is useful to know whether a legume is likely to nodulate when introduced into a new region, and an idea of whether the legume is highly specific or wildly promiscuous in its nodulation will give an indication of this. The problem then is the choice of strains used to develop such a classification. Any classification will obviously depend on the specificity or promiscuity of the strains used to generate it (for example, see the discussion of *Stylosanthes* rhizobia above). Until there is a fuller knowledge of the rhizobia present in tropical soils, it will not be possible to resolve this question.

Legume establishment and maintenance

The benefits of inoculation and N_2-fixation can be particularly pronounced in the establishment of legumes in native savannahs, or in pre-established grass swards, when competition with grasses may be severe. Various methods can be used to establish legumes in pastures and the applicability of the methods will depend on the availability of inputs such as machinery or labour. Clearing of land (and trees) and superficial cultivation generally give the best establishment but this obviously requires significant inputs and is not possible for large areas. Burning of the savannah before sowing or other mechanical methods of 'scarifying' the pasture can aid establishment. *S. guianensis* was successfully established in *Imperata* grassland dominated by alang-alang (*I. cylindrica*) by oversowing following burning (Chadokar, 1977). Heavy grazing prior to sowing has been shown to be a useful alternative to cultivation or burning for establishment of *Desmodium* spp. in Kenya (Keya *et al.*, 1971) but in Thailand the lack of persistence of *S. humilis* was attributed to heavy grazing during establishment (Shelton and Wilaipon, 1984). Climbing legumes certainly need to be allowed to flower and set seed initially (and periodically thereafter) without too severe grazing if they are to persist. Oversowing of legumes into native grassland is an attractive, low-input method but success is dependent on the moisture status after sowing and on competition from the existing vegetation. In the llanos, sowing of the legumes into fertilized strips from which the savannah has been cleared mechanically has proved to be an effective method for legume establishment. A similar approach to 'veld reinforcement' has been used successfully to introduce a range of legumes into grass savannah in southern Africa (Clatworthy *et al.*, 1986; MacLaurin and Grant, 1987).

Seeds of many legumes have a hard coat, which causes a mechanical dormancy. The dormancy is an important adaptation that allows seed to remain ungerminated in the soil for long periods, enabling survival during adverse conditions such as prolonged droughts. Moreover, in natural conditions not all of the seeds germinate at the same time, giving a greater chance for successful regeneration. In grazed pastures, legume seeds are scarified in the rumen of the grazing animal. When legume seed is sown, the mechanical dormancy must be broken by pre-treatments, such as abrasion or soaking in boiling water, to ensure uniform germination. Damage to the savannah caused by the grazing animals can provide important gaps for establishment of seedlings and also for vegetative spread of the legumes once they are established in a mixed sward. The intensity of grazing is a crucial factor in determining the long-term survival of legumes, and one that can be manipulated to advantage to encourage persistence of the legume (Spain *et al.*, 1985; Curll and Jones, 1989).

Addition of some fertilizers is often essential at sowing, as the germinating seedlings have a high requirement for nutrients but have insufficient root growth (or mycorrhizal infection) to allow them to compete effectively with the associated grasses for the small amounts of nutrients available. Phosphorus is the most commonly required nutrient addition (e.g. Lowe *et al.*, 1981), but many soils in Australia and Brazil are acutely deficient in molybdenum, which must also be added, albeit in very small amounts (e.g. 100 g Mo ha^{-1}). Whether other nutrients are required will

depend on the soil. For instance, in northeast Thailand sulphur additions improved establishment and maintenance of *Stylosanthes* in communal pastures (Shelton and Wilaipon, 1984). The most successful legumes in a comparison of 50 species in northern Australia were able to persist for 5 years after sowing when the available phosphorus in the soil had declined to its former low levels (Anning, 1982). However, it is likely that further fertilizer additions will be necessary to maintain introduced legumes under many conditions ('t Mannetje, 1986). A further alternative is to clear the land and plant a crop before establishing the improved pasture, in what is effectively an arable/ley rotation (see below).

Persistence of the legume in the sward will depend on its ability to withstand environmental stresses – and grazing – and at the same time to compete with other species present. One of the major problems with persistence of some species of *Stylosanthes* has been their susceptibility to the fungal disease anthracnose (caused by *Colletotrichum gloeosporioides*) but ecotypes have been identified that are tolerant of this disease (Irwin *et al.*, 1984).

Ley Farming

In many parts of the tropics, land left fallow between cropping seasons is grazed by cattle, but ley farming has never been widely adopted in small-scale farming systems in the tropics (Humphreys, 1994). There is comparatively little research examining the use of planted legume or legume/grass leys for animal production and the restoration of soil fertility in the tropics. Two main types of ley farming can be identified: fodder banks, in which an area of land is fenced off for forage production; and more extensive pasture/arable crop rotations.

Fodder banks

A specific type of ley farming that has received considerable focus in West Africa is the use of fodder banks to provide supplemental feed during the dry season for cattle and goats. An area close to the homestead is fenced off and animals are confined inside to graze down the vegetation and prepare the land for planting. An area of around 4 ha has been recommended, though this varies depending on the needs of the farmers (Tarawali and Mohamed-Saleem, 1994). Seed is then scarified and broadcast with single superphosphate fertilizer. Alternatively, the legume can be established by intercropping or undersowing within a cereal crop (Kouamé *et al.*, 1993; Tarawali and Mohamed-Saleem, 1994). Light grazing early in the wet season assists in controlling the growth of grasses. The main species currently used are *S. guianensis* and *S. hamata* but other species, such as *C. rotundifolia*, are potentially suitable (e.g. Tarawali and Peters, 1996). The fodder banks are then used for grazing during the dry season when fodder is scarce. An alternative approach is to grow fodder and harvest for storage as hay or process as silage (e.g. Maasdorp and Titterton, 1997; Titterton and Maasdorp, 1997).

Yields of maize, grown without N fertilizer after *Stylosanthes* fodder banks, were doubled compared with land continuously cultivated to crops (Mohammed Saleem and Otsyina, 1986; Tarawali, 1991). The benefit in cereal yield increased roughly in proportion with the time left under the legume (Mohammed Saleem and Otsyina, 1986). Because of their prolific seed production, the legumes are able to re-establish spontaneously (if grazing is not too severe), which is an important benefit in view of the costs of labour and seed availability for establishment of fodder banks.

In smallholder farming areas where there is little land for communal grazing, cattle are housed and fed in stalls. This is typical of smallholder dairy farmers around Nairobi, who produce around 80% of the milk in Kenya (Gitau *et al*., 1994). Farms are generally less than 2 ha and many farmers, including those who own no cattle, devote about one-tenth of their land area to production of Napier grass (*Pennisetum purpureum* cv. Bana), and feed it to cattle held in 'bomas'. Several legumes, such as *D. intortum* and *N. wightii*, fit well into this system, as they can be grown between the widely spaced stools of the grass; however, they are slow to establish, which may be the greatest constraint to widespread uptake by farmers (Mwangi *et al*., 2001).

Pastures in rotation with arable crops

In Malawi, 3- to 4-year-old pure pastures of 11 legumes were compared with grasses and other non-legumes for their contribution of N to subsequent maize crops, in experiments where the grasses and legumes were cut three times a year but the residues were not removed (MacColl, 1990). *D. uncinatum*, *C. pubescens* and *N. wightii* were the most useful in terms of the N contribution to the yield of maize (*D. uncinatum* contributed the equivalent of 30–40 kg N ha^{-1} $year^{-1}$) and all of the legumes resulted in better yields of the subsequent maize crop than grasses or non-legume crops. In the sandy infertile soils of farmers' upland fields in northeast Thailand, pure legume swards of *M. atropurpureum* and *S. hamata* proved to be better at regenerating soil fertility than the green manure *C. juncea* (Gibson and Waring, 1994). The N-fertilizer value of the *Macroptilium* ley in the first year was estimated to be 132 kg N ha^{-1}, and an optimum ley period was found to be 1–2 years, with a cropping phase of similar duration.

In northern Australia, *C. pascuorum* was found to be well adapted for use as a ley pasture plant (Cameron, 1996). Other legumes well suited for this role were *Alysicarpus vaginalis*, *C. rotundifolia*, *M. gracile* and *S. hamata* (Cameron, 1996). There is a clear need to match the legume to the most appropriate system of cropping. Although *C. pascuorum* was not persistent, this was an advantage in short-term rotations as competition with subsequent crops is decreased (Tarawali and Peters, 1996). For longer-term pastures, *Chamaecrista* and *Stylosanthes* persisted well. A further approach that may assist in overcoming problems due to local environmental variations is to use mixtures of legume species. When sown together, *C. pascuorum* provided rapid initial soil cover and suppressed competition from weeds, but was rapidly replaced by *S. guianensis*. Another species, *C. macrocarpum*,

established slowly but was more important in the mixture after growth for 1 and 2 years (Peters *et al.*, 1999).

In South America, systems have been developed for intensifying production on land traditionally used for pasture in the Brazilian cerrados and the Colombian llanos. In Brazil, soybeans have been introduced to the cropping system and are commonly grown on large areas by contractors (with addition of rhizobial inoculants, phosphorus fertilizers and lime – see Chapter 13). Improved pasture grasses and legumes are then grown on the residual fertility caused by fertilization and enhanced mineralization of N stimulated by tillage of the soil. The cycle is repeated roughly every 5 years. Similar systems have been developed in Colombia for production of upland rice followed by establishment of improved pasture (Thomas *et al.*, 1995). This approach became possible due to the development of rice varieties tolerant of the acid soils with high aluminium saturation, and allows the fertilization costs to be borne by the cash crop.

Inputs to the N Cycle from N_2-fixation

Although the soil under established pasture may contain a large amount of N, little of this is available for plant growth. Less than 1% of the N present in pasture soils may be mineralized in a year (Henzell, 1968) and so, contrary to the situation in many cultivated soils, depletion of total soil N is not the major problem for production. Removal of nutrients in animal products from unfertilized tropical grassland would not cause a significant decrease in the soil organic N in less than 100 years (Henzell, 1968). The result of the lack of available N is that the fodder eaten by the grazing animal usually has a very small N content, which is why the introduction of N_2-fixing legumes, with their protein-rich leaves and their effect on increasing the N content of grass grown in mixtures, has such a dramatic effect on animal production.

How much N_2 do the legumes fix?

In a mixed sward, only a small proportion of N added as fertilizer is recovered by the legume – commonly less than 10% (Vallis *et al.*, 1977). The severe competition for uptake of mineral N afforded by the native savannah leads to almost complete dependence on N_2-fixation by the legume. Estimates carried out using the ^{15}N isotope dilution method indicate that 80–95% of the legume N is derived from N_2-fixation under field conditions when other nutrient limitations are removed (Vallis *et al.*, 1977; Cadisch *et al.*, 1989; Thomas *et al.*, 1997). Thus, simple measurement of N accumulation in the legume will provide a useful guide to the amount of N_2-fixation over short time-periods in fertilized swards. In pure legume stands the proportion and amount of N_2-fixation will be related to the availability of soil N, as long as no other environmental stresses prevail (Chapter 13). This is illustrated by increases in the %N from N_2-fixation found in pasture legumes as the proportion of legume in the sward decreases and competition for soil N becomes more severe

Table 10.2. Some estimates of N_2-fixation in tropical pasture legumes growing in the field.

Species	N_2 fixed		Time period	Country	Method[a]	Ref.[d]
	kg N ha^{-1}	%				
Arachis pintoi	1–7	68–82	12 weeks	Colombia	ID	1
Calopogonium mucunoides	136–182	–	1 year	W. Samoa	Diff	2
	64[b]	–	1 year	Brazil	ID	3
Centrosema acutifolium	43	82	17 weeks	Colombia	ID	4
	1–5	90–98	12 weeks	Colombia	ID	1
	5–33	76–95	10–14 weeks	Colombia	ID	5
C. macrocarpum	41	83	17 weeks	Colombia	ID	4
	5–40	63–94	10–14 weeks	Colombia	ID	5
C. pubescens	67–87	–	1 year	W. Samoa	Diff	2
	80–280	–	1 year	Various	Diff	6
	136	–	1 year	Uganda	Diff	7
Clitoria ternatea	42	45	–	Australia	NA	8
Desmanthus virgatus	3–15	15–24	–	Australia	NA	8
	5–8	11–51	–	Australia	NA	9
Desmodium canum	90[b]	–	1 year	Hawaii	Diff	10
D. intortum	380	–	1 year	Hawaii	Diff	10
	103	–	1 year	Australia	Diff	11
	35–51	41–55	6 months	Kenya	NA	12
D. ovalifolium	64–110	–	1 year	W. Samoa	Diff	2
	25	70	17 weeks	Colombia	ID	4
	31–54	82–89	14 weeks	Brazil	ID	13
	–	30–72	–	Brazil	ID/Ureide	14
Galactia striata	31–54	81–93	14 weeks	Brazil	ID	13
Lablab purpureus	32–146	51–90	–	Australia	NA	8
Lotononis bainesii	17–92	92–94	–	Australia	NA	8
Macrotyloma axillare	9–32	44–61	6 months	Kenya	NA	12
Macroptilium atropurpureum	46–167	–	1 year	W. Samoa	Diff	2
	97–137	–	1 year	Australia	Diff	14
	29	33	–	Australia	NA	8
	15–68	43–61	–	Australia	NA	9
Neonotonia wightii	126	–	1 year	Australia	Diff	14
	2–27	18–65	6 months	Kenya	NA	12
Pueraria phaseoloides	115	88	17 weeks	Colombia	ID	4
Stylosanthes spp.	<1–100[b]	71–92	10 weeks	Australia	ID	15
	20–263[c]	–	1 year	Various	Diff	16
	39	49	–	Australia	NA	8
S. capitata	38	87	17 weeks	Colombia	ID	4
	141–179	73–88	16 months	Brazil	NA	1
	3–46[b]	–	1 year	Brazil	Diff	17
	1–40	82–95	12 weeks	Colombia	ID	18
S. guianensis	47	75	17 weeks	Colombia	ID	4
	7–42[b]	–	1 year	Brazil	Diff	17
	76–102	68–79	16 months	Brazil	NA	18
S. macrocephala	71	88	17 weeks	Colombia	ID	4
	4–18[b]	–	1 year	Brazil	Diff	17
	68–89	74–79	16 months	Brazil	NA	18
S. scabra	22–40	52–70	16 months	Brazil	NA	18
Zornia glabra	61	88	17 weeks	Colombia	ID	4

Footnotes on *opposite* page.

Table 10.2. *continued*

[a]Diff = N-difference; ID = ^{15}N isotope dilution; NA = ^{15}N natural abundance. [b]Measured under grazing. [c]Range from the literature.
[d]1: Thomas *et al.*, 1997; 2: Reynolds, 1982; 3: Seiffert *et al.*, 1985; 4: Cadisch *et al.*, 1989; 5: Cadisch *et al.*, 1993a; 6: Clements *et al.*, 1983; 7: Stobbs, 1969; 8: Armstrong *et al.*, 1997; 9: Armstrong *et al.*, 1999; 10: Whitney *et al.*, 1967; 11: Johansen and Kerridge, 1979; 12: Mwangi *et al.*, 2001; 13: Viera-Vargas *et al.*, 1995; 14: Alves *et al.*, 2000; 15: Vallis and Gardener, 1985; 16: Vallis and Gardener, 1984; 17: Thomas and de Andrade, 1984; 18: Miranda *et al.*, 1999.

(Thomas *et al.*, 1997). In fodder banks, by contrast, the opposite may occur, as N-rich leaf litter will gradually build up the soil organic N pool and N_2-fixation may be suppressed, as demonstrated with N_2-fixing trees (Chapter 12).

The estimates of N_2-fixation vary widely (Table 10.2). Henzell (1968) calculated that inputs from N_2-fixation were in the range of 30–280 kg N ha^{-1} from estimates of dry matter offtake in pastures. Most of the estimates given in Table 10.2 are for well-fertilized mown pastures, whereas much smaller amounts of N are likely to be fixed in grazed pastures (Vallis *et al.*, 1983), because of the continual defoliation of the legume and the recycling of N to the soil in animal excreta. The amount of N_2 fixed depends mainly on the dry matter yield of the legume and so larger amounts of N_2-fixation are generally found when the dry season is short and there is a long growing season. Given the rapid turnover of legume litter it is likely that virtually all of the estimates of primary productivity and N_2-fixation underestimate actual amounts (Rezende *et al.*, 1999).

When phosphorus and potassium were limiting plant growth, the proportion of the plant N from N_2-fixation was found to vary from 44 to 84% in eight pasture legumes; thus, accumulation of N in this case was not a good guide to the amounts fixed (Cadisch *et al.*, 1989). The proportion of N in the roots decreased from more than 50% to around 20% when phosphorus and sulphur fertilizers were supplied to *S. humilis* (Robinson and Jones, 1972) and this should be taken into account, particularly when measuring N_2-fixation in unfertilized pastures. In many acid soils in the tropics, micronutrients are also deficient and must be added to ensure productive growth (Chapter 13) (Cheng and Kerridge, 1982). Ideally, measurements should be made in grazed pastures to take account of the effects of defoliation and the uneven redistribution of N in dung on N_2-fixation. Whilst it might be expected that the proportion of N from N_2-fixation in the legumes might decrease with age of the pasture as more N is accumulated in the soil, experiments with several species of *Stylosanthes* under grazing showed that the %N fixed remained close to 80% over 5 years (Vallis *et al.*, 1985). Measurements of legume yields of 4–80 kg N ha^{-1} in a number of such trials with *Stylosanthes* spp. in Australia indicated that substantial amounts of N are fixed under grazing (Vallis and Gardener, 1984). To evaluate the contribution from the legume properly, further measurements of N_2-fixation in grazed pastures under realistic fertilization are required.

Accumulation of N over time within the plant/soil system provides a guide to the amounts of N_2 fixed but does not account for N losses, or for gains from other sources such as rainfall. As loss of N by volatilization of ammonia is greater under

grazing, due to the high concentrations in urine patches, simple comparison of grazed and ungrazed plots is not a reliable measure of the removal of N by the animal. Changes in soil N under pastures with different species of *Stylosanthes* varied from a decrease of 30 kg N ha^{-1} $year^{-1}$ during drought to increases of 30–106 kg N ha^{-1} $year^{-1}$, and similar results have been reported for other legumes (Vallis and Gardener, 1984). In terms of pasture production, increases in N availability are more important but may be difficult to detect by soil analysis, due to the rapid uptake of mineral N by the plants as it is released.

Cycling of the fixed N

More than 90% of the nitrogen fixed by the legume is returned to the soil in plant litter or in animal excreta if cattle are being grazed for beef production, whilst more of the N (up to 30%) is removed in milk production (Henzell, 1973). Release of N from the plant litter can be slow, with less than 30% being made available in the first year, whilst N in excreta is readily available but much more susceptible to being lost (Vallis *et al.*, 1983). Up to 50% of the N can be lost from urine patches within a few weeks, with the most rapid loss taking place immediately by ammonia volatilization – 26% of the N in a urine patch was lost in the first day but only 32% within a week (Vallis *et al.*, 1985). Losses of N from dung can be reduced by burying of faeces by dung beetles (Gillard, 1967). Below-ground transfer of N through rhizodeposition or root turnover and decay seems to be of little significance in the short term (Urquiaga *et al.*, 1995; Trannin *et al.*, 2000).

The relative importance of the two main pathways (the 'litter' and 'excreta' pathways) for cycling of fixed N depends on the palatability of the legume (Cadisch *et al.*, 1994a). Cattle tend to graze the grasses preferentially from mixed swards during the rainy season when their forage quality is good. Legumes such as *C. mucunoides* (Seiffert *et al.*, 1985; Euclides *et al.*, 1998) and *C. rotundifolia* (Clements *et al.*, 1996) are not eaten until the availability and quality of the grass declines. Exceptions are highly palatable species such as *A. pintoi*, which are consumed throughout the year (Lascano and Thomas, 1988; Carulla *et al.*, 1991). Thus with less palatable legumes such as *Calopogonium*, fixed N will become available to the associated grass through litter fall and decomposition during the wet season and N transfers through animal manure will only be of importance during the dry season. The significance of this observation was mentioned above; the transfer of N through animal manure is much more susceptible to gaseous losses of N from the system (Thomas, 1992; Cadisch *et al.*, 1994a). The proportion of the N in the plant biomass from N_2-fixation that is required to balance the N cycle, and prevent a net drain of N, is thus linearly related to the proportion of the pasture biomass that is eaten by the cattle (known as the % utilization).

The N balance under a mixed *Brachiaria*/*Calopogonium* sward was evaluated using simple models (Cadisch *et al.*, 1994a). Calculations indicated that a legume content equivalent to 31–46% of the N in the mixed sward (13–23% dry weight) is sufficient to sustain productivity if a large proportion of the legume N is derived from

N_2-fixation (Cadisch *et al.*, 1994a). Thomas (1992, 1995) estimated that 38–53% of the N (20–31% dry weight) was required to come from the legume when pasture utilization was 10–40% but that this would increase to 57–67% of the N (35–45% dry weight) at higher utilization rates of 50–70%. To maintain N stocks under grazed pastures, between 15 and 158 kg fixed N ha^{-1} are required – amounts that could readily be provided by legumes. These N balance calculations ignore contributions from below-ground biomass, which are still poorly understood but may reduce the above-ground N requirement by about 14% (CIAT, 1992). Rao (1998) estimated N content of roots in a well-fertilized *Brachiaria dictyoneura*/*C. acutifolium* sward up to 18 kg N ha^{-1} but there are indications that amounts of N contributed below ground may be greater (Chapter 5). An example of the N balance of a mixed *A. gayanus*/*Stylosanthes* pasture grazed by cattle is shown in Fig. 10.4.

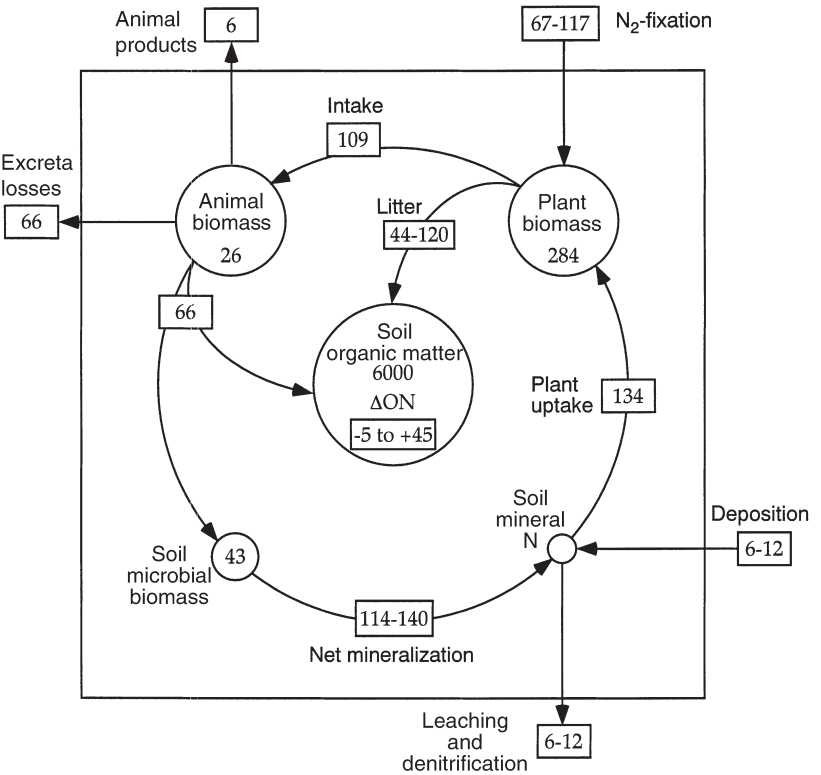

Fig. 10.4. The nitrogen cycle of a grazed mixed sward of *Andropogon gayanus* together with *Stylosanthes capitata, S. guianensis* var. *pauciflora* and *S. macrocephala* on an acid Oxisol in the cerrados of Brazil. Circles represent the main N pools with amounts in kg N ha^{-1}; rectangles are the major N fluxes, in kg N ha^{-1} $year^{-1}$; ΔON is the predicted annual change in the soil organic N pool. (Adapted from Cadisch *et al.*, 1993b.)

The rate of N mineralization from plant litter can vary widely between legume species (Thomas and Asakawa, 1993). Leaf litter from *M. atropurpureum* released much more N than that from *D. intortum* even though the N contents were similar, and this was attributed to greater concentrations of polyphenols in *Desmodium* which can form complexes with proteins, rendering them resistant to microbial attack (Chapter 5) (Vallis and Jones, 1973). Thomas and Asakawa (1993) found that litter from *S. capitata* and *A. pintoi* decomposed rapidly, much faster than *C. acutifolium*, *S. guianensis* and *P. phaseoloides*, with *Desmodium ascendens* having the slowest decomposition rates. Rates of decomposition of tropical grass litters were similar to more slowly decomposing legume litters, but nutrient release from the grass litters was much slower, due to their poor initial contents of nutrients (Thomas and Asakawa, 1993). The recalcitrance of some *Desmodium* species to decomposition and N mineralization, which is due to the presence of large amounts of reactive polyphenols, is commonly observed (e.g. Luna-Orea *et al.*, 1996). Although the initial rate of release of N from *Desmodium* is slow, it can increase the N mineralization in the longer term (Cadisch *et al.*, 1996). *Desmodium* contributed 24% of the C in the surface layer of a 6-year-old pasture (Cadisch and Giller, 1996). Uptake of N from ^{15}N-labelled residues of *D. intortum* was greater in the second year after addition (Vallis, 1983). A mixed sward took up 17% of the N from ^{15}N-labelled residues of *S. humilis* in the first year and 9% in the second year (Vallis and Gardener, 1984). Similar results were found with siratro, where 50–60% of the N from the leaves and stems was found in the soil organic N pool after 1 year (Vallis, 1983). The overall result of N_2-fixation by the legume is certainly that grasses grown in mixtures generally contain higher concentrations of N, due to the increased inputs of N to the system (Birch and Dougall, 1967).

Factors regulating availability of N in pasture soils

Disturbance of the soil due to cultivation can result in a dramatic release of available soil N 2–3 months later in acid savannah soils (Sylvester-Bradley *et al.*, 1988b). In the absence of disturbance, virtually all of the mineral N in the soil was present as ammonium, and nitrification (the microbial conversion of ammonium to nitrate), which led to substantial leaching losses of nitrate, occurred at significant rates only in soil that had been cultivated.

The reasons for the lack of mineralization and nitrification in undisturbed savannah soils are not fully understood. Decomposition studies with residues of *Panicum* indicated that mineralization was, in fact, rapid but that the N was rapidly immobilized by microbes and so there was little net release of N for plant uptake (Chapter 5) (Robbins *et al.*, 1989). The C : N ratios of root systems in mixed pastures were above 100 : 1, indicating that little N would be readily released from these tissues when they decompose (Rao, 1998). Urquiaga *et al.* (1998) found narrower C : N ratios in legume roots of between 27 and 70. All of these induced net immobilization of N for around 50 days, even when added to an Oxisol in small amounts, though immobilization was much stronger with *Brachiaria* roots, which

had a C : N ratio of 130 : 1. There are indications that productive tropical pastures could play a major role in sequestration of carbon from the atmosphere, due largely to the deep nature and poor quality of the root systems of the grasses (Fisher *et al.*, 1994, 1997).

Soil disturbance stimulates mineralization by allowing better aeration of the soil or by exposing more surfaces available for microbial attack. The pasture grass *B. humidicola* has been shown to inhibit the rate of nitrification in soil on which it grows (Sylvester-Bradley *et al.*, 1988b). Accumulation of nitrate could readily be detected in soil on which various pasture grasses (including other *Brachiaria* spp.) or legumes had been grown, or in soil that had been fallowed, when ammonium was present, but not in soil from under *B. humidicola*.

The extremely small phosphorus contents of roots from tropical grasses and legumes may restrict their decomposition (Gijsman *et al.*, 1997a). Addition of phosphorus fertilizer apparently stimulates N uptake by grasses in pure stands, leading to the suspicion that severe phosphorus deficiency prevents mineralization of N from the soil organic matter. Close study of this phenomenon indicated that the benefits of N uptake by *Brachiaria* were in fact due to enhanced capture of mineralized N resulting from the better developed root system in P-fertilized plants (Cadisch *et al.*, 1994b).

Legumes have been shown to have a stimulatory effect on the availability of soil and fertilizer N to associated grasses. In experiments where small quantities of ^{15}N-labelled fertilizers were applied to established swards of grasses or grass/legume mixtures, recovery of the applied N was three times greater in the mixed swards than in the sole grass (Vallis *et al.*, 1977). This increase in availability of N on introduction of the legume may result from reduced immobilization of N, or enhanced re-mineralization of N that had been immobilized, due to the addition of N-rich legume residues to soil. Part of this benefit could be due to the improved physical properties found when legumes are present in the sward, though a large part of the effect could be 'pool substitution' with N from the more readily decomposable legume residues (Ehaliotis *et al.*, 1998). Thus, effects of legumes on increasing the release of native organic N from the soil are likely to be small.

Other benefits and problems of pasture legumes

Not all of the benefits of legumes can be attributed to N inputs alone. Soil under grass/legume pastures had a much larger proportion of large pores than pure grass pasture or native savannah, leading to much faster infiltration of rainfall (Gijsman and Thomas, 1996). This was attributed to old root channels left by the coarse root systems of the legume and an increase in earthworm burrows; Decaens *et al.* (1994) found that earthworm biomass was doubled due to the presence of a legume in the pasture. Increased infiltration of water reduces risks of soil erosion, which is a major factor in declining soil fertility. The stimulation of biological activity due to the presence of a legume in sown pastures also resulted in increases in organic

phosphorus (Guggenberger *et al*., 1996) and more plant-available forms of phosphorus (Gijsman *et al*., 1997b; Oberson *et al*., 1999).

Long-term pastures cause progressive acidification of the soil due to a combination of factors, including: proton release from the roots; an increase in soil organic matter, CEC and exchangeable acidity; release of protons by nitrification; microbial and root respiration; inputs of acidity from atmosphere; leaching of cations; and export of cations in forage and livestock (Bromfield *et al*., 1983; Haynes, 1983; Slattery *et al*., 1991). Soils that are already mildly acid and light-textured sandy soils are the most prone to acidification, which may cause the soil pH to drop by one unit in 15–40 years (Williams, 1980; Noble *et al*., 2000a). The rate of pH decline appears to be faster under legumes, perhaps due to their capacity to fix N_2, which results in a net excretion of protons (see Marschner, 1995, for a detailed discussion). This is clearly a major concern regarding the long-term sustainability of pastures (e.g. Noble *et al*., 2000b), and indeed of other types of cropping systems.

Current Use of Pasture Legumes in the Tropics

The most successful of the tropical pasture legumes appear to be *Stylosanthes* spp.; it is estimated that they were grown on more than 800,000 ha in Australia 20 years after their introduction, and 13,000 ha only 8 years after their introduction to China (CIAT, 1991; Thomas, 1995). By 1999 more than 27,000 farmers had adopted use of improved forage legumes on 19,000 ha in West Africa (Elbasha *et al*., 1999). However, despite widespread adoption of improved pastures in the llanos of Colombia, where over 16 years the area increased tenfold, fewer than 2% of the pastures contained legumes (Smith *et al*., 1997). Where legumes had been sown, almost 90% of farmers reported that they failed to persist.

In Zimbabwe, improved pasture technologies have been widely adopted by commercial farmers but have had little impact in smallholder agriculture (Clatworthy, 1985; Clatworthy *et al*., 1986). The exceptions are where introduction of forage legumes has been linked to small-scale beef fattening schemes, or smallholder milk production where farmers already have readily marketable commodities. In Nigeria, legume growth was much poorer on farmers' fields than under experimental testing, and *Stylosanthes* was the only legume showing potential under their low-input management (Muhr *et al*., 1998). Thomas and Sumberg (1995) criticized many research programmes in Africa for duplication of effort and lack of rigour in testing under relevant conditions. Many factors come into play that limit the use of pasture legumes by smallholder farmers; communal ownership of grazing lands and free grazing of animals, costs and other demands on labour, costs of fencing and fertilizer, lack of seed supplies and poor information and extension are among them.

Elbasha *et al*. (1999), based on experience of uptake of pasture legumes in West Africa, cautioned that it may take 15 years or more before diffusion of a technology occurs. However, it is clear that much more effort must be focused on developing and adapting technologies together with smallholder farmers if pasture legumes are

to play a full role in animal production in the tropics. Farming systems where there is opportunity for intensification of livestock production and limitations on land are likely to be those where pasture legumes may have an immediate benefit (Smith *et al.*, 1997; Thomas, 2000).

Conclusions

Legumes have an essential role to play in the improvement of tropical pastures, largely due to their ability to fix most of their N_2. Apart from the direct contribution to animal production through the provision of protein-rich fodder, the legume can improve the productivity of savannah by increasing the amount of N available for uptake from the soil by the associated grasses. Future improvements in the N economy of legume/grass pastures may depend on finding ways of decreasing the losses of N, which can be especially great under grazing, such as the use of less palatable legumes.

Selection of herbaceous legumes under grazing has led to the identification of several species that show great potential for the improvement of tropical pastures. Selection of effective, competitive strains of rhizobia that are adapted to acid soils with poor fertility has resulted in large responses in N_2-fixation and growth in the field for several species of tropical pasture legumes. These selected strains have proved to be useful over a wide range of environments and further research to select strains for other legume species is certainly warranted. Persistence of the legume is a problem with many species, but can be improved by careful grazing management and moderate inputs of fertilizers, especially phosphorus.

The major challenge for tropical pasture improvement is to gain widespread adoption of these promising technologies by farmers (discussed further in Chapter 14). To this end, seed production and distribution networks have been established – but without a system for the production and extension of rhizobial inoculants.

Chapter 11

Plantation Crops: Understorey Legumes and Shade Trees

Products from plantation crops make up an important part of the economy of many countries in the tropics. The plantation crops most widely produced are coffee, tea, rubber, oil palm, cacao and coconut; several other crops (e.g. sisal) are of local importance. Rubber is produced principally in Southeast Asia, where Malaysia and Indonesia have long been the major producers, although exports from China and India are increasing rapidly (Baulkwill, 1989). Oil palm is also an important plantation crop in Malaysia and Indonesia and in several countries in West Africa (Hartley, 1988). Production of cacao has traditionally been based in Latin America and West Africa, with Brazil, Cameroon, Ghana, Côte d'Ivoire and Nigeria as the main producers, but significant amounts are also grown in Malaysia and Papua New Guinea and in Caribbean countries (Wood, 1985). These three crops are commonly established on acid Ultisols and Oxisols in areas cleared of forest.

Although all of these crops are produced mainly for profit, and it might be expected that the N requirements would readily be met by the use of fertilizers, N_2-fixing legumes do in fact have important roles to play in the production of plantation crops, both as cover crops and as shade trees. Understorey legumes can be useful as cover crops with virtually all types of plantation crops, whilst shade trees are required only for the production of coffee, tea and cacao when grown with minimal inputs.

Cover Crops in Plantations

The earliest interest in the use of cover crops in plantations undoubtedly came from the problems of soil erosion that occurred during the initial establishment of the plantation crops (Maas, 1922; Bunting and Milsum, 1928). Trees of coconut (*Cocos nucifera*), rubber (*Hevea brasiliensis*) and oil palm (*Elaeis guineensis*) are generally

planted several metres apart on cleared ground, thus affording the newly exposed soil little protection from erosion. The use of herbaceous legumes to provide soil cover and the additional benefits that they can confer over grass or other non-legume cover crops has been the subject of much research, particularly in Malaysia. Shrub and tree legumes such as *Crotalaria anagyroides*, *Flemingia macrophylla* and *Tephrosia candida* have also been used to provide soil cover and N in plantations and can be useful for strengthening contour terraces on sloping land (Bunting and Milsum, 1928; Pushparajah and Tan, 1976). In some cases grain legume crops may be cultivated between the rows of the plantation crops; for instance, *P. vulgaris* is often grown between rows of coffee bushes in northern Tanzania.

The crop cycle

All plantation crops are initially grown in nurseries until the plants are of sufficient size, often when they are 1–2 years old, to be transplanted to establish the plantation. The growth cycle of most plantation crops can be divided into two main phases (e.g. rubber, Fig. 11.1). The establishment phase is the period during which there is much open ground as the trees grow to form a canopy. This is followed by the production phase. It can take 7–9 years before rubber trees can be tapped, while oil palms begin to produce fruit 3–4 years after transplanting. In the case of crops such as coffee or tea, the young bushes are pruned in various ways to improve the yield and the ease of harvesting (Willson, 1985). The production phase can last for many years – for rubber and oil palm up to 25 or 30 years. The plantation is usually then cleared and replanted. Felled oil palms are commonly left to decay in the plantation, while timber from rubber trees now tends to be removed from the plantation and used either for fuel as wood or charcoal, or in cardboard, packing cases or building materials (Paardekooper, 1989). Wood removal constitutes a major removal of nutrients from the site and can result in severe nutrient deficiencies in the succeeding trees (Watson, 1989b).

Legume cover crops

The legumes that have been most successful as cover crops in plantations are species that are excellent forage, pasture or green manure legumes (Chapters 9 and 10). Such species include *Calopogonium mucunoides*, *Centrosema pubescens* and *Pueraria phaseoloides*, all creeping or trailing perennials which can rapidly provide a complete soil cover. This growth habit is important, not only because plants can readily spread over gaps where establishment of trees was poor, but also due to the ability of the plants to climb up and smother vigorous grasses and other weeds. Important characteristics of legume cover crops include rapid growth and a good root system to bind the soil easily. The cover plants must grow well during the establishment of the plantation crop, after which they have fulfilled their main purpose. They should be perennials and be propagated readily, preferably by seed (Bunting and Milsum,

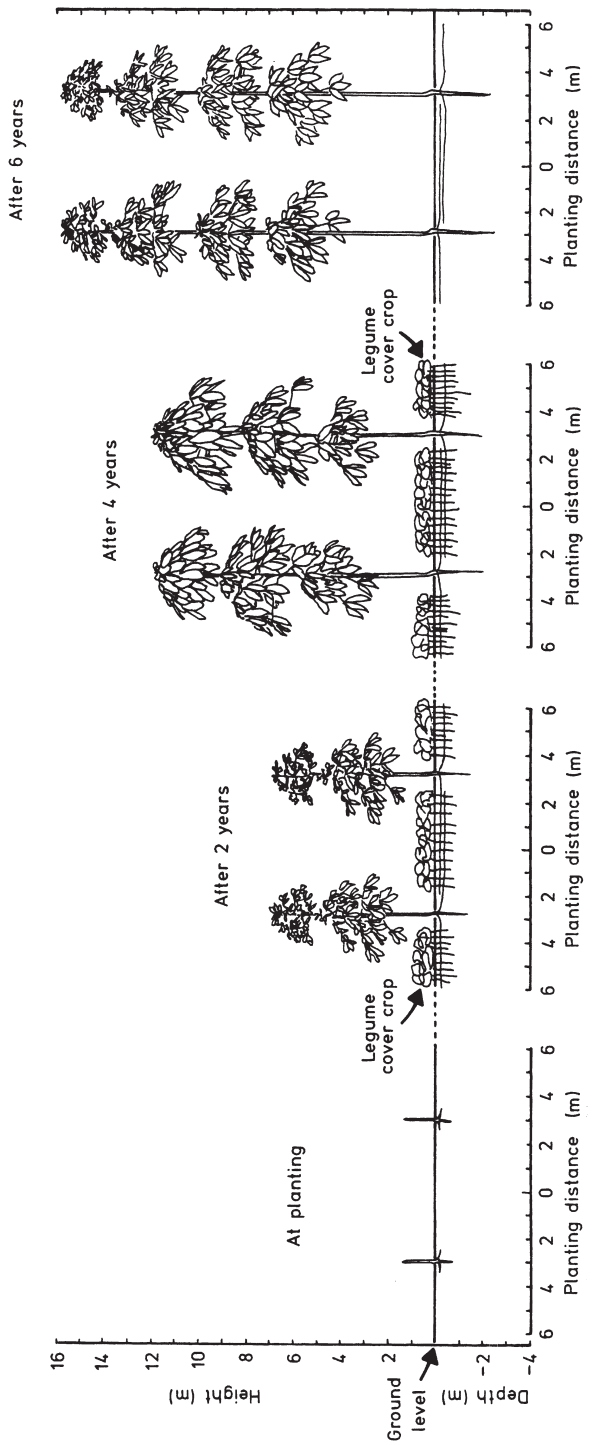

Fig. 11.1. Development of a rubber plantation and legume cover crop over the first 6 years. (After Broughton, 1977.)

1928). Practices for establishment of cover crops in plantations are described in detail by Bevan and Gray (1969). The legumes are usually sown with rhizobial inoculation after cleared ground has been weeded twice and the weeds burned (Giller and Fairhurst, 2000).

In West African plantations *P. phaseoloides* establishes naturally without seeding and must be kept in check by frequent slashing, whilst in Asia the growth is not so vigorous as to pose a problem and growth close to the trees is controlled by weeding (Corley *et al*., 1976; Hartley, 1988) (Fig. 11.2). When a mixture of cover crops is sown, *C. mucunoides* usually germinates and establishes first but is soon replaced by *Pueraria*. *Centrosema* then becomes dominant when the plantation crops begin to shade the ground. Most cover crop species die out after 4–5 years as shading becomes more dense, but some species, such as *Calopogonium caeruleum* (Tan *et al*., 1976) (Fig. 11.3) or *F. macrophylla* (Soong and Yap, 1976), can grow well under shade and persist longer. *Desmodium ovalifolium* is also quite shade-tolerant but slow to establish, requiring intensive weeding during the first 6 months (Juan and Chew, 1982). *M. pruriens* var. *utilis*, *Psophocarpus palustris* and *P. tetragonolobus* are other examples of useful ground covers. *Mucuna* is an effective cover plant that establishes quickly, partly due to its large seed, but it must be planted with perennials to ensure persistent cover throughout the immature period of oil palm growth. *Pueraria triloba* occurs frequently in plantations but is not recommended as much of its growth reserves are devoted to a large underground tuber and it is not thought to fix N_2 as actively as other species. Interestingly, *Senna cobanensis*, a legume that does

Fig. 11.2. A cover crop of *Pueraria phaseoloides* growing under rubber in Sri Lanka. (Photograph: J.M. Anderson.)

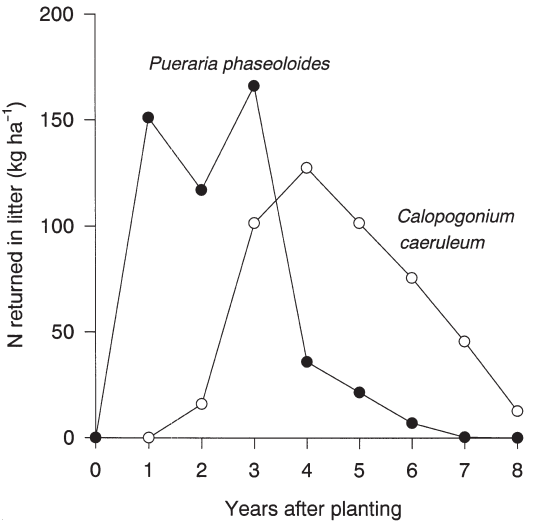

Fig. 11.3. Annual nitrogen return from two legume covers (*Pueraria phaseoloides* and *Calopogonium caeruleum*) grown in a mixture under rubber trees. (Drawn from data in Pushparajah, 1981.)

not nodulate, was found to decrease growth of rubber and is considered to be an undesirable weed.

The forage groundnut *Arachis pintoi* has been used successfully under oil palm in Central and South America, under coconut in Indonesia (Mullen *et al.*, 1997) and in *Macadamia*, papaya and banana orchards in Australia (Dwyer *et al.*, 1989; Firth, 1993; Firth and Wilson, 1995). *A. pintoi* is well adapted to acid soils rich in aluminium (Grof, 1985; Lascano and Thomas, 1988; Kerridge and Hardy, 1993) and is shade-tolerant, which allows it to persist for longer under oil palm than other cover crop species. Other forage legumes selected for tropical pastures through an intensive research programme in South America over the past 20 years (Schultze-Kraft, 1986) may also warrant more widespread evaluation as cover crops for plantations.

Benefits and management of legume covers

Benefits of including cover crops in plantations are commonly seen in terms of increased yields. Rubber trees grown with legume cover crops on replanted, infertile sites grew faster and reached a size at which they could be tapped 18 months before trees grown with grass or other non-legume covers; thus they gave much greater yields over the first 4 years of production (Mainstone, 1963). In fact the benefits of legume covers in increased yields are high over the first 10 years of tapping and persist up to 20 years, even though the legumes die out after only 4–5 years (Broughton, 1977; Ismail *et al.*, 1979). However, in drier climates legume covers can cause a decrease in yields of oil palm, due to competition with the crop for water during the dry season (Ochs and Daniel, 1976). Similarly, groundcover of *A. pintoi* reduced banana yields due to competition for water (Johns, 1994).

Use of cover crops has not been generally adopted in cacao or coffee plantations. In Southeast Asia cover crops such as *Centrosema* are used with cacao (Wood, 1985). Competition for soil moisture can be a problem in regions where arabica coffee is grown. In higher rainfall areas where robusta coffee is grown, root competition and the labour required to prevent the cover crops from climbing over the coffee bushes have been the major drawbacks (Wrigley, 1988). *Flemingia* has shown promise for use with coffee in Cameroon, where its use resulted in bushes coming into production earlier and giving more than double the yield of coffee in some cases (Bouharmont, 1978). In Tanzania, cover crops of *P. phaseoloides* gave consistently better yields of sisal than the natural vegetation and were particularly beneficial on soils poor in N or where there were pernicious weeds (Hopkinson, 1969).

Strong responses to fertilization are often found in cover crops, as plantations are frequently established on highly weathered, phosphorus-fixing soils. In Southeast Asia, reactive rock phosphate is applied both at sowing (200 kg ha^{-1}) and again after 2 years (100 kg ha^{-1}), and ideally small amounts of mixed fertilizer containing N, P, K and Mg should also be applied a few weeks after germination (Watson, 1989a). The extra expense of fertilizer is readily justified by the additional benefits in cover crop growth and N_2-fixation – in one case unfertilized legume covers produced only 12 kg N ha^{-1} compared with the 124 kg N ha^{-1} found in phosphorus-fertilized plots (RRIM, 1961). Rock phosphate has the advantage, over other types of phosphorus fertilizers, of maintaining the supply of phosphorus for both the legume cover and the plantation crop over several seasons (Pushparajah *et al*., 1977).

There are a number of insect pests that can cause severe damage and reduce the ability of the cover crops to suppress weeds (Wood, 1976). This is another reason for using mixtures, as the risk of devastation by pests and diseases is reduced. In replanted oil palm plantings, legume cover plants often regenerate spontaneously when dormant seeds are disturbed by the tracks of heavy machinery used to fell old palms and shred trunks.

Contributions from N_2-fixation

Legume cover crops, consisting of a mixture of *C. mucunoides*, *C. pubescens* and *P. phaseoloides*, accumulated 151 kg N ha^{-1} $year^{-1}$ more than non-legume cover crops or natural vegetation (Broughton, 1977). This figure is a mean over the first 5 years of the establishment phase in a rubber plantation and the legumes in fact accumulated most N over the first 2 years (Fig. 11.4).

There are few direct measurements of N_2-fixation by cover crops in plantations. *P. phaseoloides* was estimated by ^{15}N isotope dilution to fix between 60 and 80% of the N it accumulated in a 2-year-old oil palm plantation, amounting to 151 kg N ha^{-1} $year^{-1}$ (Zaharah *et al*., 1986) – precisely the same figure as that found above. A mixture of *C. pubescens* and *P. phaseoloides* was estimated to contribute 150 kg N ha^{-1} $year^{-1}$ in a study of the nitrogen cycle in an oil palm plantation in Malaysia (Agamuthu and Broughton, 1985) – again a similar estimate. Indeed all of these studies are likely to have underestimated the amounts of fixed N, as all were based on

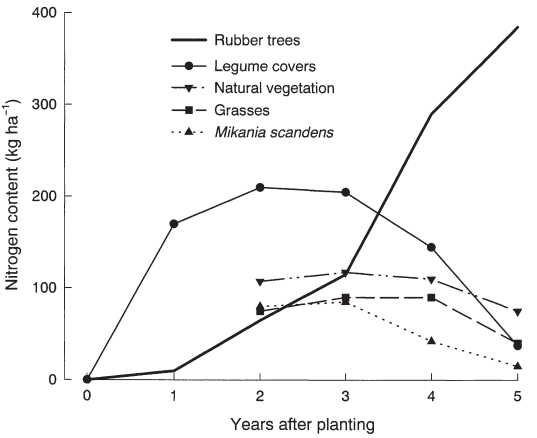

Fig. 11.4. Amounts of nitrogen in rubber trees and cover plants during the first 5 years after planting. (After Watson, 1963.)

harvests of standing plants and did not account for the N continually added in fallen leaves. Legume covers form a very dense canopy and become 'self-mulching' as the shaded leaves senesce and add substantial amounts of N to the soil (van Noordwijk and Purnomisidi, 1992).

It might be expected that the build-up of available N in the soil caused by decomposition and mineralization from the legume litter would lead to a suppression in N_2-fixation, though in a study under N-limited conditions *P. phaseoloides* litter actually stimulated growth and N_2-fixation by *P. phaseoloides* (Vesterager *et al.*, 1995). Suggestions that significant amounts of N might be transferred below ground between legume covers and plantation plants by mycorrhizal strands interconnecting roots have not been supported by experimental evidence (Ikram *et al.*, 1994).

Other evidence of the importance of N_2-fixation may be gained from the amount of N fertilizer required to attain comparable yields in the plantation crop when a legume cover crop is not used. To achieve similar yields of rubber to those found with an understorey legume cover crop (again a mixture of *Calopogonium*, *Centrosema* and *Pueraria*), fertilizer-N had to be applied to grass cover at rates of nearly 1100 kg N ha^{-1} over a 5-year period, or 840 kg N ha^{-1} over a 9-year period in a separate study (Pushparajah and Tan, 1976).

Little research has been conducted on nodulation and N_2-fixation by legume cover crops in plantations. With *C. caeruleum*, no response to inoculation with several rhizobial strains was found in four field experiments in Malaysia, even though the inoculant strains formed over 70% of the nodules in some cases (Ikram, 1983). In further experiments, inoculation of *C. caeruleum* and *Pueraria* with different strains of *Bradyrhizobium* also failed to improve their growth (Wahab *et al.*, 1989; Ikram *et al.*, 1993). In some cases, failure of cover crop establishment has been linked to the lack of suitable inoculants. For example, poor growth of *Stylosanthes guianensis* (a legume with a more specific requirement for rhizobia) on coastal soils of Malaysia was due to absence of compatible rhizobia (Gray and Kean, 1968).

Additional benefits of legume cover crops

Maintaining an understorey of legumes has been shown to reduce soil erosion, reduce weed problems and reduce the incidence of fungal root diseases. Reductions in soil erosion are due in part to improved aggregation of soil particles and increased water infiltration rates resulting from the increase in organic residues added to the soil (Soong and Yap, 1976). Leaching losses under legume covers were estimated to be 63 kg N ha^{-1} $year^{-1}$ less than losses under natural regrowth (Agamuthu and Broughton, 1985).

Legume covers compete less strongly for soil N with oil palm than many weeds (Turner, 1981). This is partly due to their ability to fix N_2 from the atmosphere, which reduces their dependence on soil N, but also due to the fairly deep rooting habit in comparison with many weed species – year-old plants of *Pueraria* and *Centrosema* rooted to more than 1 m depth, while *Axonopus compressus* had shallow roots (Chandapillai, 1968). This is consistent with observations that *Axonopus* prevents free surface rooting of rubber (Watson *et al.*, 1964).

Cattle have traditionally been used to control the height of the understorey vegetation in coconut plantations, and plantations provide an important resource for animal production (Shelton *et al.*, 1987; Kuan and Chee, 1990). The productivity of pastures under coconut plantations can be high when legumes are present, and coconut yields were increased with higher stocking rates of cattle (Rika *et al.*, 1981). Only a limited proportion of the rubber or oil palm plantations are grazed and there is certainly room for further introduction of animal production into such areas (Humphreys, 1991). Of a range of forage legume species tested, *A. pintoi*, *A. repens*, *S. guianensis* and *S. scabra* yielded well and were persistent under moderate shade (Ng *et al.*, 1997). Cattle may, however, cause some damage in oil palm plantations by grazing fronds in the lower canopy, although such damage can be minimized by carefully regulating the number of cattle (Kuan and Chee, 1990). Under rubber, *P. phaseoloides* and various grasses were progressively replaced by *C. caeruleum*, an unpalatable species, due both to selective grazing by sheep and shading (Chong *et al.*, 1997). Productivity of the pastures was poor when shading by rubber became intense. Thus inclusion of livestock should only be recommended with plantation trees in immature plantations, where production is less than optimal, or where palm density is intentionally reduced to allow better fodder production (Hartley, 1988). A more appropriate system practised by many smallholders is to 'cut and carry' fodder from underneath the plantation crop for livestock stabled at home.

Legume Trees for Shade

Shade trees have been used traditionally in plantations of coffee, tea, cacao and some other crops, such as quinine. In fact, shade trees are not essential for production of these plantation crops, but they can confer advantages over production of the crops without shade, and 'temporary' shade is essential for establishment of cacao. Crops grown without shade tend to grow faster and to be more nutrient demanding, thus

requiring greater inputs of fertilizers. If the unshaded crops are grown with sufficient amounts of fertilizer they tend to become self-shading, thus allowing production of the fruit in the lower parts of the canopy. However, if the crops are grown without large fertilizer additions they do not produce a sufficiently dense canopy to provide the shade necessary for crop production and so shade trees are required.

In addition to shading the plantation crop from direct sunlight, shade trees can provide other benefits, including protection from wind and frost, weed control, reduction of pest and disease attack, improvement of yield stability and crop quality, and in the case of some legume (e.g. *Leucaena*) or non-legume (e.g. *Grevillea robusta*) trees, can help to diversify production by providing timber. A summary of the properties required in shade trees is given by Beer (1987), and a review of their beneficial and negative effects can be found in Beer *et al*. (1998). Although the ability to fix N_2 is not a prerequisite for selection of trees for shade, it is certainly beneficial.

Leguminous shade trees

The use of legumes as shade trees is one of the earliest examples of the use of N_2-fixing trees in agroforestry (Chapter 12). Several species that were found to be useful as shade trees have found further applications in agriculture. *L. leucocephala* and *Calliandra calothyrsus* were used as shade trees for coffee, tea and quinine in Indonesia. *Leucaena* was used extensively for shade for cacao in Papua New Guinea but has been blamed for harbouring insect pests. The 'madre de cacao', *Gliricidia sepium*, is widely used as a shade tree for cacao and coffee in Central America and Southeast Asia (Siebert, 1987). Other N_2-fixing trees widely used in Latin America include species of *Inga*, *Erythrina*, *Paraserianthes* and *Acacia*. In Ceylon, *Gliricidia*, *Acacia* spp. and *Erythrina lithosperma* were widely used in tea plantations before nitrogen fertilizers became freely available in the 1960s. After this a policy of uprooting shade trees was introduced to improve yields (Marby, 1972). In Ethiopia, species of *Acacia*, *Erythrina* and *Leucaena* are widely used for shade (Teketay and Tegineh, 1991).

There have been few detailed studies of N_2-fixation by leguminous shade trees in plantations. In Mexico, assessments of nodulation and ARA of *Inga jinicuil* grown as a shade tree for coffee indicated large seasonal fluctuations in N_2-fixation, with two periods of intense activity which seemed to coincide with times of high N demand for leaf and pod development (Roskowski and van Kessel, 1985). As part of a study of nutrient cycling in plantations of cacao, *Erythrina poeppigiana* was estimated to contribute 18 kg N ha^{-1} $year^{-1}$ from N_2-fixation, though the results of this study were confounded by large differences in initial soil N contents and large annual fertilizer applications (Fassbender *et al*., 1988). Nodules of *E. poeppigiana* were found to be restricted to the surface 12 cm (Lindblad and Russo, 1986), and individual trees were estimated to have a biomass of nodules of up to 106–288 g, all of which died and senesced after pollarding of the trees (Nygren, 1995; Nygren and Ramirez, 1995). The nodules were estimated to provide 9% of the 137–238 g N contributed to the soil by each tree, the remainder coming from litterfall (24%) and the prunings

(67%). This would amount to a total input of up to 72 kg N ha^{-1} per pruning cycle (144 kg N ha^{-1} $year^{-1}$), assuming typical tree populations of up to 300 trees ha^{-1}. Others have estimated that not more than 60 kg N ha^{-1} comes from N_2-fixation in plantations of coffee and cacao with *Erythrina* shade trees in Central America, although more than 340 kg N ha^{-1} can be returned in tree litter (Beer, 1988; Beer *et al.*, 1998). Given the difficulties involved in measurement (Chapters 4 and 12), the inputs from N_2-fixation by shade trees and the effects on productivity of plantations certainly warrant further investigation.

Conclusions

Cover crops have a well-established and important role in the crop cycle of rubber and oil palm plantations, and legumes are the best choice, due to the inputs from N_2-fixation. Use of shade trees in plantations of crops such as coffee and cacao has advantages, particularly if markets for produce are uncertain, or on small holdings where large inputs of fertilizers for the growth of the plantation crop cannot be guaranteed. Under such conditions trees that provide a source of timber can be valuable, so that income is not solely dependent on the cash crop. The ability of legume cover crops or trees to grow fast in infertile soils is due, at least in part, to their ability to fix N_2.

Identification of a cover crop that can persist and fix N_2 under the shade of mature palms remains a target that may be hard to achieve (Corley *et al.*, 1976), though *A. pintoi* is one candidate species which deserves more widespread testing under plantation crops in Asia and Africa. Even if a shade-tolerant legume cover is identified, it is clear that, despite their many benefits, legume covers cannot wholly substitute for the amounts of N fertilizer required to maintain productive plantations.

Chapter 12

Agroforestry: N_2-fixing Trees in Integrated Agriculture

In its simplest definition, agroforestry is the cultivation of trees and crops together. The use of this term indicates the deliberate cultivation of trees within an agricultural setting, rather than forestry simply for wood production. Agroforestry is nothing new. Farmers have always depended on trees in slash-and-burn or shifting cultivation for the regeneration of soil fertility. A definition that summarizes the currently accepted view of agroforestry as a discipline (Young, 1997) is:

> Agroforestry is a collective name for land-use systems in which woody perennials (trees, shrubs, etc.) are grown in association with herbaceous plants (crops, pastures) and/or livestock in a spatial arrangement, a rotation, or both; there are usually both ecological and economic interactions between the tree and other components of the system.

This definition embraces the many types of agroforestry practice (Table 12.1) that are described, together with many more permutations on ways in which trees can be used in agriculture, by Combe and Budowski (1979) and Young (1997). Some types of plantations described in Chapter 11 clearly fall within the general definition of agroforestry when trees are used for shade, mixed with other crops or used for grazing.

Agroforestry is in no way restricted to the use of N_2-fixing trees, but these have a special role to play, particularly on degraded lands. Before detailed consideration is given to the uses of N_2-fixing trees it is worth describing briefly some of the more important species.

N_2-fixing Trees

N_2-fixing trees fall into two main groups: the nodulated legumes and the actinorhizal trees. Within the *Leguminosae*, the majority of the trees used for agroforestry are

nodulated members of the *Papilionoideae* and the *Mimosoideae*, but there are a few species in the *Caesalpinioideae* (many of which do not form nodules – see Chapter 2) which have been used experimentally in agroforestry. Detailed information on many more species is given by Allen and Allen (1981).

Nodulated legume trees for agroforestry

Acacia *(*Mimosoideae*)*

Acacia is the largest and most diverse genus of legume trees, containing approximately 1200 species, which are distributed throughout the tropics and subtropics (Brewbaker, 1987). Most species are found in the semiarid tropics and they are generally resistant to drought – due, at least in part, to having very deep root systems. *A. senegal* is now the major source of gum arabic (first produced from *A. arabica*), which has a wide variety of industrial uses, and is a major crop in Sudan. The black

Table 12.1. The main classes of agroforestry practices. In many cases there will be considerable overlap between the crop and livestock-based systems of agriculture. (After Young, 1997.)

Predominantly agrosylvicultural (trees with crops)
Rotational:
shifting agriculture
managed tree fallows, including relay intercropping
taungya (growing agricultural crops whilst establishing plantations)
Spatially mixed:
trees on cropland
perennial crop/tree combinations
multistrata systems (agroforests), including forest gardens, home gardens
Spatially zoned:
boundary planting (for hedges and biomass transfer)
trees on erosion control structures
windbreaks and shelterbelts (also sylvopastoral)
hedgerow intercropping (alley cropping), including tree-row intercropping
contour hedgerows
biomass transfer (cut-and-carry mulching)
Sylvopastoral (trees with pastures and livestock)
Spatially mixed:
trees on pastures (parkland systems)
perennial crops with pastures (including orchards)
Spatially zoned:
hedges and live fences
fodder or protein banks
Trees predominant
Farm and village forestry (including woodlots)
Reclamation agroforestry

wattle (*A. mearnsii*), which is frost-tolerant and can grow at high altitudes in the tropics, is an important source of tannins for leather treatment. Other species noted for their potential or actual use in agroforestry are *A. aneura*, *A. auriculiformis*, *A. holoserica*, *A. mangium* and *A. tortilis*, and there are many additional species that occur naturally in Australia and Southeast Asia that could be of widespread use in arid regions of the tropics (Turnbull, 1987).

The majority of *Acacia* species examined can form nodules although, as with *Prosopis*, these are often at considerable depths in the soil (several metres below the surface). Some species are unable to nodulate (e.g. *Acacia brevispica*) (Odee and Sprent, 1992) and this seems to be a particular feature of species that grow in very dry regions. Different species of *Acacia* fell into at least three cross-inoculation groupings when tested against ten strains of rhizobia isolated from West African soils (Dreyfus and Dommergues, 1981). In this study *A. mearnsii* and *A. albida* nodulated only with *Bradyrhizobium* strains (in fact, *A. albida* is no longer considered to be an *Acacia* species – see *Faidherbia* below); *A. senegal* nodulated only with *Rhizobium* strains; and *A. seyal* nodulated with both *Bradyrhizobium* and *Rhizobium* strains. The fast-growing *Rhizobium* strains isolated from acacias all nodulated *L. leucocephala* effectively, and conversely strain NGR8 isolated from *Leucaena* nodulated the *Acacia* species of the second group effectively. In another study, *A. mangium* and *A. auriculiformis* nodulated effectively with *Bradyrhizobium* strains from a wide range of tropical countries but formed ineffective nodules with fast-growing strains, and were said to belong to the *A. mearnsii* group (Galiana *et al.*, 1990; see also Turk and Keyser, 1992). In Kenyan soils, of nine *Acacia* spp. assayed, only nodules of *A. nubica* and *A. tortilis* yielded slow-growing isolates, and the majority of isolates from all species were (very) fast-growing (Odee *et al.*, 1997). Rhizobia isolated from the roots of *A. senegal* and *Prosopis chilensis* in Sudan were also mainly fast-growing (Zhang *et al.*, 1991). Analysis using partial 16S rRNA gene sequences indicated that one strain belonged to the *R. huakuii* branch whilst the majority were *Sinorhizobium*, showing affinities to *S. fredii*, *S. meliloti*, *S. terangae* and *S. saheli* (Haukka *et al.*, 1996). There were also two distinct clusters among the *Sinorhizobium* strains, which led to the description of a further two species: *S. arboris* and *S. kostiense* (Nick *et al.*, 1999a). Other strains from this collection, together with isolates from *Acacia* spp., *Prosopis juliflora*, *Leucaena* spp. and *Chamaecrista ensiformis* from Senegal and Brazil, led to the description of *Mesorhizobium plurifarium* (de Lajudie *et al.*, 1998b).

Calliandra *(*Mimosoideae*)*

C. calothyrsus, which originated in Central America, was introduced into Java in 1936 by foresters in the search for an alternative to *Leucaena* as a shade tree for higher altitudes. *C. calothyrsus* is a tall shrub (4–6 m) often found in thickets on steep slopes from Mexico to Panama (Anon., 1983). Of the 100 or so other *Calliandra* species that occur in Central and South America, only *C. tetragona* has been widely tested for its use in forestry. As this species was found to be slow growing, it has been neglected in favour of *C. calothyrsus*. Farmers in Java have widely adopted *C. calothyrsus* and use it for both fuel wood and animal fodder (Fig. 12.1). It responds readily to coppicing and is said to have roots that penetrate deep into the soil and also roots in the upper

Fig. 12.1. Farmers in Java carrying maize and *Calliandra* cut to feed stalled cattle.

soil horizons. *Calliandra* is suited to growth in humid areas with annual rainfall of 2000–4000 mm on soils with good drainage, as waterlogging can rapidly kill the trees. It is among the best-adapted N_2-fixing trees for acid soils (Powell, 1998). In Java it grows best at altitudes from 250 to 800 m, although it can grow at up to 1800 m in its native Central America.

Calliandra nodulates predominantly with fast-growing rhizobia and shares a loose cross-inoculation group with *Leucaena* and *Gliricidia* (Turk and Keyser, 1992; Lesueur *et al*., 1996; Bala and Giller, 2001). Compatible indigenous rhizobia that nodulate *Calliandra* are widespread in soils across the tropics and include representatives of the fast-growing genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Bala, 1999).

Erythrina (Papilionoideae)

Several species of this large pan-tropical genus have been traditionally used for shade and soil improvement (Chapter 11) (Neill, 1993). In Central America *E. fusca* and *E. poeppigianna* are widely used for shade in coffee and cacao as well as for fuelwood, fodder and live fences (Russo, 1993; Kass, 1994). The soil-improving properties of *Erythrina* have long been recognized in plantations in Sri Lanka (Chapter 11), but although many species of this genus occur in East Africa, few have been actively exploited in agroforestry (Jaenicke and Owino, 1993).

Erythrina spp. are nodulated by *Bradyrhizobium* (Allen and Allen, 1936; Gross *et al*., 1993) and are not infected by fast-growing rhizobia (Nakao *et al*., 1993). The roots are infected both through root hairs and through epidermal cracks (de Faria, 1993).

Faidherbia (Mimosoideae)

Faidherbia albida (syn. *Acacia albida*) is the only species in this genus and is found throughout the drier regions of sub-Saharan Africa (Wickens, 1969). It has the unusual characteristic of shedding its leaves during the rainy season, known as 'reverse phenology', and in many countries is maintained within fields by farmers who plant crops underneath the trees as well as in the open field. The leaves are shed on to the soil at the same time as the crop is planted and so there is a less dense shade under the trees during the cropping season. The leaves and pods are a valuable source of fodder during the dry season.

F. albida nodulated only with slow-growing *Bradyrhizobium* strains in soils from West Africa (Dreyfus and Dommergues, 1981; Lesueur *et al*., 1993; Dupuy *et al*., 1994). Isolates from *F. albida* nodules in Kenyan soils were fast and slow-growing in roughly equal proportions (Odee *et al*., 1997). Some isolates from *Gliricidia* nodules nodulated *Faidherbia* effectively (Bala and Giller, 2001).

Flemingia (Papilionoideae)

The genus *Flemingia* (syn. *Moghania*) contains 40 species indigenous to tropical regions of Asia, Africa and Australia. *F. macrophylla* (syn. *F. congesta*) is the species in which there is the greatest current interest for soil improvement in agroforestry, although *F. strobilifera* has been used as a cover crop in coconut plantations in Trinidad and *F. lineata* was used in Malaysia as a green manure (Allen and Allen, 1981). *F. macrophylla* is reported to be well adapted to acid soils with high aluminium saturation. As *Flemingia* is found within the tribe *Phaseoleae* it is likely that it transports its fixed N as ureides (Chapter 4). This gives rise to the potential for using the ureide method for measurement of N_2-fixation in species of this genus, although this appears not to have been tested. *Flemingia* is effectively nodulated by *Bradyrhizobium* strains (Turk and Keyser, 1992) but only formed ineffective nodules with a few fast-growing isolates tested (Bala and Giller, 2001).

Gliricidia (Papilionoideae)

The genus *Gliricidia* is indigenous to tropical America, where the small trees are commonly found up to altitudes of 2000 m. The pink-flowered *G. sepium* is the most widely known species and has often been treated as being synonymous with the white-flowered *G. maculata* (Simmons and Stewart, 1994). *Gliricidia* has been used as a shade tree for coffee and cacao, as its common Latin American name 'madre de cacoa' testifies. The bark and seeds have been used as poisons for rats and the value of the foliage for fodder is the subject of disagreement. It appears that the leaves are toxic to horses but can provide high-quality feed for cattle and goats. The ease with which *Gliricidia* regenerates when short sticks are planted in the ground makes it an ideal species for 'live fencing'.

G. sepium nodulates and fixes N_2 effectively with fast-growing rhizobia. Slow-growing strains formed ineffective nodules (Akkasaeng *et al*., 1986). Isolates from nodules of *Gliricidia* were mainly related to *R. tropici*, but some isolates were placed in the genera *Sinorhizobium* and *Agrobacterium* (Bala, 1999).

Leucaena (Mimosoideae)

The taxonomy of this Central American genus has been extensively revised and 22 species are now recognized (Hughes, 1998). Four of these species are tetraploids, three of which appear to have arisen by interspecific hybridization. Species that have excellent agroforestry potential include *L. leucocephala*, *L. diversifolia*, *L. esculenta* (Pound and Martinez-Cairo, 1983; Anon., 1984a) and *L. pallida*, of which all but *L. esculenta* are tetraploid. Earlier reports on adaptation and productivity of *L. esculenta* and *L. pallida* may be confused, as *L. pallida* was considered a subspecies of *L. esculenta*. Within *L. leucocephala* three distinct subspecies are recognized: the bushy (to 5 m) 'Common' type is ssp. *leucocephala*; the tall (to 15 m) extensively branched 'Peru' type and the tall (to 20 m) trees with unbranched trunks known as the 'Salvador' type or 'Hawaiian Giant' are both placed within ssp. *glabrata*; and a variant recently discovered in northern Guatemala is ssp. *ixtahuacana* (Hughes, 1998).

The Hawaiian type was introduced into the Philippines, probably in the 16th century, via the Spanish galleon trade route and spread through Southeast Asia and the Pacific Islands (Brewbaker and Hutton, 1979). By the end of the last century *Leucaena* was widely distributed in the tropics from Hawaii to South Asia, Africa and the Caribbean islands. *Leucaena* has many uses. It is fairly certain that the Spanish used *Leucaena* as fodder and bedding for animals and its fast growth led to its widespread use as a shade tree for plantation crops (e.g. coffee, quinine) in the 19th century. The wood is useful as timber, for fencing posts and for charcoal and the seeds are eaten as a delicacy in Mexico. *Leucaena* has a high nutritive value as fodder, although the large content of the alkaloid mimosine can lead to problems of toxicity (see below). The prolific seed production of *Leucaena*, which flowers year-round, means that it can be a problematic weed (Hughes and Jones, 1998).

The strong, deep root system allows *Leucaena* to tolerate drought and, once established, it can survive in areas with only 600 mm annual rainfall but it does not tolerate extended flooding. *L. leucocephala* is sensitive to low temperatures whereas *L. diversifolia* and *L. esculenta* show better growth at altitudes above 1500 m. One of the major limitations of *L. leucocephala* is its poor growth on acid soils. Although improvement of acidity tolerance has been achieved by crossing with more acid-tolerant accessions of *L. diversifolia* (Hutton, 1990), widespread testing of many types of *Leucaena* led Mullen *et al*. (1998b) to conclude that effort to identify agroforestry species for acid and infertile soils would be more effective if focused on other genera (see, for example, Evans and Szott, 1995). *Leucaena* was devastated in many countries by a sucking psyllid insect (*Heteropsylla cubana* – from the Caribbean, as its name suggests) a surprisingly long time after the initial introduction of the plant, since when no serious pests had occurred. In Southeast Asia a balance seems to have been established between the psyllid and its natural predators such that this is unlikely to be a serious long-term problem. The psyllid arrived in Africa in the early 1990s and caused massive damage as it rapidly spread through many countries.

L. leucocephala can nodulate profusely and nodule masses of 51 kg ha^{-1} have been recorded in the top 70 cm of soil, with the majority of the nodules at a depth of 10–30 cm (Högberg and Kvarnström, 1982). The young indeterminate nodules are spherical but these elongate and branch to form coralloid nodules up to 3 cm in

diameter (Halliday and Somaseragan, 1982). Growth of *Leucaena* is improved by rhizobial inoculation on some soils of Australia (Diatloff, 1973), Colombia (Halliday and Somaseragan, 1982), Africa (Högberg and Kvarnström, 1982; Hodges *et al.*, 1983; Sanging *et al.*, 1986), Malaysia (Chee *et al.*, 1989) and Thailand (Homchan *et al.*, 1989a), although in several cases indigenous rhizobia are present which can nodulate *Leucaena* (e.g. Bushby, 1982; Homchan *et al.*, 1989a). *Leucaena* is nodulated by fast-growing rhizobia isolated from several tropical hosts but generally not by most temperate *Rhizobium* species or by slow-growing rhizobia (Trinick, 1968; Jarvis, 1983; Lewin *et al.*, 1987).

Fast-growing *Leucaena* rhizobia inoculated into acid soils were found to survive well, suggesting that the lack of tolerance in the plant rather than the bacterium is the cause of poor growth and nodulation under acid conditions (Halliday and Somaseragan, 1982), but the results of Norris (1973) suggest that this acid-tolerance is not shared by all *Leucaena* rhizobial strains. There is a degree of host species by strain interaction for effectiveness of N_2-fixation between species of *Leucaena* (Mullen *et al.*, 1998a). One slow-growing alkali-producing strain was isolated from *Leucaena* – i.e. *Bradyrhizobium* sp. (*Leucaena*) – which gave good nodulation on *Leucaena* grown in an acid soil (pH 5) whilst the strain then recommended for *Leucaena* in Australia only nodulated if lime was added (Norris, 1973). Sanginga *et al.* (1989b) reported that some slow-growing, alkali-producing strains of rhizobia (*Bradyrhizobium* spp.) isolated from *Tephrosia vogelii* and *Faidherbia albida* could nodulate *Leucaena* and one of these was effective in N_2-fixation.

The fast-growing rhizobia that effectively nodulate *Leucaena* include strains of *R. tropici* (Martínez-Romero *et al.*, 1991), *R. etli* (Hernandez-Lucas *et al.*, 1995) and *S. fredii* (Trinick, 1980). *L. leucocephala* isolates from Mexico (Wang *et al.*, 1999a) and in soils across the tropics (Bala and Giller, 2001) were placed in the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*.

Prosopis (Mimosoideae)

The 44 species of *Prosopis* are found in semiarid and arid regions throughout the tropics (Anon., 1979). A number of species are of interest for use in agriculture largely due to their ability to withstand extreme drought, and their pods are an important animal fodder in many countries (Felker, 1979). *P. pallida* grows naturally along the dry coastline of Ecuador and Peru. In India, *P. cineraria* and *P. juliflora*, originally from Central America, are commonly planted on field boundaries, where they provide an important source of firewood and fodder. Most species of *Prosopis* produce seed prolifically and can be difficult to control, particularly thorny species, which are more resistant to heavy grazing. Wood from *Prosopis* spp. makes excellent charcoal.

Although nodules may not be found on *Prosopis* roots in the surface soil, this does not mean that the plants are not nodulated. The ability of these plants to grow in arid regions is due to their ability to reach and exploit water deep in the soil, and nodulated roots have been reported at 4 m below the soil surface, where the numbers of compatible rhizobia may be much greater (10^4 cells g^{-1} soil) than in the surface soil (< 10 cells g^{-1} soil) (Virginia *et al.*, 1986). *Prosopis cinerarea* is nodulated by many

fast-growing rhizobia that are also effective on *Leucaena*, *Gliricidia*, *Calliandra* and *Acacia* spp. (Herrera *et al*., 1985; Bala and Giller, 2001). There are also reports of nodulation of *Prosopis* spp. by slow-growing rhizobia (Jenkins *et al*., 1987). As indicated above, *Prosopis* and some species of *Acacia* share similarities in rhizobial affinities; *Sinorhizobium arboris*, *S. kostiense* and *Mesorhizobium plurifarium* have all been isolated from *Prosopis* nodules in African soils (de Lajudie *et al*., 1998b; Nick *et al*., 1999b).

Sesbania *(*Papilionoideae*)*

The 60 or so species of *Sesbania* are distributed throughout the tropics, with representatives native to all continents (Gillett, 1963; Evans and Rotar, 1987; Evans, 1990), and vary from annuals to short-lived perennials. *S. bispinosa* (syn. *S. aculeata*), *S. cannabina*, *S. grandiflora*, *S. sesban*, *S. speciosa* and *S. rostrata* have traditionally been used or have been investigated for use, in agroforestry. Differences between *S. bispinosa*, *S. aculeata* and *S. cannabina* are small and these are sometimes considered to be a single species (Evans and Rotar, 1987). The large white flowers of *S. grandiflora* are eaten as a vegetable in Indonesia and this perennial species is widely used for fuelwood production. Most species of *Sesbania* are palatable to livestock. A major advantage of *Sesbania* is that many species are tolerant of waterlogging and of saline and alkaline conditions. The stem-nodulating species *S. rostrata* has excited intense interest due to its ability to grow and fix N_2 in waterlogged conditions and its fast growth rate.

Rhizobia nodulating roots of *Sesbania* spp. are generally fast-growing strains, and often exhibit rapid growth, producing large (> 4 mm) colonies in less than 24 h, but a few slow-growing isolates have been recorded (Odee, 1990; Odee *et al*., 1995). There can be marked host specificity for isolates between different *Sesbania* species. The young root nodules are round, but older nodules are multi-lobed (Harris *et al*., 1949). Two species of *Sinorhizobium* were described from root nodules of species of *Sesbania* and *Acacia* in Senegalese soils: *S. saheli* and *S. terangae* (de Lajudie *et al*., 1994). *S. saheli* consists solely of isolates from nodules of several *Sesbania* species; *S. terangae* comprises isolates from both *Sesbania* and *Acacia*. A further species, *Rhizobium huautlense*, represents a narrow genetic group of isolates from nodules of *Sesbania herbacea* grown in Mexican soils (Wang *et al*., 1998). Isolates from nodules of *S. sesban* grown in a wide range of African soils predominantly belonged to the genus *Mesorhizobium*, although *Rhizobium*, *Sinorhizobium* and *Allorhizobium* were all represented (Bala, 1999).

The symbionts isolated from the stem nodules of *S. rostrata* are particularly interesting as they belong to a distinct genus, *Azorhizobium*, containing the single species *A. caulinodans* (Dreyfus *et al*., 1988) (Chapter 2). It was later found that *S. saheli* and *S. terangae* can also induce stem nodules on this legume, but there appear to be no reports of *Sinorhizobium* strains isolated from stem nodules in the field.

Tephrosia *(*Papilionoideae*)*

Two species of this large genus, which contains some 400 species, are widely used in agroforestry: *T. candida* and *T. vogelii*. Both species have been widely used to provide

shade for tea and coffee, and for soil improvement. *T. vogelii* is better known in Africa and *T. candida* in Asia. Both species are widely known as the 'fish bean', due to the high content of rotenoid alkaloids, which can kill fish at low concentrations. *Tephrosia* is nodulated by *Bradyrhizobium* strains (Turk and Keyser, 1992) but appears not to be effectively nodulated by most fast-growing rhizobia (Bala and Giller, 2001).

Other nodulated legume trees

The species considered above by no means constitute an exhaustive list of all the nodulated legume trees that have been used or that may be used in the future. Other species of N_2-fixing legume trees and shrubs that show potential for use in agroforestry are *Albizia lebbeck*, *Cajanus cajan* (described in Chapter 8), *Inga* spp. and *Paraserianthes* (syn. *Albizia*) *falcataria* (Zabala, 1994).

Non-nodulating legume trees for agroforestry

The non-nodulating trees that have been used in agroforestry mainly belong to the *Caesalpinioideae* – and more specifically to what was formerly the genus *Cassia*. Taxonomic revision of the genus *Cassia* led to the recognition of three separate genera: *Cassia*, *Senna* and *Chamaecrista* (the latter contains only nodulating species) (Irwin and Barneby, 1981). The species *Cassia siamea* and *C. spectabilis* are now placed within the genus *Senna* (and will therefore be referred to as *S. siamea* and *S. spectabilis*), from which no species have been found to nodulate. Despite the lack of nodulation, these two species have been tested in agroforestry and other species now placed within this genus (e.g. *S. cobanensis*) have been used as shade plants in tea and coffee plantations (Allen and Allen, 1981). The vigorous growth of *S. tora* was once the source of speculation that it might be able to fix N_2 in symbiosis with bacteria without forming nodules, but all attempts to demonstrate bacteria in root tissues proved negative (Allen and Allen, 1933). There are no reports of nodulation within the species of the tribe *Cassieae* that have been used in agroforestry.

Actinorhizal trees for agroforestry

Of the actinorhizal trees, the alders (*Alnus* spp.) are used at high altitudes for timber in several Central American countries (e.g. Holdridge, 1951; Russo, 1990, 1995) and the Himalaya (Cairns and Garrity, 1999) but these trees are not adapted to the climates of the lowland tropics. Species of *Casuarina* are the most important non-leguminous N_2-fixing trees in the lowland tropics.

Casuarina (Casuarinaceae)

Four genera are now recognized within the *Casuarinaceae*: *Allocasuarina*, *Casuarina*, *Gymnostoma* and *Ceuthostoma*, which together contain about 90 species native to

Australasia, Malaysia and Polynesia (Diem and Dommergues, 1990). The highly reduced leaves and photosynthetic branchlets assist in the remarkable drought tolerance of casuarinas and, together with their cone-like inflorescences, give them the superficial appearance of gymnosperms, although they are of course angiosperms (Anon., 1984b). The most widely used species is *C. equisetifolia*, which is tolerant of saline soils and of winds; two other quite widely cultivated species are *C. cunninghamiana* (which is sensitive to salinity) and *C. glauca* (Anon., 1984b). The substantial variability between different provenances of the same species suggests that improved types could be selected. Casuarinas are readily propagated by seed or from cuttings.

Marked differences in effectiveness of N_2-fixation have been found both between different strains of *Frankia* (Reddell and Bowen, 1985) and between different accessions of *Casuarina* (Sanginga *et al.*, 1990b). In a comparison of three clones of *C. equisetifolia* inoculated with three *Frankia* strains, the host-plant genotype was dominant in determining the amount of N_2-fixation (Sougoufara *et al.*, 1992).

Requirements for inoculation

As discussed elsewhere (Chapters 8, 10 and 14), only those legume species that are very specific in their requirements for rhizobia will generally need inoculation to ensure nodulation. Where compatible rhizobia are present, responses to inoculation are unlikely unless the number of compatible rhizobia is small ($< 50\ g^{-1}$ soil) or unless a rigorous selection for adapted, competitive strains has been carried out. Rhizobial counts can be used to assay whether inoculation is necessary (Turk and Keyser, 1993; Turk *et al.*, 1993). Of the legume trees described here, *Leucaena*, some *Acacia* and some *Sesbania* species have more specific rhizobial requirements and may need inoculation when introduced into new regions. Of the most commonly used fast-growing trees, *C. calothyrsus*, *G. sepium* and *L. leucocephala* have a limited ability to cross-nodulate, whereas *S. sesban* falls into a completely discrete group (Turk and Keyser, 1992; Bala and Giller, 2001). Among the first three species, it appears that *Gliricidia* and *Leucaena* most often need to be inoculated (Bala, 1999), though this has rarely been done. *Sesbania* more commonly requires inoculation when planted on upland soils that are outside its common habitat and this requirement is discussed in more detail below. Inoculation may also assist in establishment and early growth of N_2-fixing trees in nurseries (Sutherland *et al.*, 2000).

It is notable that in few research projects in which N_2-fixing trees have been evaluated for growth in new areas has care been taken to ensure that the trees are nodulated, and so species that require inoculation, but are otherwise suitable for use, may well have been overlooked. *Casuarina* species often benefit considerably from inoculation with *Frankia* when first planted in a soil, particularly as they are often used on very marginal lands (Reddell *et al.*, 1988). In the first 3 years after establishment, inoculation doubled wood production in some *C. cunninghamiana* provenances.

How Much N_2 Can Trees Fix?

Of all the N_2-fixing symbioses, trees are the most problematic for measurement of the amounts of N_2 fixed. Difficulties encountered in measuring N_2-fixation in grain and pasture legumes have already been discussed at length (Chapter 4) and these are magnified enormously when we consider trees. Root systems may go very deep into the soil (this is one of the main criteria used to select N_2-fixing trees for agroforestry) and it is nearly impossible either to recover roots and nodules from depth to assess the total N content of the trees or to label the soil with ^{15}N fertilizer evenly to sufficient depths. It is likewise difficult to select suitable reference crops, given the wide variety of rooting patterns among tree species. It is possible to satisfy the assumptions of the methods where root growth is restricted in large pots, but this risks making experiments unrealistic. Few trees used in agroforestry transport their fixed N as ureides, so application of this method is limited to a few species in the tribe *Desmodieae* (Table 4.3) although, as mentioned above, *F. macrophylla* is a member of the tribe *Phaseoleae* and probably transports its fixed N as ureides.

N accumulation may be indicative of the possible benefits from N_2-fixation but cannot actually distinguish N_2-fixation from the ability to scavenge soil N from depth. The figure of 500–600 kg N ha^{-1} $year^{-1}$ fixed for *Leucaena* is commonly quoted, but these are measurements of total N accumulated above ground by densely planted saplings and are not necessarily due to N_2-fixation (Hutton and Bonner, 1960; Guevarra *et al*., 1978). Comparison of N accumulation between inoculated and uninoculated *Leucaena* in the field indicated that 224–274 kg ha^{-1} of symbiotically fixed N were harvested in prunings after 6 months (Sanginga *et al*., 1986), but again inoculation could also have increased uptake of soil N by increasing the size of the rooting system.

Other measurements of N_2-fixation in trees grown in Leonard jars (Van Kessel and Nakao, 1986) or in pots (Sanginga *et al*., 1990a,b,c) have been made using ^{15}N-labelled fertilizers. Comparisons of *S. rostrata* and *S. sesban* grown in pots containing 33 kg of ^{15}N-labelled soil indicated that *S. rostrata* fixed between 80 and 110 kg N ha^{-1} in 60 days whilst *S. sesban* fixed only 7–18 kg N ha^{-1} (Ndoye and Dreyfus, 1988). Some studies have used concrete cylinders buried in the soil to restrict lateral root growth (Sougoufara *et al*., 1990) or have enclosed plots within concrete-filled trenches (Baker, 1990). Whilst these may be useful methods for comparing rates of N_2-fixation in different tree species or between different accessions of the same tree, they do not tell us how much N is fixed by the trees growing naturally in the field. This approach was effective in demonstrating that N_2-fixation is progressively suppressed when substantial quantities of legume pruning N are returned to the soil (Kadiata *et al*., 1998).

In field plots where the roots were not restricted, *Leucaena* was estimated to fix 134 kg N ha^{-1} when inoculated with strain IRc1045 and 76–98 kg N ha^{-1} with strain IRc1050 (between 34 and 39% of shoot N), over 6 months' growth using an uninoculated treatment as a reference for both N-difference and ^{15}N isotope dilution calculations (Sanginga *et al*., 1989c). The ^{15}N-labelled microplots used in this study measured only 1.5 m across, but *Leucaena* roots commonly spread more than 1 m

from the tree trunk in the surface soil (Hairiah and van Noordwijk, 1986). Even where unharvested border rows were included in ^{15}N-labelled microplots (e.g. Peoples *et al.*, 1996), or where a strip 3 m wide was applied along a hedgerow (Hairiah *et al.*, 2000), the trees sampled were still less than 1.5 m from the edge of the plot. There are many other examples where small microplots have been used to measure N_2-fixation in the field, and an equal number which show that root systems of many legume trees can extend at least 4–8 m in the surface soil. Given these concerns, the natural abundance method appears to be the best choice for measuring N_2-fixation by trees growing in the field (Boddey *et al.*, 2000).

Yoneyama *et al.* (1990b) used ^{15}N natural abundance to estimate that between 60 and 100% of the N in leaves of N_2-fixing trees growing in Thailand was derived from N_2-fixation. Similar $\delta^{15}N$ estimates of N_2-fixation in trees and shrubs in Southeast Asia and Australia showed wide variation in the proportion of N from N_2-fixation (Table 12.2) (Peoples *et al.*, 1991b). The ^{15}N natural abundance method has the advantage that it enables estimates of N_2-fixation in mature trees already growing in the field by using non-N_2-fixing neighbouring shrubs or weeds as reference plants. Such estimates are only likely to be approximate, but then so are ^{15}N isotope dilution or N-difference estimates from even the most carefully controlled experiments. At least this approach has the virtue of reflecting the real, field situation.

However, in assessing the benefits derived from N_2-fixation by trees in the long term, we need to know whether trees continue to fix N_2 throughout their growth. This is problematic. With time, the soil under the trees will become enriched with N that was initially derived from fixation and was then returned to the soil by decay of plant leaves and roots (Van Kessel *et al.*, 1994). The fact that some of the mineralized soil N, which now has a $\delta^{15}N$ characteristic of fixed N, will be absorbed by the plant, coupled with the fact that new leaves will be formed partly from N stored within the plant, means that ^{15}N natural abundance estimates may not be useful for indicating current rates of N_2-fixation. The ureide method would be ideal for testing whether mature trees fix substantial amounts of N, as it provides an indication of the proportion of the N in the xylem stream that comes from current N_2-fixation (Chapter 4), but, as indicated above, few legume trees transport N in the xylem in the form of ureides.

There is still a dearth of information on the amounts of nitrogen fixed in the field by trees, mainly due to the enormous task of destructively harvesting and then analysing mature trees to estimate their total N content. In particular, there are no ^{15}N or N-difference based estimates of the amounts of N_2-fixation in the field which take full account of the amount of fixed N in the roots and trunk of the plants. Allometric methods are available that can be applied to estimate biomass of large trees (e.g. Nygren *et al.*, 1993; Ketterings *et al.*, 2001), so that destructive assays of whole trees may not be needed when estimating contributions from N_2-fixation. Pot experiments have indicated that as much as 60% of the total N can be in the roots of young *Leucaena* saplings (Sanginga *et al.*, 1990d), which suggests that amounts of N fixed may have been substantially underestimated. Indeed, roots of N_2-fixing trees contained 60 and 133 kg N ha^{-1} in the surface 60 cm of soil (Schroth *et al.*, 1995a), although some species such as *G. sepium* may contain little N below ground (Schroth

Table 12.2. Estimates of amounts of N_2-fixation in trees and shrubs (see also Tables 8.2 and 9.1).

	N_2 fixed					
Species	kg N ha^{-1}	%	Time period	Assay conditions	Method[b]	Ref.[c]
Legumes						
Acacia auriculiformis/ mangium	–	52–66	–	Leaves of mature trees	NA	1
A. erioloba		21		Leaves of mature trees	NA	2
A. hebeclada		16		Leaves of mature trees	NA	2
A. hereroensis		49		Leaves of mature trees	NA	2
A. holoserica	3–6	30	7 months	Prunings	NA	2
A. karroo		25		Leaves of mature trees	NA	2
A. kirkii		19		Leaves of mature trees	NA	2
A. melanoxylon	46	43	2 years 3 months	Total biomass of establishing saplings	NA	3
A. mellifera		71		Leaves of mature trees	NA	2
A. mucronata	116	48	2 years 3 months	Total biomass of establishing saplings	NA	3
A. reficiens		22		Leaves of mature trees	NA	2
A. tortilis		13		Leaves of mature trees	NA	2
Cajanus cajan	–	65	–	Regrowth	NA	1
	71–118	53–72	6–9 months	Biomass of fallows	NA	4
Calliandra calothyrsus	–	0–14	–	Regrowth	NA	1
	1026	24–84	2 years 3 months	Prunings	NA	5
	4–21	10–40	6 months	Biomass of 6-month old fallows	NA	4
Cordariocalyx gyroides	–	48–86	–	Prunings	NA	5
	–	39–94	–	Prunings	Ureides	5
Crotalaria grahamiana	116–162	36–80	6–9 months	Biomass of fallows	NA	4
Desmodium rensonii	–	68–84	–	Regrowth	NA	1
Faidherbia albida		2		Leaves of mature trees	NA	2
		32–52		Leaves of mature trees	NA	6
Flemingia macrophylla	27	24	1 year	Prunings	NA	7
Gliricidia sepium	–	26–75	–	Regrowth	NA	1
		30–55	–	1–3-year-old trees	NA	8
	1063	56–89	2 years 3 months	Prunings	NA	5
	146–204	54–92	1 year	Prunings	NA	9
	19	37	1 year	Prunings	NA	7
	70	44–58	203 days	Prunings	NA	10
Leucaena leucocephala	224–274	56	–	Prunings	Diff	11
	76–133	34–39	–	Prunings	Diff	12
Paraserianthes falcataria	–	60–100	–	–	NA	13
	–	55		Leaves of mature trees	NA	1
Prosopis glandulosa		30		Leaves of mature trees	NA	2
Sesbania grandiflora/ sesban	–	78–86		Regrowth	NA	1
S. sesban						
	7–18	–		Pot plants grown in 20 kg soil; 2 months old	Diff/ID	14

Table 12.2. *continued*

Species	N_2 fixed kg N ha^{-1}	N_2 fixed %	Time period	Assay conditions	Method[b]	Ref.[c]
S. rostrata	83–109	–		Pot plants grown in 20 kg soil; 2 months old	Diff/ID	14
	–	93–100		–	NA	13
Tephrosia vogelii Actinorhizal trees	98–124	58–73	6 months	Biomass of fallows	NA	4
Casuarina equisetifolia	40–60	39–55		Plants grown in 1m^3 soil; 11 months old	Diff/ID	15
	4–15[a]	54–76		Pot plants grown in 50 l of soil; 12 months old	Diff	16
	2–11[a]	34–60			ID	16
	2–25[a]	25–75		Pot plants grown in 10 l soil; 9 months old	ID	16
	45	24–39	3 years	Young plantation trees	Diff/NA	18
	–	65–90		–	NA	13
C. cunninghamiana	4–29[a]	14–76		Pot plants grown in 10 l soil; 9 months old	ID	17

[a]mg N per plant.
[b]NA = natural abundance; Diff = N-difference; ID = ^{15}N isotope dilution.
[c]1: Peoples *et al.*, 1991b; 2: Schulze *et al.*, 1991; 3: Hamilton *et al.*, 1993; 4: Gathumbi *et al.*, 2001a; 5: Peoples *et al.*, 1996; 6: Phombeya *et al.*, 2001; 7: Hairiah *et al.*, 2000; 8: Ladha *et al.*, 1993; 9: Nygren *et al.*, 2000; 10: Rowe *et al.*, 1999; 11: Sanginga *et al.*, 1986; 12: Sanginga *et al.*, 1989a; 13: Yoneyama *et al.*, 1990b; 14: Ndoye and Dreyfus, 1988; 15: Gauthier *et al.*, 1985; 16: Sougoufara *et al.*, 1990; 17: Sanginga *et al.*, 1990b; 18: Mariotti *et al.*, 1992.

and Zech, 1995; Nygren and Cruz, 1998). In Indonesia, destructive harvesting showed that 26% of the N in *G. sepium* managed under alley cropping on an acid Ultisol was in the roots, 13% in the trunk and 61% in the small branches and leaves (Rowe, 1999).

Uses of N_2-fixing Trees in Agroforestry

Many N_2-fixing trees can grow rapidly on poor soils, due at least partly to their ability to fix N_2, and are thus chosen to form the tree component of agroforestry systems. As with any other tree species, they may be selected either because they produce a needed product – timber, fuelwood or fodder – or simply because of their overall contribution to soil fertility. Each of these will be considered in turn.

Fuelwood and timber

Of the actinorhizal trees, casuarinas are the only species that are widely used in the tropics specifically for fuelwood production. The wood is dense and has a high calorific value (Anon., 1984b). As casuarina wood splits and warps on drying, it does not have much value for timber, though the poles are strong and can be used for construction work. *Casuarina* trees are often grown as windbreaks on farms as well as being widely used for stabilizing coastal sand dunes or for reclaiming degraded lands (Diem and Dommergues, 1990). In Sabah, Malaysia, fast-growing legume trees are used extensively for replacement of overcropped, degraded forests. *Acacia mangium* and *Albizia lebbek* are two of the species used for production of wood chips (Jones, 1983). In Rwanda, farmers are keen to grow tree legumes such as *C. calothyrsus*, *S. sesban* and *L. diversifolia* as they provide long stakes when coppiced, which are needed to support climbing varieties of *Phaseolus vulgaris* (see Fig. 8.1). Many other species are used to provide fuelwood, including several *Acacia* and *Prosopis* spp. (Ryan, 1994).

Trees as food and forage producers

Over 200 species of tropical legume trees and shrubs are reported to be useful for fodder production and most of these fix N_2 (Brewbaker, 1987; Gutteridge and Shelton, 1994). The high protein content of foliage and the protein and carbohydrate content of pods of many species of N_2-fixing trees makes them important complementary feedstuffs (Blair *et al.*, 1990; Topps, 1992). However, several species (e.g. *Prosopis*, *Acacia*) are heavily armed with thorns to protect them from grazing (particularly while the young plants establish) and others contain toxins. Some of these toxins are antifeedants and antinutritive compounds (such as tannins and cyanogenic compounds), which deter animals from grazing, and others are directly poisonous to animals. *C. calothyrsus* contains large amounts of highly reactive polyphenols, which reduce protein digestibility of the forage (Norton and Ahn, 1997). The leaves of some trees such as *Gliricidia* are not readily accepted by animals as fodder when first encountered but can become a preferred choice once the initial rejection is overcome by feeding the animals on a pure diet of *Gliricidia* (Atta-Krah and Sumberg, 1988). *L. leucocephala*, which is the most widely used forage tree legume, contains large amounts of mimosine, an amino acid that is highly toxic to non-ruminants (Shelton and Brewbaker, 1994). Mimosine is broken down in ruminants to produce DHP (3-hydroxy-4(1h) pyridone), which is a potent goitrogen (Jones and Bray, 1983). Cattle in India, Indonesia, Hawaii, Brazil and the Philippines are able to break down DHP and an inoculum from their rumens can transfer this ability to Australian cattle (Jones, 1986; Quirk *et al.*, 1988).

Trees can be important sources of fodder or 'browse' in natural vegetation (Le Houérou, 1980). Trees can also be incorporated into extensive grazing systems as hedgerows or 'live fences' (e.g. *Gliricidia*), either by including trees within the pasture for direct browsing by the animals, or by maintaining areas of trees as 'fodder banks' that the animals are periodically allowed to graze. Where animals are kept in stalls,

tree foliage is often cut and carried to the animals and can be used for silage (Tjandraatmadja *et al*., 1993). *A. lebbeck* has been identified as a promising species for inclusion in pastures in Australia (Prinsen, 1986; Lowry, 1989; Lowry *et al*., 1994). Naturalized *Calliandra* and *Leucaena* provide an important feedstuff for stalled cattle in Java (Fig. 12.1). However, the usefulness of trees as forage is not limited solely to N_2-fixing species. Many non-nodulating legume trees, such as *Bauhinia* spp., *Ceratonia siliqua*, *Cassia* and *Senna* spp., are also excellent sources of fodder, as are many non-legumes (Brewbaker, 1987). Detailed discussion of uses of N_2-fixing trees for fodder throughout the tropics can be found in Daniel and Rochetko (1998) and Gutteridge and Shelton (1994).

Some tree legumes produce edible fruits, pods or leaves and are grown on farms primarily for this purpose (Zimsky, 1990). The flowers, leaves and pods of *S. grandiflora* are eaten as vegetables in Indonesia and the pods of *Inga edulis* provide an important source of protein in Andean countries. Wild tree legumes may provide sources of human food in times of extreme drought. In Somalia, the ye-eb nut (*Cordeauxia edulis*), which grows in very dry areas, has traditionally been gathered and eaten when the crops failed, but overgrazing and cutting has led to a great reduction in the distribution of this tree (Miege and Miege, 1978; Drechsel and Zech, 1988).

Inclusion of trees to promote soil fertility

Trees can make a significant contribution to the maintenance and restoration of soil fertility. The role of trees within agricultural fields can range from the use of improved fallows, planted to increase the speed and success of soil fertility regeneration, to the integration of perennial trees within fields used for continuous cropping.

Soil erosion is a major problem throughout the tropics, resulting in declining crop yields due mainly to the loss of soluble nutrients and organic matter as the soil is washed or blown away. Trees help to slow this process in several ways. They provide additional soil cover, particularly when prunings are added back to the soil in the form of mulch. These mulches, like other green manures (Chapter 9) increase the nutrient status of the soil, can help to suppress weeds (Salazar *et al*., 1993) and, in time, can gradually improve the soil structure (Hulugalle and Kang, 1990), due to effects on aggregation of soil particles and on the stimulation of biological activity in soil. Increased soil fauna activity, especially that of earthworms, can aid infiltration of water and the gradual increase in soil organic matter content can increase water retention in the soil (Lawson and Kang, 1990). The tree roots help to bind the soil together and can also promote the infiltration of water. However, the effects of trees on soil erosion are not all positive, as water splash can be increased under tree canopies and can have much more erosive power than direct rainfall.

The maintenance of deep rooting systems in the soil can help to close up nutrient cycles, as the trees act as 'nutrient pumps' (Nye and Greenland, 1960), reducing losses by leaching and returning nutrients to the soil in leaf litter. The ability of trees to survive the dry season and maintain their green leaves means that

there will be active roots in the soil at the start of the rains, when there is often a tremendous flush of mineralized nitrogen. This can be very susceptible to leaching but the presence of active tree roots can prevent much of it from being lost from the cropping system, so acting as a 'safety net' (van Noordwijk, 1989; Rowe *et al.*, 1999). The trees can also assist in recycling of other nutrients, such as cations (Schroth *et al.*, 1995b). Deep-rooted trees can capture nitrate from subsoils; for instance, *Sesbania* and *Calliandra* were highly effective in recycling nitrate from depths of 2–4 m (e.g. Jama *et al.*, 1998a).

Observations of an abundance of nodules on the roots of legume trees when in close proximity to roots of other plants has led to the suggestion that there may be substantial underground transfer of N (van Noordwijk and Dommergues, 1990). Effects on root nodulation are most likely to occur through competition for soil N, making the legume tree more dependent on N_2-fixation (Chapter 5). Increases in nodulation on parts of the root system of a legume tree seem unlikely, considering the mobility of N within the plant in relation to autoregulatory control of nodulation (Chapter 3). As with other legume/non-legume intercrops, there is little evidence for substantial transfer of N from roots of legume trees to companion grasses, unless the tree is cut back (Rao and Giller, 1993). Nodule senescence can lead to release of substantial amounts of N from some species of *Erythrina* (Nygren and Ramirez, 1995) (Chapter 11).

In evaluation of the contributions from N_2-fixation in agroforestry systems it is important to bear in mind all of these possible benefits (Chapter 5). Of course, not all effects of including trees in mixed systems with crops and animals are positive. The ecological interactions between trees and crops are discussed in detail by Ong (1996) and Ong *et al.* (1996) and share much in common with legume/cereal or legume/grass mixtures. The balance of these interactions, together with the social and economic benefits and costs, drive the ultimate success or failure of agroforestry systems and these are considered below.

Legume Trees in Agroforestry Systems

Trees in fields of the arid zones

It is common practice in many regions to leave trees growing either on the field boundaries or actually within the fields. Throughout the Sahel from Senegal to Sudan and Ethiopia, *F. albida* is maintained by farmers within cropping fields and has also been used in revegetation programmes on degraded lands (Kirmse and Norton, 1984; Breman and Kessler, 1995). *Faidherbia* is mainly found close to watercourses in eastern and southern Africa, where uniform stands of large trees may dominate croplands in areas such as the river floodplains close to Lake Malawi (Weil and Mughogho, 1993). The soil beneath the trees develops a greater content of organic matter, and hence N, and an improved ability to retain moisture (Dancette and Poulain, 1969; Bernhard-Reversat, 1982; Kamara and Haque, 1992; Rhoades, 1995). These effects are seen beneath *Acacia* trees, which are widespread in the dry

savannahs of East Africa (Belsky *et al.*, 1989, 1993). Several non-legume trees also show enhanced soil fertility under their canopies, indicating that at least part of the increase in soil fertility is due to the contributions from resting animals and birds (Rhoades, 1997). The positive effects on crop yields under the canopies of *F. albida* trees are well documented. Groundnut and millet yields were doubled beneath trees in Senegal (Dancette and Poulain, 1969); yields of maize were doubled in Malawi (Saka *et al.*, 1994); and yields of sorghum and maize were over 50% greater beneath *Faidherbia* trees in eastern Ethiopia (Poschen, 1986). *Faidherbia* loppings and pods are also important sources of fodder for livestock during the dry season (Breman and Kessler, 1995).

Acacia and *Prosopis* have been shown to have incredibly vigorous tap roots, which grow rapidly to great depths in the soil. Two extreme examples are the 'tenere' tree (*A. tortilis*) in the southern Sahara, which is reported to have roots reaching 35 m (Fagg, 1991), and roots of *Prosopis* spp. have been founds at 53 m depth (Philips, 1963). These trees are 'phreatophytes' whose ability to grow in arid conditions is dependent on the ability to reach a deep water table in the dry season. This has a major advantage for mixing with crops, as the trees do not compete for water from the surface soil horizons (Roupsard *et al.*, 1999). In Niger, provenances of *Faidherbia* from southern Africa showed better early sapling growth than provenances from West Africa, but the southern African provenances invariably died during the dry season (Vandenbeldt, 1991). The better establishment and survival of the West African provenances was shown to be due to faster early root development, and these differences in adaptation fit well with the much deeper dry-season water tables found underneath *Faidherbia* stands in West than in southern Africa. It also raises the danger that damage to the tap root of seedlings raised in nurseries can be a major problem for establishment of the trees and direct sowing of *Faidherbia* seed in fields has been recommended (Bunderson *et al.*, 1995). Better establishment of *Faidherbia* trees appeared to be due to planting where soil fertility had been enhanced by termite activity (Geiger *et al.*, 1994), raising the possibility that part of the 'albida' effect was not caused by presence of the tree. This did not seem to be the case in Malawi, where there was no evidence that *Faidherbia* established in fertile spots (Rhoades, 1995).

Although it is widely assumed that *F. albida* fixes N_2 poorly, substantial variability in N_2-fixation was observed between provenances at the seedling stage (Sanginga *et al.*, 1990a), and young seedlings nodulate profusely in Malawian soils. Observations of nodulation on mature trees are impossible: *Bradyrhizobium* strains have been isolated from soils more than 30 m below *Faidherbia* (Dupuy and Dreyfus, 1992; Dupuy *et al.*, 1994) and much of the nodulation and N_2-fixation of large trees may occur at depth. In Namibia, *Faidherbia* derived negligible amounts of N from N_2-fixation (Schulze *et al.*, 1991), whereas mature *Faidherbia* trees in the lakeshore region of Malawi were estimated to derive 32–52% of their N from N_2-fixation (Phombeya *et al.*, 2001). Although these trees were large – over 25 m tall, with canopies of 800 m^2 – they were found to be only 25 years old by tree ring analysis (Phombeya *et al.*, 2001). It has been suggested that 20–40 years of growth of *Faidherbia* are required before substantial soil fertility benefits will occur (Poschen, 1986; Rhoades, 1997). However, Phombeya *et al.* (2001) found that soil fertility was

enhanced under some trees that were only 9–10 years old, indicating that planting of *Faidherbia* will yield benefits for farmers in a shorter time.

Prosopis cineraria is traditionally used in a similar way to *Faidherbia* in the arid regions of northern India (Tejwani, 1987). Despite its initial slow growth (the trees take 10–20 years to reach full size) it is planted both within fields and on field boundaries and can be maintained for a long time – some trees are as much as 200 years old. In addition to the benefits of improved soil fertility for crop production, the branches of *P. cineraria* are lopped to provide fodder and fuelwood, the roots provide hardwood for agricultural implements and the pods are used as a vegetable.

In very dry regions of the Sahel, where water tables are more than 80 m deep and beyond the reach of *Faidherbia* roots, other species of *Acacia* survive by efficient use of water in the upper soil horizons. *A. seyal* has a funnel-shaped canopy that catches rainfall and directs it down the trunk so that it can be captured by the deep tap root (Soumaré *et al.*, 1993; van Noordwijk *et al.*, 1996). This species also has superficial roots that may extend over 25 m to capture moisture and nutrients and although these roots will obviously compete for resources with crops or forage, the concentration of resources close to the tree canopy can sometimes outweigh any negative effects. However, in Pakistan, negative effects on wheat of *A. nilotica* growing in fields were found up to 8.5 m from the trees (Khan and Ehrenreich, 1994).

A further promising way that trees can be used in arid lands is as 'live hedges', where one or more rows of trees are grown around home gardens or animal kraals to control livestock. A live hedge is a dense planting – compared with 'live fences', where often trees are simply used as support for barbed wire – and is a practice that appears to have substantial potential in the arid zones of West Africa (Ayuk, 1997).

Alley cropping

Alley cropping, or hedgerow intercropping, is the growing of crops in alleys formed by trees or woody shrubs. In essence it is a more organized use of trees in cropping fields than those just discussed, and has emerged as a possible response to the need for shortened fallow cycles (Wilson and Kang, 1981). During the fallow period the land is rested, nutrients accumulate in the vegetation and the soil, and the land is then rejuvenated and can be used in another cropping cycle. The pressures of increased population density and the need to intensify agricultural production led to the identification of alley cropping as a possible alternative to leaving land fallow.

In alley cropping the regenerating tree fallow is essentially maintained within the field alongside the crops so that the trees can take immediate advantage of any period between crops. The trees help to enhance crop production in several ways. In particular, as discussed above, the leafy branches can be lopped and added to the soil as a mulch or green manure to enhance the growth of crops in the alleys (Wilson *et al.*, 1986). This also reduces competition for light, water and nutrients between the hedgerow trees and crops. Recovery of N from tree prunings can be substantial; uptake of N by maize was increased by amounts equivalent to over 30% of the N supplied in a mulch of *Leucaena* (Sanginga *et al.*, 1986). Maize yields were increased

markedly, particularly when the prunings were incorporated into the soil (Kang *et al.*, 1981). *S. rostrata* could even be used for alley cropping in flooded rice paddies in Nigeria (Mulongoy, 1986b).

The advent of alley cropping stimulated intense research interest throughout the tropics in the 1980s (e.g. Kang *et al.*, 1990; Szott *et al.*, 1991). The concept of deep-rooted trees leading to tightening of nutrient cycles and offering the opportunity to regenerate the fertility of the soil without having to take the land out of production is highly attractive. Alley cropping could also be a mixed crop and fodder production system (Kang and Gutteridge, 1994). Considerable interest of donors and agricultural development organizations was attracted and in some African countries the wave of enthusiasm created led to widespread promotion with smallholder farmers. As alley cropping was tested more widely, a number of concerns arose.

Accumulating problems with alley cropping

In arid or semiarid regions, it was quickly realized that intense competition from the hedgerow trees for soil moisture led to strong depression of crop yields in the alleys (Rao *et al.*, 1992). Although it was suggested that losses in crop yields may be offset to some extent by the wood and fodder production from the trees (Singh *et al.*, 1989), further promotion of alley cropping was largely restricted to regions with wetter climates. Alley cropping also appeared to give little benefit on infertile soils; yields of crops grown in alleys gradually declined on an acid Ultisol unless fertilizer inputs were added (Evensen *et al.*, 1995). On strongly acid Oxisols in northern Zambia, yields of maize were increased only where the growth of *Leucaena* had been promoted by large (and uneconomic) additions of lime (Matthews *et al.*, 1992). Even on more fertile soils in southern Zambia, *Leucaena* was much more productive than *Flemingia*, but yields were greater with *Flemingia* as it did not compete as strongly with crops for moisture (Chirwa *et al.*, 1994). This is a clear example where the soil fertility benefits were smaller than, and did not compensate for, the negative effects due to tree/crop competition (for other examples see Sanchez, 1995).

Many of the suggested benefits of alley cropping and other agroforestry practices depended on an assumption that the tree species are deep rooted. *L. leucocephala* is a species with a strong tap root and there is direct evidence that it exploits water from deeper soil horizons than the intervening alley crops (Lawson and Kang, 1990). However, trees such as *Leucaena* with strong tap roots can also have a well-developed superficial root system which will compete with the alley crop for soil moisture (Jonsson *et al.*, 1988). Studies with *Gliricidia* in Nigeria indicated that it can also be deep rooted, but studies in Sumatra showed that roots extended for more than 6 m from the plant just beneath the soil surface and there was little tap root penetration (Hairiah *et al.*, 1992a). This is due to differences between the soils on which these studies were conducted – the acid Ultisols in Sumatra have a thin organic surface horizon beneath which the soil has a high aluminium saturation, which prevents the penetration of *Gliricidia* roots. Nodules found in the surface soil close to a non-nodulating tree, *Peltophorum*, actually belonged to *Gliricidia* trees growing 4 m away.

The realization that many experiments on alley cropping had been seriously flawed raised further concerns. The majority of experiments had been established on relatively small plots to allow a number of treatments to be compared within a single experiment. When root systems of the trees were examined it was realized that trees were exploiting soil from far outside the plots in which they were planted, and often from the control plots. A depression of yields in the control plots and overestimation of the benefits of alley cropping resulted, which was often severe with N_2-fixing as well as non-nodulating species (see below).

Alley cropping with non-nodulating legume trees

In a comparative study of the suitability of three tree legumes for alley cropping, the amounts of the major nutrients N, P and K in the initial cutbacks and subsequent prunings were compared (Table 12.3). The initial contribution of N was greatest with *S. siamea*, which does not nodulate or fix N_2, but *Gliricidia* maintained a substantially greater production of N in subsequent prunings. Decomposition of the initial cutbacks was fastest with *Gliricidia* and slowest with *Flemingia*, but subsequent *Flemingia* prunings decomposed faster than those of *Senna* (Yamoah *et al.*, 1986b). However, the slow decomposition, and thus more persistent mulch, of *Senna* leaves resulted in more effective weed control during the cropping period. In the first two seasons, maize yields were improved to the same extent over the control plots in the alley-cropping treatments with either *Senna* or *Gliricidia*, but it is likely that the ability of *Gliricidia* to regrow after cutting would give higher yields in the long term.

Much of the earlier research was conducted without questioning the source of the N in the non-nodulating trees, and in some cases undoubtedly due to the simple

Table 12.3. Nutrient content of prunings of three legume trees compared in an alley cropping experiment at Ibadan, Nigeria. The first pruning was made in April 1983, 2 years after planting, and the further prunings were 6, 22 and 30 weeks later. Tree spacing was 0.5 m within hedgerows with 4 m wide alleys. (From Yamoah *et al.*, 1986a.)

		Nutrient content in prunings (kg ha^{-1})				
		First cutting				
Hedgerow tree	Nutrient	Prunings	(Litter)	Second	Third	Fourth
Gliricidia sepium	N	126	(23)	119	144	88
	P	8	(2)	7	9	5
	K	86	(7)	75	12	52
Flemingia macrophylla	N	62	(44)	46	25	23
	P	6	(4)	5	3	3
	K	40	(7)	35	16	23
Senna siamea	N	274	(102)	16	78	5
	P	27	(13)	2	10	1
	K	123	(40)	12	59	5

assumption that *Senna* species would fix N_2 because they were legumes. The ability of *Senna* species to maintain rapid growth and accumulate large amounts of N-rich biomass is dependent on efficient extraction of N from a large soil volume. Roots of *S. siamea* were found to extend 15 m in the surface soil, taking nutrients from an area more than six times the plot size (Hauser, 1993). This raised serious doubts as to the validity of many experiments, and attempts to reduce the problem by using barriers to prevent lateral root growth were only partially successful. Confinement of the tree root systems led to increased extraction of moisture from deeper soil horizons within the restricted zone (Hauser and Gichuru, 1994), and was only partly successful in containing the tree roots. Within a season, roots were observed to grow underneath the barriers and up into adjacent plots (Hauser, 1993; Itimu, 1997). Such non-nodulating legumes have been described as 'plunderers' of N (Giller, 1998), due to the strong competition exerted within the cropping system. For example, alley cropping with *S. siamea* in the Gambia reduced yields of upland rice (Danso and Morgan, 1993).

In Malawi, alley cropping was tested by more than 100,000 farmers as a result of a 'food for work' programme in the early 1990s. Most of the farmers were supplied with seedlings of *S. spectabilis*, as this species had been shown to give substantial benefits in promoting maize yields by efficiently recycling and concentrating N and other nutrients around the emerging crop on fertile soils of the experimental stations. Unfortunately, benefits were minimal on poor soils more representative of smallholder farmers' fields (Itimu, 1997), and yields were no better or worse than in plots without trees. The reason that *Senna* was promoted in Malawi was largely due to the lack of sufficient supplies of seed of *Gliricidia*, and severe damage to *Leucaena* from termites and the recently arrived psyllid.

Contrasting opinions remain as to the benefits of non-nodulating trees in alley cropping. *S. siamea* accumulated more N than *Leucaena* and *Gliricidia* on infertile soils in Bénin and Togo, presumably due to accessing N from the relatively fertile subsoil (Aihou *et al.*, 1999; Tossah *et al.*, 1999). *Peltophorum dasyrrachis*, a relatively slow-growing non-nodulating tree, has been shown to have advantages in acid Ultisols of Sumatra, Indonesia (Hairiah *et al.*, 1992a). This native tree has the ability to invade anthropic *Imperata cylindrica* monocultures, leading to development of secondary forest, and its roots penetrate deeper into the aluminium-rich subsoil (van Noordwijk *et al.*, 1991). The slow growth of *Peltophorum* gives minimal shade or demand on labour, and these benefits, coupled with polyphenol-rich litter that provides long-term soil fertility, make this an option for controlling the growth of *I. cylindrica* (van Noordwijk, 1996). Ultimately, non-nodulating trees can only assist in improving crop yields by enhancing the efficiency of N recycling, or by controlling weeds, as no extra N is added to the system.

Management to reduce competition

It is unfair to state simply that alley cropping does not work. There are many examples where legume trees have given substantial benefits in crop yields (reviewed by Rao *et al.*, 1998), although poor experimental designs may have overemphasized some of the benefits. Alley cropping appears to be most successful on fairly fertile soils where rainfall is adequate, so that strong tree/crop competition is avoided.

Strength of competition between trees and crops in alley cropping can be managed by the choice of tree, and details such as the optimum alley width and the timing and frequency of pruning. Optimal management differs, depending on the soil and climate. Pruning of trees prior to crop planting reduces shade and demand for uptake of water and nutrients; for example, after pruning, *Leucaena* was unable to compete for mineral N from the soil (Vanlauwe *et al*., 1998). Increasing the frequency of pruning may increase yields of associated crops (Duguma *et al*., 1988), but reduces the amounts of prunings produced. A common response of trees to pruning is to have a higher root density in the topsoil (Szott *et al*., 1991). More severe pruning reduces the apical dominance of root systems, so that they become more branched and superficial (Hairiah *et al*., 1992a). Management to reduce above-ground competition may thus inadvertently increase the severity of competition underground. Pruning also causes shedding of nodules and fine roots, which may release N for crops growing in the alleys (Fownes and Anderson, 1991) but will reduce the rate of N_2-fixation until growth and nodulation of the tree is re-established.

Intensity of management required and competing demands for labour at critical points in the cropping calendar suggest that only the most enterprising, or well-endowed, farmers will use alley cropping in the way it was originally envisaged (Hoekstra, 1994; David, 1995). Only in cases where the benefits of alley cropping in improved soil fertility and other products such as forage, wood or grain are large is the investment likely to be rewarded. Pigeonpea (*C. cajan*) compared favourably with *Leucaena* and *S. siamea* in alley cropping trials in Malawi in terms of the yield of interplanted maize, and was also a useful fuelwood source (Chiyenda and Materechera, 1989). Farmers in Ghana were interested in growing late-maturing pigeonpea in alley cropping as the yield of pigeonpea seed more than compensated for the reduced land area available in the alleys (Sipkens, 1989), although yields of pigeonpea in alley cropping were poor in Benin (Böhringer and Leihner, 1997). As *Cajanus* is a grain legume, this is essentially a form of 'strip intercropping' and other spatial arrangements would probably better suit mixing of pigeonpea and maize (Chapter 8) (Sakala *et al*., 2001). Although the potential for widespread adoption of alley cropping appears to be limited (Swinkels and Franzel, 1997), primarily due to the intensive management and labour required, one case where a form of alley cropping has been successful is when erosion barriers are planted on sloping lands.

Barrier fences

Dense planting of woody legumes (notably *Leucaena*) in hedgerows along contours on steeply sloping fields has been successful in reducing soil erosion (Celestino, 1985). The trees act as a permeable barrier, slowing the rate of water flow so that soil particles are deposited and infiltration of water into the soil is increased. The build-up of soil behind the hedgerow can gradually lead to development of terraces (Fig. 12.2), though this is often at the expense of 'scouring' of soil from the upper part of the terrace (van Noordwijk and Garrity, 1995). For this system to be successful, dense hedgerows are required – a few large tree trunks can do little to

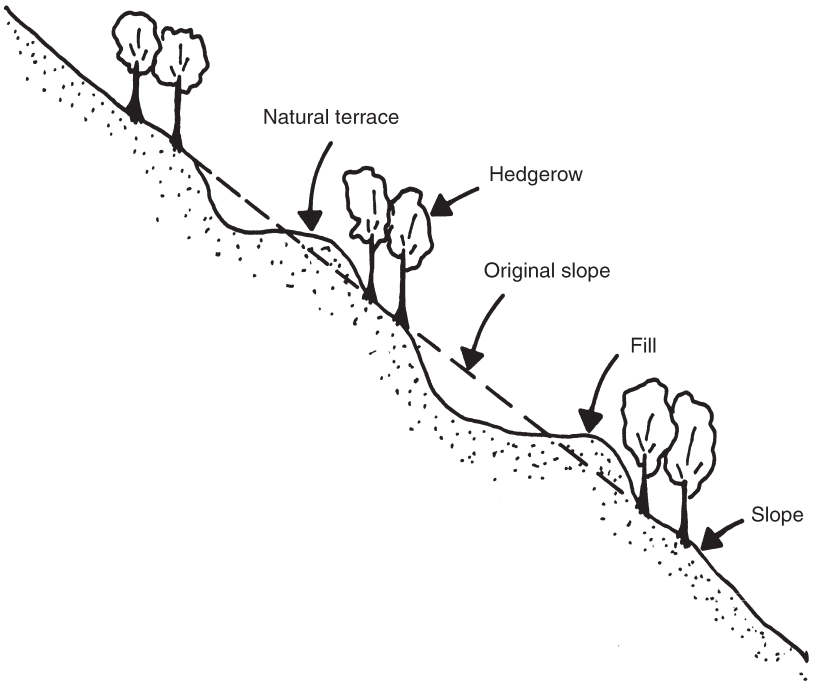

Fig. 12.2. Natural development of terraces behind hedgerows of trees on sloping land. (After Celestino, 1985.)

reduce the runoff of water. The barriers can also be principally formed with grasses, with legume trees planted along the strips. For example, *L. diversifolia* is planted on contours of Guatemala grass (*Tripsacum laxum*) in the Usambara Mountains of northern Tanzania (Pfeiffer, 1990), and *C. calothyrsus* on contours of *Pennisetum* in Burundi (Akyeampong, 1996). *Calliandra* hedges reduced erosion risks on steep fields by increasing infiltration (Perret *et al.*, 1996).

Improved fallows

Fallowing of land is an age-old practice of allowing soil fertility to regenerate (Chapter 1), but food demands of increasing populations inevitably lead to periods of fallow far shorter than those required to maintain soil fertility (e.g. Trenbath, 1989). One approach to improving a bush fallow is to enrich the vegetation with particular tree species, by selective removal or interplanting of trees. In central Zambia, farmers chose to enrich bush fallows dominated by *Acacia polyacantha* with trees that provided fruit or poles and timber for building (Chidumayo, 1988). An alternative strategy is intensifying the rate of soil fertility improvement, by planting of fast-growing leguminous trees in 'improved' or planted fallows (Gichuru, 1991). In improved fallows, trees are used in a similar way to green manures (Chapter 9) to give a large amount of high-quality organic residues that release nutrients for subsequent

crops. Tree fallows have the added advantage of providing stakes or fuelwood, and can be more effective than alley cropping in preventing soil erosion on sloping lands (Hoang Fagerström *et al.*, 2001b).

In the Philippines, planting *L. leucocephala* allowed farmers to halve their fallow period to 2–4 years without any observable loss in soil fertility (MacDicken, 1991). Fallows of *S. sesban* were introduced in eastern Zambia where the duration of bush or grass fallows was becoming progressively shorter (Kwesiga and Coe, 1994). *S. sesban* is indigenous to this region, where it grows mainly in low-lying, wet habitats. Farmers in western Kenya traditionally leave trees of this species to grow within their fields (Swinkels and Franzel, 1997). It was found to be well adapted to the upland soils in eastern Zambia and grows prolifically, reaching a height of 5–6 m within 1 year (Fig. 12.3). Productivity of *Sesbania* is difficult to estimate as it sheds a lot of

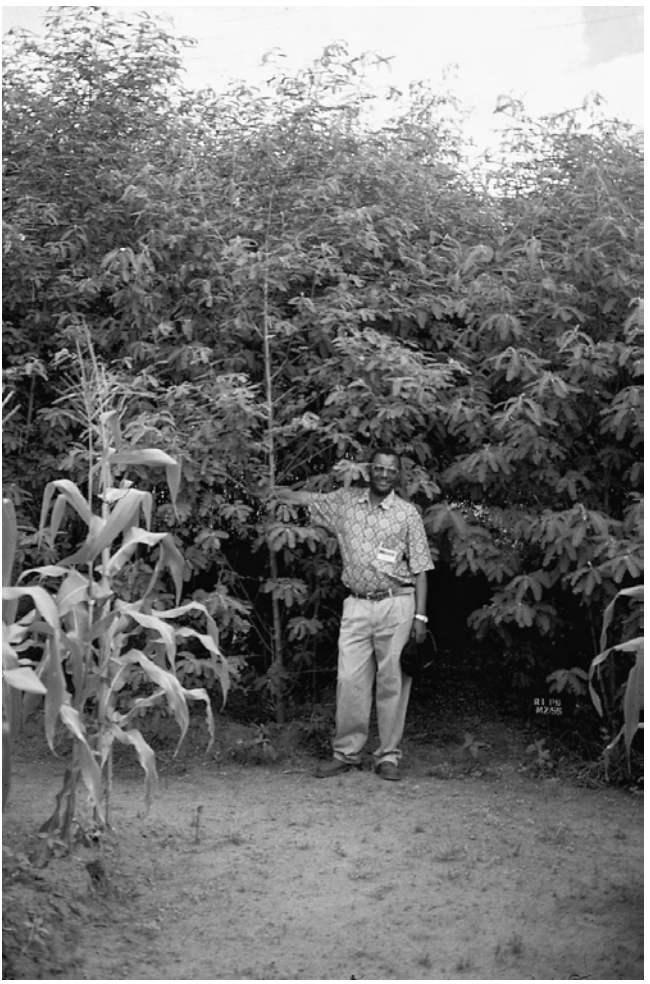

Fig. 12.3. A 1-year-old stand of *Sesbania sesban* in eastern Zambia.

leaves, particularly during drier periods, but when estimates of fallen leaves were included above-ground N inputs under *Sesbania* fallows amounted to 25–44 kg N ha^{-1} for 1-year fallows and 71–95 kg N ha^{-1} for 2-year fallows (Torquebiau and Kwesiga, 1996). *Sesbania* roots were 7 m deep after 2 years and reached the water table, but the majority of the roots were in the top 50 cm. The surface metre of soil contained a root biomass of 3 t roots ha^{-1} which contained 21–25 kg N ha^{-1} (Torquebiau and Kwesiga, 1996). In western Kenya, *Sesbania* roots penetrated to below 4 m in a strongly acid Oxisol, and were able to use deep moisture and capture N that was leached from below the rooting zone of maize (Mekonnen *et al.*, 1997). The N input from the legume fallow to the cropping system may come from both N_2-fixation and capture of N from depth, both of which are contributions to the cropping system that are not possible when the tree is not present. *Sesbania* fixed roughly 60% of its N, amounting to 70 kg N ha^{-1}, in a 6-month period in western Kenya and recovered 54 kg N ha^{-1} from the soil (Gathumbi *et al.*, 2001a). Under the same conditions *C. cajan*, *C. grahamiana* and *T. vogelii* all grew more rapidly and fixed more N than *Sesbania*. The largest amounts were found in *Crotalaria*, which accumulated 199 kg N ha^{-1} in 6 months, three-quarters of which was estimated to come from N_2-fixation (Table 12.2). Although *Sesbania* had a deeper root system than other fallow shrubs, and was shown to recover greater amounts of N from depth using ^{15}N labelling of the soil N, all of the species recovered similar amounts of soil N.

Mixtures of different fallow species also show promise. When the shrubby *C. grahamiana* was grown together with *S. sesban*, which has a complementary canopy, the amounts of N accumulated (120 kg N ha^{-1}) were greater than pure *S. sesban* (83 kg N ha^{-1}) but less than the pure stand of *C. grahamiana* (230 kg N ha^{-1}) (Gathumbi *et al.*, 2001b). There was substantially more root biomass in all soil horizons down to 1.5 m under the mixed fallow than with the sole species, due to the complementarity of their rooting patterns. As indicated above, rooting patterns and distributions are strongly influenced by the soil conditions. In northern Rwanda, where the acid soils are rich in aluminium, *S. sesban* roots hardly penetrated below a depth of 50 cm and spread over 6 m from the trees in the surface soil (Rubaduka, 1994).

Yields of maize after 1–3-year *Sesbania* fallows were two to four times greater than yields where maize was grown continuously (Kwesiga and Coe, 1994). Cumulative yields over a 4-year cycle were substantially increased with *Sesbania* fallows of 1–2 years where no N fertilizers were applied, while the large yields of over 5 t ha^{-1} after a 3-year *Sesbania* fallow just compensated for the lack of production in the previous years (Fig. 12.4). By contrast, when the maize received N fertilizer no substantial benefits in cumulative maize yields were seen over a 4-year cycle.

Many farmers have experimented with improved *Sesbania* fallows in eastern Zambia and a detailed extension manual on how to establish and manage the fallows is available (Kwesiga and Beniest, 1998). To improve the initial rate of growth of *Sesbania*, which has very small seeds and is slow to establish, seedlings are raised in a nursery and transplanted. Instead of sowing seedlings in pots or polythene tubes, these can be raised in nursery beds in low-lying areas (dambos or vleis which have

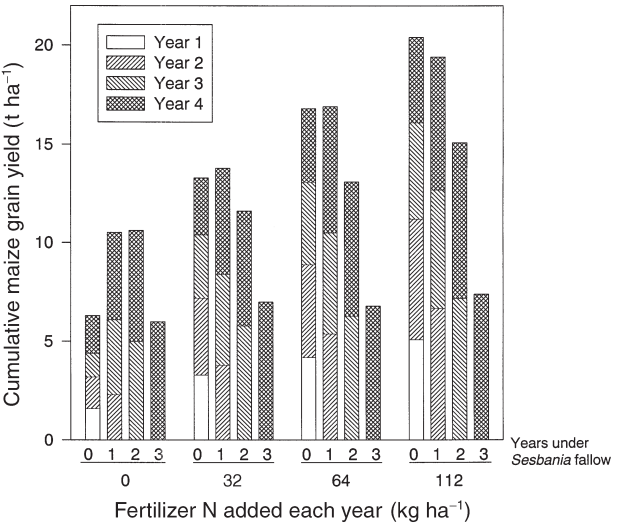

Fig. 12.4. Cumulative maize grain yields (t ha^{-1}) in plots continuously cropped to maize or where maize was grown after improved fallows of *Sesbania sesban* of 1, 2 or 3 years in duration (drawn from data in Kwesiga and Coe, 1994). The yields of the first maize crop under continuous cropping were estimated as the mean of the 3 succeeding years in the relevant fertilizer treatment, as no yields were given for this year.

sufficient moisture during the dry season) and transplanted as 'bare-rooted' seedlings by digging up and separating the seedlings for planting (Kwesiga and Beniest, 1998; Kwesiga *et al*., 1999). As laboratory-prepared inoculants were not readily available, seedlings were inoculated with soil from well-established stands of *Sesbania*, though the risks of transporting nematodes and seeds of weeds such as *Striga* was acknowledged. A survey of soils from Malawi and Zimbabwe indicated that *Sesbania* rhizobia were present in only 20% of the soils (Bala, 1999). The soils where *Sesbania* nodulated successfully were all alluvial soils or soils from along water courses – the typical habitat of this species. Other researchers have not inoculated *Sesbania* when evaluating it for agroforestry systems in southern Africa. This may partly explain its poor growth in some areas, although *Sesbania* is poorly adapted to the sandy soils of southwestern Zambia and Zimbabwe. By contrast, inoculation of *Sesbania* is not necessary in western Kenya (Odee *et al*., 1995).

Assessment of the benefits of improved fallows is complex and cannot be based solely on crop yields. In the study by Kwesiga and Coe (1994), the fallows also produced 15–20 t firewood ha^{-1} when they were cleared, and the fallows may have other benefits in improving soil physical properties or weed control. Whether improved fallows are useful for farmers depends largely on competing demands for land and labour, including the potential for off-farm income, and on prices and availability of fertilizers. As the *Sesbania* trees are spaced 1 m apart, and at least one weeding is required to ensure establishment, the labour demands for planting 10,000 plants ha^{-1} are substantial. A study in western Kenya indicated that labour used

for *Sesbania* fallows was less than when maize was cultivated, and that use of the fallows was economically favourable where the trees grew well (Jama *et al.*, 1998b). If pressure on land is severe, households employing improved fallows must plan carefully to ensure continuous supplies of sufficient maize.

In wetter climates, where there are two rainy seasons each year, *Sesbania* or other species can be established by undersowing within a cereal crop in the long rains and then allowed to grow on through the short rains to minimize the time when no crops are grown. In Cameroon, intercropping of ten different fast-growing tree species with groundnut did not reduce the growth during establishment of the trees (Duguma *et al.*, 1994). Groundnut and the forage legume *Macroptilium atropurpureum* both grew and yielded well in an establishing fallow *S. sesban*, which has an open canopy well suited to intercropping with low-growing species (Gathumbi *et al.*, 2001c), and farmers showed great interest in these mixtures.

In other regions, such as the unimodal climates of southern Africa, growth of *Sesbania* is too slow to give sufficient biomass unless it is sown very early in the rainy season. Under these conditions *T. vogelii* and *C. grahamiana*, which have larger seeds than *Sesbania* and can be established by direct sowing, appear to be more promising options for farmers. These shrubs are also being evaluated by farmers in western Kenya, who appreciate the labour savings of direct sowing (Franzel, 1999).

Fodder banks, woodlots and cut-and-carry for mulch

For farmers in regions where livestock are a major component of the farming systems, or where wood is a scarce resource, fodder banks or woodlots may be useful. Due to the costs of establishment, and the high value placed on the fodder, farmers in Zimbabwe maintain the fodder banks on the same land, rather than rotating with crops, which would give a strong soil fertility benefit. Fodder banks and other cut-and-carry systems lead to export of N, which can be replaced by N_2-fixation, but there is also export of large amounts of other nutrients (Lehmann *et al.*, 1999). If these are not replaced then productivity will inevitably decline in the long term, but nutrient removal is much less significant if only the wood is removed.

Cut-and-carry systems have also been evaluated as a means to provide mulch for soil fertility improvement (Kormawa *et al.*, 1999). Although maize yields were doubled using prunings from *L. leucocephala* or *G. sepium*, the large amount of labour and scarcity of land made the system unprofitable. The conflicting requirements for labour at planting time appeared to be the main constraint to use of *T. candida* mulch grown outside the fields for rice production in northern Vietnam (Hoang Fagerström *et al.*, 2001a).

N_2-fixing trees for reclamation of degraded land

Due to their rapid growth on soils depleted in organic matter, N_2-fixing trees are especially useful for reclamation of degraded or polluted land. In the forests of

Southeast Asia, *Acacia mangium*, which can grow extremely rapidly, is often used to stabilize the soils on abandoned 'log-landings' after extraction of timber. A number of legume trees grow prolifically on aluminium-rich wastes from bauxite mining in Amazonia (notably *Acacia* spp., of which *A. mangium* was the fastest), stabilizing the soil and preventing further erosion – which has severe consequences for siltation and pollution of the rivers (Franco and de Faria, 1997). Trees have been used in a similar way to recolonize lagoon ash in China (Cheung *et al*., 2000).

Conclusions

The possibilities for use of N_2-fixing trees in agriculture are widely recognized and agroforestry is likely to be increasingly adopted in future, if only in response to the acute lack of fuelwood in many countries. Although alley cropping is an ideal experimental system to explore the ecological interactions between trees and crops, and has undoubtedly led to a much deeper understanding of agroforestry systems, it seems unlikely to become a widespread practice in the tropics. A literature search of a scientific citation database in the middle of the year 2000 revealed 1202 journal articles using the keyword 'agroforestry' out of more than 18 million articles published in science since 1981. Of these, 262 were related to 'alley cropping' or 'hedgerow intercropping' but only 36 were related to 'tree fallows' or 'improved fallows' and 45 were found using the term 'fallows and agroforestry'. This search was conducted on words in the title, keywords or abstract of the papers and will not find all related articles, but it is indicative of the uneven balance of research in this field. Much more research is warranted on finding ways of integrating trees into cropping systems, and will only be successful through close partnership with farmers.

By fixing N_2, leguminous trees clearly satisfy what has been coined as the 'central agroforestry hypothesis' (Cannell *et al*., 1996), that 'the trees must acquire resources that the crop would not otherwise acquire'. Whether agroforestry systems based on N_2-fixing trees are successful depends on benefits from N_2-fixation outweighing competition with crops for other resources. Large amounts of N may be contributed to cropping systems by N_2-fixing trees and these can have a major impact on agricultural productivity.

The major constraints to adoption of agroforestry practices are the lack of secure land tenure of many farmers in the tropics and the availability of land and labour for devoting to management of the trees. Agroforestry may not yield major benefits in the short term and there will be little incentive to farmers to adopt practices unless they can realize some of the benefit themselves. Loss of land to trees in agroforestry must be compensated for rapidly by increased yields of crops or in terms of firewood or fodder. However, in the absence of mineral fertilizers or sufficient animal manures, agroforestry is a practical means of replenishing N (and perhaps other nutrients from deeper soil horizons) in exhausted soils.

Part III

Optimizing N_2-fixation

Chapter 13

Environmental Constraints to N_2-fixation

The ability of free-living or symbiotic N_2-fixers actually to fix N_2 in the field is strongly influenced by the prevailing environmental conditions. As indicated in Chapter 1, although the tropics contain some of the most productive environments in the world, they also contain their fair share of hostile environments. The susceptibility and adaptation of free-living bacteria and *Azolla* to environmental stresses were considered in Chapters 6 and 7; the discussion here will focus on the legume/rhizobial symbiosis. One factor that has a strong influence on the proportion of N_2 fixed in most N_2-fixing organisms is the availability of mineral N, and this was discussed in Chapters 3, 5 and 8.

The main environmental stresses that occur in the tropics can be divided into predominantly physical factors (temperature, moisture) and into chemical factors, which include toxic effects (acidity, aluminium) and nutrient deficiencies. Such environmental stresses can act at several different levels. They may reduce the survival or rate of growth of microorganisms in the free-living state, or interfere with the process of plant infection or nodule development or they may affect the fixation of N_2 once the symbiosis has been established. A single environmental stress may, of course, affect one or all of these processes.

Genetic variability in tolerance to most environmental stresses has been shown in both host legumes and rhizobial strains (reviewed by Hungria and Vargas, 2000). Moreover, the two partners in a symbiosis can differ markedly in their tolerance to particular stresses. There is an enormous body of literature concerning the selection of bacteria and plants for N_2-fixation in adverse environments. Here the discussion will be restricted to the effects of the major stresses on survival of both symbiotic partners and on N_2-fixation, and to approaches to alleviating the problems.

Physical Constraints

Temperature

In parts of the tropics the surface soil temperature can occasionally reach 65–70°C and temperatures above 50°C can be found at 5 cm depth (Dudeja and Khurana, 1989) – sufficiently high to inhibit germination of seeds and to kill many bacteria. Many species of cyanobacteria can form spores (akinetes) that are highly resistant to desiccation but most heterotrophic free-living N_2-fixers and rhizobia do not possess this capability. Excessive soil temperatures can therefore kill the majority of the bacteria in the surface layers of soil, though some rhizobia can survive periods at 70°C in dry soil (Marshall, 1964). Survival of bacteria in soils at high temperature appears to be improved by the presence of clay particles and soil organic matter, but many of the soils where high temperatures are experienced are sandy. In Samaru, northern Nigeria, populations of rhizobia of only 4 to 40 cells g^{-1} soil were found in the surface 5 cm of soil, whilst populations of up to 10^4 cells g^{-1} soil were found at a depth of 20–25 cm below the surface (Day *et al.*, 1978). In general, bacteria are less tolerant of high temperatures in moist than in dry soil.

High temperatures can prevent nodulation or, if nodulation does occur, can inhibit the activity of N_2-fixation in legumes (e.g. Day *et al.*, 1978) even though the root nodules will be insulated from the highest temperatures by the soil (Piha and Munns, 1987b). Genotypes of soybean appeared to differ in their sensitivity to growth at 35°C but no genetic variability in adaptation to temperature was apparent in *Phaseolus vulgaris* (Piha and Munns, 1987b). Conversely, cool temperatures lead to delayed development of plants, including delays in the formation of nodules, and so decreased rates of N_2-fixation. The optimum temperatures for growth and N_2-fixation vary widely between legume species and reflect their environmental adaptation.

Differences in environmental adaptation to high temperatures have been demonstrated between rhizobia isolated from different climatic zones. More than 90% of cowpea rhizobia isolates from soils of the hot, dry Sahelian savannah in Niger were able to grow on media at 40°C whilst few of the isolates from cooler, humid regions of West Africa could grow at this temperature (Eaglesham and Ayanaba, 1984). Only the high-temperature tolerant isolates retained, or increased, their effectiveness in N_2-fixation in symbiosis with cowpea when the day temperatures were kept above 40°C. Fast-growing rhizobia isolated from cowpea nodules in Zimbabwe could grow at a wider range of temperatures (28–47°C) than slow-growing isolates (32–44°C) (Mpepereki *et al.*, 1996). Although more of the fast-growing isolates could grow at temperatures above 40°C, there was no relationship between the climate from the soils where the isolates originated and tolerance to high temperatures.

Drought

The numbers of rhizobia in soil decline drastically as soil dries. Comparisons of survival in drying soil have indicated that *Bradyrhizobium* strains are more tolerant

of desiccation than strains of *Rhizobium* over short periods (Bushby and Marshall, 1977). Other workers found no simple relationship between the desiccation tolerance of fast- or slow-growing rhizobia but they did find that specific strains of each survived in much greater numbers than others (Mahler and Wollum, 1981). Rhizobia generally survive in smaller numbers on drying in soils that contain only small amounts of clay or organic matter (Chao and Alexander, 1982). Pronounced variability exists between both chickpea and *Phaseolus* rhizobia in the ability to survive in dry soils (Issa and Wood, 1995). Strains that survive under greater water stress are those that retain less water within the cells (Bushby and Marshall, 1977; Al-Rashidi *et al.*, 1982).

Drought stress also has drastic effects on N_2-fixation in legumes. Rates of N_2-fixation are more sensitive to reductions in soil water content than other processes such as photosynthesis, transpiration, leaf growth rates or nitrate assimilation (Sinclair *et al.*, 1987; Serraj *et al.*, 1999). Even in species that are grown in arid regions and are considered to be tolerant of drought (e.g. moth bean, *Vigna aconitifolia*), slight changes in the plant water potential cause a marked reduction in both the rate of N_2-fixation and in the translocation of the products of N_2-fixation to the shoot (Rao and Venkateswarlu, 1987; Venkateswarlu and Rao, 1987).

Tolerance to drought in the field will be strongly influenced by the ability of plants to capture water – that is, by the extent and distribution of the root system. Highly drought-tolerant trees and shrubs, such as species of *Prosopis* and *Acacia*, have rooting systems that can reach water at great depths (Chapter 12). In *P. glandulosa* growing in the Sonoran Desert, more than 90% of the water used by the trees was taken up from a permanent water table at a depth of 5 m below the surface (Nilsen *et al.*, 1983). Many drought-tolerant, shrubby legumes have very small leaflets, presumably to reduce the surface area from which water may be lost, and they may also have physiological adaptations to drought stress, such as the ability to adjust the osmotic potential in their tissues and to regulate the sensitivity of stomata to vapour pressure deficits (Nilsen *et al.*, 1983).

Grain legumes with deep rooting systems, such as cowpea, are preferentially grown in climates with limited rainfall, as they can withstand prolonged periods of drought – as long as the roots have managed to penetrate sufficiently deep into the soil before the drought begins. The ability of cowpea to give a better yield than soybean or black gram under moisture stress in the field was attributed not simply to differences in rooting pattern, but to a greater adaptability in development that allowed a delay in the onset of reproduction under stress (Nilsen *et al.*, 1983). The greater drought resistance of *P. acutifolius* compared with *P. vulgaris* was attributed to the ability to maintain lower osmotic potentials in its leaves (Parsons and Howe, 1984). It has been postulated that plants with indeterminate root nodules may be able to recover from drought more rapidly than plants with determinate nodules (Eaglesham and Ayanaba, 1984) because they retain an active meristem. However, ureide-exporting legumes, the majority of which have determinate nodules, appear to be more sensitive to water stress than legumes that export their products of N_2-fixation as amides (Serraj *et al.*, 1999). As substantial genetic variation in response of N_2-fixation to water stress exists within legume

species, there appears to be potential for breeding and selection for drought tolerance.

Saline and sodic soils

Saline soils are formed under hot, arid conditions due to an accumulation of salts in the topsoil and can form naturally or as the result of poorly managed irrigation (Nortcliff, 1988). Salinity is commonly measured as electrical conductivity (EC) and soils are classified as saline if the EC is greater than 4 dS m^{-1}. Sodic soils are those sufficiently rich in sodium to interfere with the growth of most plants and they may or may not also be saline. The pH value of saline and sodic soils is usually above 8.5, and this can result in reduced availability of phosphorus, iron, zinc, manganese and boron for plant growth, but the primary stresses encountered in saline soils are those of water availability due to the osmotic potential in the soil.

Whilst the stress imposed by drought in soils is essentially temporary, so that bacteria need only to be able to survive until the drought is over, saline stresses are more permanent; thus the microorganisms must be able both to survive and to grow (Sprent, 1984). Amongst the free-living N_2-fixing bacteria, some species such as *Azospirillum halopraeferens*, isolated from the rhizosphere of plants growing in a highly saline environment, have some tolerance to salt. There are marked differences between strains of rhizobia in adaptation to saline conditions and in fact the host legumes are generally much more sensitive to salinity than the bacteria. Some strains of rhizobia can actually grow in solutions with salinities as high as 43 dS m^{-1} – the equivalent of 92% of the salinity of seawater (Singleton *et al.*, 1982). This is perhaps not surprising in view of the fact that in the symbiotic state rhizobia live within cells that have much greater solute concentrations than those generally experienced in soils (Sprent, 1984). Salt stress to rhizobia is more severe at alkaline pH, and chloride ions are particularly toxic to rhizobia (Elsheikh and Wood, 1989a,b).

The process of root hair infection of legumes is particularly sensitive to saline stress, perhaps due to the common cessation of root hair growth in these conditions (Sprent, 1984). It may also be caused by the bacterial partner, as different strains of rhizobia were found to show marked differences in the ability to infect and form nodules on pigeonpea under saline conditions (Subbarao *et al.*, 1990b). Reduced nodulation of soybean was shown to be due to interference with the early stages of infection such that nodulation was almost completely suppressed at a salinity of 8 dS m^{-1} (Singleton and Bohlool, 1984). No differences in nodulation of soybean were observed between salt-sensitive or salt-tolerant strains of *Bradyrhizobium* across a range of salt concentrations (Elsheikh and Wood, 1995). Other species are less sensitive to salinity. There was no reduction in nodule formation by *Atylosia platycarpa*, a wild relative of pigeonpea, at salinities (sodium and calcium chlorides) up to 8 dS m^{-1} and effective nodules were still formed at 12 dS m^{-1}, whilst numbers of nodules on pigeonpea were reduced at 4 dS m^{-1} (Subbarao *et al.*, 1990a). Nodulation in groundnut is also relatively insensitive to salinity. It has been suggested that this may be related to the direct mode of infection by rhizobia (Sprent, 1984).

Once nodules are formed, the process of N_2-fixation is less sensitive to salinity in soybean than, for example, leaf expansion, and effects on N_2-fixation are therefore indirect (Singleton and Bohlool, 1984). Similarly pigeonpea, following initial nodulation, grew in saline treatments (up to 8 dS m^{-1}) with no harmful effects on the subsequent development or functioning of nodules (Subbarao *et al.*, 1990a). Variation in sensitivity of growth and N_2-fixation to salinity has been found among genotypes of chickpea (Soussi *et al.*, 1999) and soybean (Ranga Rao *et al.*, 1990), which may be due to variation in the ability to avoid uptake of Cl^- ions.

Waterlogging

In addition to the period during the cultivation of wetland rice when the soil is deliberately kept waterlogged, rice paddies can also be prone to flooding when other rainfed crops are being grown. The intensity of rainfall in the humid tropics can be so high that even upland, sandy soils can become inundated for some time; Lal (1974) recorded 47 storms in a single season where the intensity of the rain exceeded 75 mm in an hour. The length of time that the soil remains flooded will depend on how freely draining the soil (and the site) is.

If temperatures are high and there is much organic substrate present, free oxygen can be rapidly used up in waterlogged soil and so the soil becomes anaerobic within hours. Whilst rhizobia are normally aerobic organisms, some strains of *Bradyrhizobium* and *S. meliloti* possess a dissimilatory nitrate reductase that can function as an electron acceptor, and thus enable the bacteria to survive under anaerobic conditions (Zablotowicz *et al.*, 1978; Daniel *et al.*, 1982). These are the conditions that *Bradyrhizobium* needs to fix N_2 in the free-living state (Kurz and LaRue, 1975; McComb *et al.*, 1975; Pagan *et al.*, 1975).

Survival of rhizobia during long periods of flooding is of particular importance in cropping systems in which legumes are grown in rotation with rice. In Thailand, the size of the populations of rhizobia nodulating siratro in sandy soils sampled from the field was generally larger (10^2–10^4 cells g^{-1} soil) when the soil was moist or fully waterlogged than when the soil was dry (< 10–10^3 cells g^{-1} soil) (Toomsan, 1990). In a heavier textured soil, numbers of soybean bradyrhizobia multiplied during flooded cultivation of a rice crop but declined when the soil was drained (Simanungkalit *et al.*, 1995). Roughley *et al.* (1995) confirmed the ability of soybean bradyrhizobia to survive for more than 5 years in soils that were periodically flooded for rice cultivation. In contrast, large reductions in the numbers of rhizobia nodulating chickpea – which are generally fast-growing rhizobia and may not possess a dissimilatory nitrate reductase – have been found after paddy rice (Toomsan *et al.*, 1982).

Lack of oxygen is also a major problem for root respiration and can rapidly result in loss of nitrogenase activity (Sprent and Gallacher, 1976; Witty *et al.*, 1986). Even in legumes that grow well in waterlogged conditions, such as species of *Aeschynomene* and *Sesbania*, root nodules do not develop under water, though stem nodules are unaffected when submerged, presumably as oxygen is transported to the nodules through lacunae in the shoots (Eaglesham and Ayanaba, 1984). Other legumes can

transport some oxygen from their shoots to the roots (de Willigen and van Noordwijk, 1989) and if the plants develop under wet conditions where the oxygen supply to the roots from the soil is poor, the porosity of the roots may increase – indicating an adaptive response to increase the supply of oxygen from the shoot to the root. Depression of growth in soybean plants caused by waterlogging was greater in plants dependent on N_2-fixation than plants supplied with nitrate (Bacanamwo and Purcell, 1999), suggesting that N_2-fixation is more sensitive to effects of waterlogging than plant growth *per se*. The degree of tolerance to waterlogging may differ widely between cultivars of legumes, as has been demonstrated for pigeonpea (Chauhan *et al*., 1997). Nodules can develop a thick cortex and enlarged 'lenticels' in response to waterlogging, which can also help in gaseous exchange across the nodule surface (Minchin and Summerfield, 1976).

Other problems of waterlogging can result from the rapid release into the soil solution of large concentrations of iron and manganese, which are normally present in relatively insoluble oxidized forms in all but the most acid soils. These cations are highly toxic to both rhizobia and plants if present in sufficient concentrations, and toxicity of iron and manganese to *Phaseolus vulgaris* growing in soils of near-neutral pH subjected to only transient waterlogging by intense rainstorms has been observed (Giller *et al*., 1992).

Chemical Constraints

Toxicities

Given the large proportion of tropical soils that are acid, problems associated with low pH are of widespread importance. These problems can arise from two causes. The first is the simple problem of survival in a medium of low pH. The second category of problem results from other chemical changes in the soil caused by strong acidity, particularly the large amounts of aluminium and also iron and manganese that may come into solution, the corresponding decreases in availability of phosphorus and molybdenum, and the lack of calcium in most acid soils (Chapter 1).

The bacterial symbionts in particular may be directly affected by the acidity of their environment. Bacteria with a greater capacity to regulate their internal pH show increased survival rates at low pH (O'Hara *et al*., 1989), and calcium moderates acidity problems (Dilworth *et al*., 1999). Aluminium toxicity, probably the most severe component of stress in acid soils, also has a major impact on rhizobial survival, and strains of rhizobia that can tolerate a pH of 4.5 are not necessarily tolerant of aluminium toxicity (Keyser and Munns, 1979a,b). Some considerable variation was found between strains, however, with the broader host-range strains of *Bradyrhizobium* tested being more tolerant of aluminium than *B. japonicum* strains (Keyser and Munns, 1979b). The chemistry of aluminium is complex and the precise identity of the toxic ionic species of aluminium remains unclear (Kinraide, 1991). The ready complexation of aluminium ions by organic compounds and phosphorus means that experiments in bacterial growth media or on agar plates are difficult to

interpret (Flis *et al.*, 1993). By comparing results from 20 studies, Flis *et al.* (1993) concluded that *Bradyrhizobium* strains are generally more tolerant of acidity and aluminium than fast-growing rhizobia. Tolerance to aluminium observed in growth media appears not to be due to the presence of exocellular polysaccharides (Kingsley and Bohlool, 1992) (which, in any case, might not be produced to any large degree in the oligotrophic environment of the soil). The suggestion that toxicity of aluminium to rhizobia may be due to inhibition of DNA replication because of binding of aluminium to DNA (Johnson and Wood, 1990) is probably based on an artefact due to the experimental conditions. The ability of aluminium to bind to phosphate is well known and DNA is likely to be a major binding site for aluminium ions once cells lose their integrity (Flis *et al.*, 1993). Rhizobia may also survive in aluminium-rich soils within microsites or in soil horizons rich in organic matter, in a similar manner that some host legumes avoid aluminium toxicity (see below). Other problems of acid soils such as manganese toxicity and calcium or phosphorus deficiency appear to have a lesser effect on rhizobial survival (Keyser and Munns, 1979a,b).

The *R. tropici* strain CIAT899 (UMR1899) appears to be among the most tolerant of acidity of all rhizobia tested (Graham *et al.*, 1994). The rhizobial population able to nodulate *P. vulgaris* in an acid soil in Kenya was dominated by *R. tropici* whereas a soil of near-neutral pH was dominated by other rhizobial species (Anyango *et al.*, 1995), suggesting that *R. tropici* may be generally more tolerant of acid soils. However, the most acid soils in a comparison of plots that had been limed 6 years previously in Brazil yielded mainly strains of *R. leguminosarum* bv. *phaseoli*, and *R. tropici* was more abundant in soils that had been limed (Andrade *et al.*, 2001), tending to dispel this hypothesis.

Low pH *per se* is not the principal cause of toxicity to plants. Rather, the problems of plant growth in acid soil result from aluminium and manganese toxicity. Aluminium decreases initiation of nodules at concentrations that have no discernible effect on growth of the legume or functioning of nodules (Robson, 1983). Among the grain legumes, cowpea and groundnut are more tolerant of soil acidity than are soybean or *P. vulgaris* (Munns, 1977). Groundnut does suffer some problems in acid soil, however, as it is highly susceptible to calcium deficiency because the developing pods need to absorb adequate concentrations of calcium directly from the soil to yield well (Bledsoe *et al.*, 1949). Bambara groundnut (*V. subterranea*) can occasionally produce pods above ground, and appeared to be wholly dependent on root uptake of calcium (van der Straten *et al.*, 1995).

In general, legumes (and grasses) that have been selected for use in tropical pastures have actually been chosen as a result of screening in the acid soils in which they must be used rather than, as so often happens, on the basis of growth under optimum conditions. Thus, most species selected do exhibit some tolerance to acidity. However, species initially selected for the Australian tropics have not always been successfully transferred to other countries, as soils in Australia do not have the same problems of aluminium toxicity that are common elsewhere – for instance, in the savannahs of South America (Sanchez and Isbell, 1979).

Large differences in sensitivity to the toxic effects of acid soils have been found between different species of tropical pasture legumes (Andrew *et al.*, 1973;

Andrew, 1976; de Carvalho *et al.*, 1981). Several species of *Stylosanthes* are tolerant of concentrations of aluminium in solution that severely depress nodulation and plant growth of other species. Even between different species of *Stylosanthes* there are marked differences in aluminium tolerance (Fig. 13.1) (de Carvalho *et al.*, 1981). *Stylosanthes* is nodulated by direct infection (Chapter 2) and the toxic effects of aluminium on nodulation may be related to a reduction in lateral root formation reducing the number of possible infection sites (de Carvalho *et al.*, 1982). Aluminium is known to reduce meristematic activity if present in toxic concentrations (Clarkson, 1965) and this may also explain the reduction in nodule initiation and development commonly seen in *Stylosanthes* and other legumes.

Legumes are also able to grow in acid soils by concentrating their roots in the organic-rich surface horizons, where aluminium toxicity is meliorated. Mucuna (*M. pruriens* var. *utilis*) is moderately tolerant of aluminium toxicity in solution culture (Hairiah *et al.*, 1992b) and addition of phosphorus overcame the avoidance of aluminium in solution culture (Hairiah *et al.*, 1993). Although this suggested that mucuna might be encouraged to root more deeply into aluminium-rich subsoil in the field if phosphorus was applied, this proved to be not the case and roots remained confined to the surface horizons (Hairiah *et al.*, 1994, 1995).

Nutrient deficiencies

Several of the nutrients essential for growth of plants or bacteria play specific roles in nodulation and/or N_2-fixation (Table 13.1). Deficiencies in these nutrients, or other nutrients essential for the growth of bacteria or plants, can cause reductions in the numbers and size of nodules formed and in the amount of N_2 fixed.

Acid soils are normally highly weathered and leached and thus are deficient in a range of nutrients (Chapter 1). Phosphorus and molybdenum deficiencies are common in acid soils as they are bound into forms not available for uptake, whilst other nutrients (e.g. iron, zinc) are unavailable when the soil pH is high. Deficiencies can occur even in soils of near-neutral pH simply due to depletion of nutrients by leaching and repeated cropping. The problems associated with deficiencies of nutrients will be discussed first and subsequently approaches to diagnosing and alleviating these deficiencies.

Phosphorus

There is substantial variation in the ability of rhizobia to grow in low concentrations of phosphorus (Beck and Munns, 1984), which appears to be due to variation in the efficiency of phosphorus uptake systems (Smart *et al.*, 1984). Strains of rhizobia also differ in their ability to store phosphorus, as polyphosphate (Smart *et al.*, 1984) and as bacterioferritin (St Pierre *et al.*, 1999). Even in the most efficient case the amount of phosphorus stored can only support growth for three to four generations after removal of all phosphorus from the medium (Cassman *et al.*, 1981; Beck and Munns, 1984). High concentrations of calcium are required for growth of rhizobia in low concentrations of phosphorus (Beck and Munns, 1985; Watkin *et al.*, 1997) but

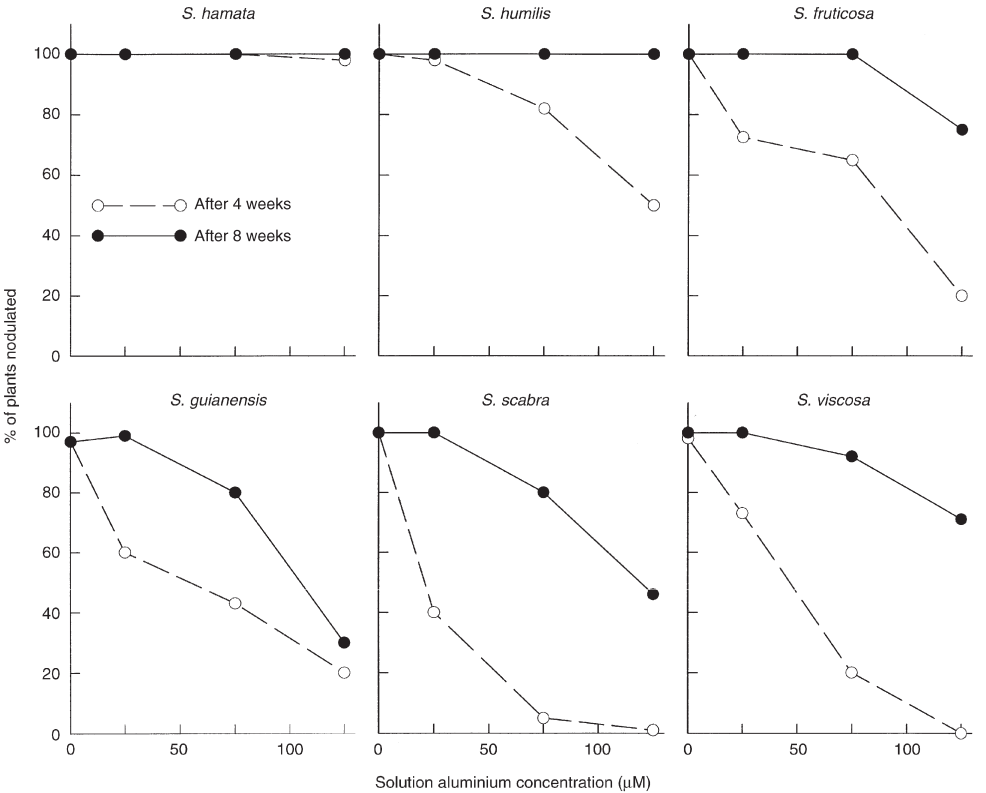

Fig. 13.1. Differences in the ability to nodulate between different species of *Stylosanthes* when grown in sand and irrigated with solutions containing increasing concentrations of aluminium. (From de Carvalho *et al.*, 1981.)

Table 13.1. Micronutrients with an essential role in legume nodulation or N_2-fixation (for further information see Robson, 1983; O'Hara *et al.*, 1988a; O'Hara, 2001).

Micronutrient	Effect of deficiency	Role	Ref.[a]
Boron	Reduction in number and size of nodules	Cell division and cell wall development during nodule formation	1
Cobalt	Reduction and delay in nodule initiation, reduced multiplication of rhizobia in the plant, N deficiency	Cobalamin-dependent enzymes in rhizobia	2
Copper	Reduced N_2-fixation	Unclear	3
Iron	Reduction in nodule initiation, nodule development and N_2-fixation rate	Constituent of nitrogenase proteins and of leghaemoglobin	4
Molybdenum	Ineffective nodules, N deficiency	Constituent of the Mo-Fe protein of nitrogenase	5
Nickel	Nodulation delayed, plant growth reduced	Urease activity in legumes and uptake hydrogenase in rhizobia	6
Selenium	Reduced hydrogenase activity and autotrophic growth in free-living *Bradyrhizobium*	Constituent of uptake hydrogenase in *Bradyrhizobium*	7
Zinc	Reduction in number and size of nodules	Possibly involved in leghaemoglobin synthesis	8

[a]1: Mulder, 1948; Bonilla *et al.*, 1997; 2: Ahmed and Evans, 1960; Dilworth *et al.*, 1979; Riley and Dilworth, 1985; 3: Snowball *et al.*, 1980; Seliga, 1993, 1998; 4: O'Hara *et al.*, 1988b; Tang *et al.*, 1990, 1992; 5: Smith, 1999; 6: Eskew *et al.*, 1983; Klucas *et al.*, 1983; 7: Boursier *et al.*, 1988; 8: Demetrio *et al.*, 1972; Marsh and Waters, 1985.

calcium deficiency often accompanies phosphorus deficiency in acid soils. Although *Bradyrhizobium* strains appear not to synthesize alkaline phosphatase, other fast-growing rhizobia appear to produce both acid and alkaline phosphatases (O'Hara, 2001). Rhizobia are well adapted to survive periods of nutrient starvation and can respond quickly when nutrients become available once more (Thorne and Williams, 1997).

Acute deficiency of phosphorus can prevent nodulation by legumes. Phosphorus and sulphur are required for nodule metabolism and tend to be concentrated in the nodules when the plant is deficient in these nutrients (O'Hara *et al.*, 1988a). It has been suggested that, as nodulated plants often have less well-developed root systems than non-nodulated plants, the ability of nodulated plants to capture nutrients, particularly phosphorus, is decreased (Cassman *et al.*, 1980).

Most legumes are heavily dependent on mycorrhizas for efficient uptake of phosphorus (Hayman, 1986). Mycorrhizas assist in uptake of phosphorus (and

possibly other nutrients that are poorly mobile in the soil) by increasing the volume of soil effectively explored by the plant. Most herbaceous legumes and some trees and shrubs are infected by vesicular–arbuscular (VA) mycorrhizas. Other woody legumes form ectomycorrhizas, specifically members of the tribe *Amherstieae* and some genera (*Afzelia*, *Intsia*, *Eperua*) in the tribe *Detarieae* of the *Caesalpinioideae*, the tribe *Mirbelieae* of the *Papilionoideae* and other members of the *Papilionoideae* that have similarities to the *Caesalpinioideae* (Alexander, 1989). Some species can form both VA mycorrhizas and ectomycorrhizas. The degree of dependence on the mycorrhiza for capture and uptake of phosphorus is partly determined by the root geometry of the legume; legumes with a poorly branched root system with few root hairs – e.g. *Leucaena* (Munns and Mosse, 1980), and *Centrosema pubescens* and *S. guianensis* (Crush, 1974) – tend to be more dependent on mycorrhizas than those plants with many long root hairs – e.g. *Lotus pedunculatus* (Crush, 1974). Nodulation and growth of legumes growing on phosphorus-deficient soil are often stimulated by mycorrhizal inoculation (Sieverding, 1991). Given the similarities between the behaviour of molybdate and phosphate ions in soil, it is likely that VA mycorrhizas also improve the uptake of molybdenum from soil. In addition, some ecto-mycorrhizas have been shown to possess proteolytic enzymes that allow the fungus to supply the plant with nitrogen from organic compounds in the soil (Abuzinadah and Read, 1986; Bending and Read, 1996).

The formation of dense clusters of rootlets or 'proteoid' roots by *Casuarina*, which is thought to be induced by microorganisms, can also aid phosphorus uptake by increasing the surface area of the roots (Malajczuk and Bowen, 1974). Host plant growth in *Casuarina* appears to be more sensitive to deficiency of phosphorus than nodulation or N_2-fixation *per se* (Yang, 1995; Reddell *et al.*, 1997).

Other major nutrients

In acid soils, deficiencies of the major cations calcium and magnesium are common, due to the small amounts of these cations present and to the secondary effect of high concentrations of hydrogen ions inhibiting uptake of these cations by plants (Andrew, 1978). The importance of calcium for the ability of rhizobia to infect legumes has been well documented (O'Hara *et al.*, 1988a; O'Hara, 2001). Its functional role is not certain, but it has been shown to be of importance in the initial attachment of rhizobial cells to root hair tips. *Rhizobium* cells grown in low calcium concentrations (the conditions, incidentally, that prevail in the commonly used growth medium, TY) show decreased attachment ability (Smit *et al.*, 1987) and this has been correlated with the calcium dependence of a proteinaceous cell-surface component of *R. leguminosarum* bv. *viciae*, believed to be required for rhizobial attachment to the root (Smit *et al.*, 1989a,b). This molecule, called rhicadhesin, belongs to a particular class of 'adhesin' molecules that appear to be limited to the *Rhizobiaceae* and may be essential for the rhizobial infection of legumes (Smit *et al.*, 1989b). Both calcium and magnesium ions appear to be involved in a specific, tight binding of rhizobia to roots of the host legume in the early stages of infection (Lodeiro *et al.*, 1995). Calcium may also act as a secondary messenger in Nod factor transduction in root hairs (Niebel *et al.*, 1999).

The infection process also appears to be affected by calcium deficiency from the plant side, particularly in plants infected through root hairs (Andrew, 1976). Given the importance of calcium in plant cell walls, it seems likely that deficiency of calcium causes problems in formation and growth of infection threads (Sethi and Reporter, 1981).

Potassium is mobile in soil and is readily leached, and is also taken up by plants in large quantities so that deficiencies are likely in soils subject to repeated cropping. Sulphur deficiency is widespread in the tropics, particularly on sandy soils. No direct role of potassium or sulphur on N_2-fixation has been reported but, as with phosphorus, addition of sulphur can increase nodulation of legumes on deficient soils (Andrew, 1977).

Micronutrients

Molybdenum deserves special consideration here as one of its major requirements in plants is for N_2-fixation. In fact, Mo is a constituent of the enzymes nitrate reductase, required for assimilation of nitrate from the soil, and nitrogenase, so that Mo deficiency is manifested as a deficiency of plant N. If a symbiosis is established, unusual proliferation of nodules is often observed when legumes are deficient in Mo, presumably in response to the ensuing N deficiency of the plant. Less Mo is required for nitrate reduction than to support N_2-fixation (Parker and Harris, 1977) so that legumes are particularly susceptible to Mo deficiency when relying on N_2-fixation. It is presumably for this reason that Mo is very efficiently concentrated in the nodules of Mo-deficient plants (Brodrick and Giller, 1991a). The alternative nitrogenases which do not require Mo (Chapter 3) have not been found in legume root nodules (Dilworth and Loneragan, 1991). Deficiencies of Mo have been reported in crops in many parts of the tropics – e.g. Australia (Anderson, 1956), Brazil (Franco and Day, 1980), Malaysia (Kerridge, 1981; Cheng and Kerridge, 1982), Senegal (Martin and Fourrier, 1965) and Zimbabwe (Tanner, 1982). Mo deficiency is common in acid soils that have a strong capacity to immobilize phosphorus, as molybdenum is also bound in an unavailable form in such soils. Deficiencies can also occur in very sandy acid soils, where addition of phosphorus has actually been found to depress uptake of Mo by groundnuts (Rebafka *et al*., 1993b).

Deficiencies of selenium and nickel are unlikely to occur, as these are generally present in large quantities in soil, but deficiencies of other micronutrients are common in the tropics. Deficiencies of any micronutrient will ultimately result in reduction of growth and N_2-fixation but particular roles in the process of nodulation and N_2-fixation can be assigned for some micronutrients (Table 13.1). The roles of micronutrients in rhizobia, both in the free-living state and in development of symbioses with legumes, are reviewed in detail by O'Hara (2001).

Cobalt is unusual in that it is used in the cobamide electron transport pathways of rhizobia and is thus essential for N_2-fixation in legumes, though it is not required for the growth of plants that are not dependent on symbiotic N_2-fixation. There is evidence that supplies of cobalt to bacteroids in nodules can be inadequate even when the host plant contains sufficient cobalt (Riley and Dilworth, 1985). Effects of cobalt deficiency on agricultural production are manifested higher up the food chain in lack

of growth in animals, for which cobalt is an essential nutrient (e.g. 't Mannetje *et al.*, 1976).

Deficiencies of boron, iron, manganese and zinc most commonly occur in alkaline soils, as these elements are less available at high pH, but zinc and copper deficiencies are also frequently encountered in acid soils (Sanchez and Salinas, 1981). Boron is readily leached and is often deficient in sandy soils under high rainfall in the tropics. However, iron and manganese deficiencies are rare except in calcareous soils and in fact the opposite problem is often encountered as waterlogging increases their availability, which can lead to toxic concentrations of iron and manganese in solution. Nevertheless, some strains of *Bradyrhizobium* produce siderophores, which will assist in uptake of iron at low concentrations (Nambiar and Sivaramakrishnan, 1987; Guerinot, 1991), and this may explain why two *Bradyrhizobium* strains differed markedly in their ability to nodulate and fix N_2 in an iron-deficient soil (O'Hara *et al.*, 1993). Strains of fast-growing rhizobia also differ in their ability to scavenge iron (Carson *et al.*, 2000). Iron, and other micronutrients, appear to be transported into nodules in the phloem (Brodrick and Giller, 1991a; Parsons *et al.*, 1995).

Diagnosis and correction of nutrient problems

Where nutritional problems are suspected, a combination of plant and soil analysis can provide evidence about the probable limiting factors, but field experiments are essential to prove the causes of growth limitation (Sanchez, 1976). Once the causes have been diagnosed, possible ways of alleviating the problem can be devised. The simplest approach is to use mineral fertilizers but in many parts of the tropics they are either not available or are prohibitively expensive for use in subsistence agriculture (Sanchez and Salinas, 1981). These factors necessitate a low-input approach. Selection of adapted plants will be considered in Chapter 14 and attention is paid here to alleviation of constraints with low inputs.

Acidity

In highly weathered, acid soils it is generally impractical to consider increasing the pH to 6.0 or more, as is the practice in many countries in temperate regions. Many tropical crops grow well when the soil is acid as long as aluminium is not present in toxic concentrations, and in some cases too much lime can have harmful effects on crop growth by inducing magnesium deficiency. Where aluminium toxicity is a problem, addition of small amounts of lime to reduce the aluminium saturation of the soil and to correct calcium deficiency is often sufficient to improve the growth (and nodulation) of plants without substantially altering the pH of the soil (Kamprath, 1970). The amount of lime required to reduce the aluminium saturation to an acceptable level can be calculated on the basis of the amount of exchangeable aluminium in the soil (Kamprath, 1970; Oates and Kamprath, 1983). However, even the use of small amounts of lime may be impossible in areas remote from natural sources and in such regions the only option is to use crop plants that are highly

tolerant to aluminium, though it may still be impossible to avoid calcium deficiency without the addition of some lime (200 kg ha^{-1}). As with other plants, the degree of tolerance of legumes to soil acidity, and hence the amount of lime required to correct the problems of soil acidity, varies considerably (Munns and Fox, 1977). Animal manure (Grant, 1967) and crop residues (Rebafka *et al.*, 1993a) can provide cations and ameliorate problems of soil acidity on sandy soils.

Phosphorus

In the case of nutrients that are deficient due to their lack of availability for plant uptake from the soil, direct addition of fertilizers to the soil may not work well, as the fertilizer itself will become unavailable. This is often the case for phosphorus and molybdenum. The capacity of soils to adsorb phosphorus irreversibly can be reduced by green manuring, thus increasing the long-term effectiveness of phosphorus fertilization (Le Mare *et al.*, 1987; Nziguheba *et al.*, 1998). Pelleting of legume inoculants on to seed with lime and small amounts of molybdenum and phosphorus and pelleting of seed with rock phosphate have been used as methods of ensuring that phosphorus is supplied directly to the germinating seed (Norris, 1967).

There are also soils that do not have a large capacity to remove phosphate from solution but show deficiency due to a simple lack of phosphorus. In such cases addition of phosphate fertilizers can be successful and has been shown to improve growth and nodulation of *P. vulgaris* in neutral soils in Kenya (Ssali and Keya, 1983) and in Tanzania (Giller *et al.*, 1998).

Rocks rich in phosphorus can provide a cheap source of fertilizer. There are many sources of rock phosphates in the tropics though they differ enormously in the amount and form of phosphorus (Hammond *et al.*, 1986; Rajan *et al.*, 1996). The availability of phosphorus from rock phosphates depends upon the rate of solubilization and is increased by grinding the rocks into a fine powder before they are added to soil. As rock phosphates are generally complex calcium phosphates, they dissolve more rapidly under acid conditions where the concentration of calcium is low – so acid tropical soils are ideal in this respect. Legumes tend to acidify their rhizosphere soil when actively fixing N_2, due to uptake of more cations than anions, with a consequent net loss of protons from the roots. This acidification can help to dissolve rock phosphate when the soils are not particularly acid and addition of a small amount of soluble phosphate to aid initial establishment of the legume can enhance this effect (Aguilar and van Diest, 1981; de Swart and van Diest, 1987).

Pigeonpea appears to be unusual in its ability to access forms of phosphorus normally poorly available in the soil. Root exudates of pigeonpea contain an organic acid, piscidic acid, that has a specific ability to solubilize iron phosphates (Ae *et al.*, 1990). Although the significance of this observation is unproven in soils where organic acids may be rapidly metabolized by microorganisms, it may partly explain why pigeonpea can grow well on infertile soils. Exudation of organic acids is only one of the adaptative mechanisms by which legumes can capture phosphorus when concentrations are limiting. Other mechanisms involve an increase in the volume of soil explored, through a finely branched rooting system, and through infection with mycorrhizas (Rao *et al.*, 1999a). Adaptative growth under conditions of phosphorus

limitation can also result from more efficient utilization of the phosphorus taken up by the plant for growth. Forage legumes such as *Arachis pintoi* appear to be better adapted to access phosphorus in acid soils than grasses such as *Brachiaria decumbens* (e.g. Rao *et al*., 1999b).

Adaptation to growth under phosphorus-limiting conditions in *P. vulgaris* appears to result from better acquisition of phosphorus rather than differences in the efficiency with which phosphorus is utilized (Lynch and Beebe, 1995). Genotypes with a superior ability to capture phosphorus from the soil appear to be those with more highly branched rooting systems. A wide variability between genotypes of *P. vulgaris* in phosphorus capture suggests that breeding and selection for enhanced growth in phosphorus-limiting soils may be worthwhile (Yan *et al*., 1995; Araújo *et al*., 1997). An empirical approach to selection of *P. vulgaris* for yield in poor-fertility soils in Africa has identified several promising varieties (Wortmann *et al*., 1995). Multi-locational testing in a range of soils throughout East, Central and southern Africa identified varieties that consistently outyield the local varieties commonly grown by smallholders. While part of the enhanced yields are probably due to better acquisition of phosphorus, an enhanced ability of these genotypes to fix N_2 is also likely to play a major role, together with better disease resistance and other traits.

In contrast, genotypic variation in mycorrhizal colonization has been highlighted as a major adaptation for phosphorus acquisition in soybean, mucuna and *Lablab purpureus* (Nwoko and Sanginga, 1999). As indicated above, VA mycorrhizas are important for uptake of phosphorus, largely through increasing the volume of soil explored. Thus, if soils do not contain an abundance of infective VA mycorrhizal propagules, or if the native VA mycorrhizal fungi do not form an effective symbiosis, it is possible to improve the establishment of root infection, and hence uptake of phosphorus, by inoculating with mycorrhizae (Mosse, 1977). This has been demonstrated in the field in Senegal where inoculation of soybeans with *Glomus mosseae* improved N_2-fixation and yield of soybean growing in a phosphorus-deficient soil that had only a small population of indigenous VA mycorrhiza (Ganry *et al*., 1985). Similar growth benefits have been found in field-grown tropical pasture legumes inoculated with VA mycorrhiza (Medina *et al*., 1990).

Enhancement of soil populations of VA mycorrhiza by inoculation is probably an unrealistic proposition on a large scale for tropical field crops or pasture legumes, due to the problems of producing inoculants in sufficient quantities. VA mycorrhizal fungi are obligate symbionts and cannot be cultured axenically, and so the fungi must be propagated on plant roots. Thus, despite some improvements in the ease of VA mycorrhizal inoculum production by the use of clays as growth media for the host plants, and storage of the clays (containing mycorrhizal spores and hyphae) for later use as inoculants, the large quantities of inoculants required in the field cannot easily be produced. It has been suggested that VA mycorrhizas could be multiplied on a sufficient scale for use on large areas by initially inoculating small 'starter' areas of fields and growing a plant that is generally heavily infected by VA mycorrhiza (e.g. *Brachiaria*). Once the mycorrhiza have been multiplied sufficiently, the soil from the starter areas can then be spread across the field.

The population of mycorrhizal propagules in soil can also be manipulated by rotation of arable crops (Sieverding and Leihner, 1984). In India, leaving the land fallow or growing a non-mycorrhizal crop (mustard) led to a reduction in the number of infective propagules of VA mycorrhizas whereas the number increased when cowpea was grown (Harinikumar and Bagyaraj, 1988). Likewise, mycorrhizal infection of cowpea and *S. guianensis* was greater when grown after either cassava, *Pueraria*, *Brachiaria dictyoneura* or sorghum than when grown on previously undisturbed savannah (Dodd *et al.*, 1990a). This research indicates the possibility for enhancement of infection by VA mycorrhizas without the need for inoculation, particularly as some crop species tend to promote the multiplication of particular species of mycorrhizal fungi (Dodd *et al.*, 1990b).

Other major nutrients

Other major nutrient deficiencies can also be corrected by the use of chemical fertilizers. Potassium deficiency can be alleviated using low-cost fertilizers made from rocks such as feldspars, which are rich in potassium (Sanz Scovino and Rowell, 1988). Sulphur deficiencies used to be ameliorated as an indirect result of the application of single superphosphate, the form of phosphorus fertilizer formerly used, which contained a substantial amount of sulphur. However, the form now widely used because of its greater phosphorus content – triple superphosphate – contains little sulphur. Sulphur supplies may also be replenished if nitrogen is supplied in the form of ammonium sulphate fertilizer. When sulphur is added deliberately to correct deficiency, it is usually added as gypsum (calcium sulphate) or elemental sulphur.

Micronutrients

Although molybdenum is essential for N_2-fixation, and molybdenum-deficient soils do occur (see above), the amounts required are so small that the seed of grain legumes can contain sufficient molybdenum for the growth of one generation of plants (Harris *et al.*, 1965). Thus it may be feasible to import seed grown on soils rich in molybdenum, which are often found nearby (Brodrick *et al.*, 1992, 1995; Jongruaysup *et al.*, 1997). Some genotypes of *P. vulgaris* are much more efficient than others in translocating molybdenum into the seed (Franco and Munns, 1981; Brodrick and Giller, 1991b) and may be particularly useful for production of seed for planting in deficient soils. Liming to raise the pH can correct the problem of molybdenum deficiency in most cases but, as discussed earlier, this is often impractical in the tropics. Soaking of the seed in molybdenum can be a sufficient alternative to correct deficiencies and is certainly more effective than applying molybdenum fertilizers to the soil (Reisenauer, 1963).

Most micronutrient deficiencies can be readily corrected by the use of foliar sprays, as long as the deficiency is not so acute as to prevent initial establishment of the plants. For instance, deficiency of boron in *P. vulgaris* and *V. radiata* growing on a high pH Mollisol in Colombia was readily corrected by spraying with borax (Howeler *et al.*, 1978).

Pollution

There is evidence that at least some of the myriad pesticides used in agriculture can have adverse effects on the survival of rhizobia or on nodulation of legumes (Edwards, 1989; Roberts, 1992), and on cynanobacteria in ricefields (Roger, 1995c). Particular attention must be paid if legumes are to be inoculated with rhizobia by seed coating when agrochemicals are also applied to the seed surface (Graham *et al.*, 1980).

Pollution of agricultural soils caused by the addition of heavy-metal contaminated sewage sludges has been shown to suppress N_2-fixation completely in white clover (*Trifolium repens*), due to the toxicity of the heavy metals to *Rhizobium* (Giller *et al.*, 1989; Chaudri *et al.*, 1993). Such problems are likely to occur in areas with much heavy industry in the tropics, particularly as pollution is often not as strictly controlled as in countries that have been industrialized for longer. Given the rapid rate of urbanization in much of the tropics, it is likely that additional man-made stresses are likely to be of increasing concern in the future.

Biological Factors

Growth and survival of rhizobia (and free-living N_2-fixing bacteria) will be influenced by competition and antagonism from other organisms. Some microorganisms, including fungi and other bacteria, will compete for nutrients, while direct antagonistic effects include production of toxic bacteriocins, lysis by bacteriophages, predation by protozoa or parasitism by *Bdellovibrio* (Roughley, 1985). Such phenomena can be demonstrated in laboratory media but the extent of their importance in soil is unknown. Grazing of rhizobia in soil by protozoa has been shown to reduce the populations of rhizobia in soil (Danso *et al.*, 1975). Soils that contain more clay afford some protection against grazing by protozoa, as the rhizobia may take refuge in micropores of soil aggregates (Heijnen *et al.*, 1991). Susceptibility of strains to bacteriophages may also result in their poor survival (Barnet, 1980).

Damage to plants by pests and diseases and grazing by animals will have deleterious effects on plant growth and thus indirectly on N_2-fixation. Specific damage to root nodules is caused by some insects in soil. The larvae of the weevil *Sitona* bore directly into root nodules, presumably recognizing them as a rich source of protein. Similar damage to nodules of pigeonpea by larvae of *Rivellia angulata* has been reported to be particularly severe in Vertisol soils in southern India (Kumar Rao and Sithanantham, 1989).

Multiple Stresses

In the real world, it is likely that many physical, chemical and biological stresses will come into play in a single field at the same or different times. Constraints during the production phase are obviously important. Likewise, factors that influence

the survival of microorganisms between cropping seasons may result in subsequent failures of the symbiosis. Attempts have been made to estimate the relative importance of different environmental factors using multi-factor models (e.g. Woomer *et al.*, 1988). In this study of different environments across a range of altitudes and soil and vegetation types in Hawaii, the major factors that correlated with the numbers of rhizobia in soil were rainfall, legume cover and shoot biomass, soil temperature, soil pH and phosphorus retention. Obviously this covers a wide range of variables, many of which are interdependent, and it is not easy to assign relative importance to each of the multitude of stresses likely to be encountered in the field. But it is certainly important that we remain constantly aware of the complexity of natural environments.

Conclusions

In the tropics, there are large areas of soils that are ill-suited for plant growth and where it is not feasible to alter the environment for crop production. Stresses such as excessive temperatures and moisture loss from soil can be reduced by improvement of the organic matter content of soils, and this will also help to reduce the problems of acidity and nutrient availability.

Unfortunately, unlike carbon and nitrogen, other nutrients cannot be fixed from the atmosphere and thus improvement of the general nutrition of plants for N_2-fixation must rely on better conservation and more efficient use of nutrients within cropping systems. Even then, there will be an inevitable net decrease of soil nutrients, particularly phosphorus and potassium, and so ultimately, unless we are prepared to accept ever-decreasing yields, these will have to be replenished in the form of fertilizers. Ironically, if crop yields are increased through N_2-fixation, the removal of other nutrients is also increased.

Chapter 14

Approaches to Enhancing N_2-fixation

Research on N_2-fixation continues to thrive: the most recent International Congress on N_2-fixation at Foz da Iguaçu, Brazil, in 1999 attracted almost 700 participants, and the literature on all aspects of free-living and symbiotic N_2-fixation continues to grow at an alarming rate. But what impact has this had on tropical farming systems? Research designed to increase the contribution of N_2-fixation has focused on a few aspects: inoculation with rhizobia; improvement of N_2-fixation by individual rhizobial strains; selecting or manipulating the host legume genotype; and changes in farming practices. The success of these approaches will be discussed in turn.

Inoculation with Rhizobia

The first patents on rhizobial inoculation were filed at the end of the 19th century, very shortly after the recognition that legume root nodules were the site of N_2-fixation by symbiotic bacteria, rather than pathological galls, and so the concept of manipulating N_2-fixation by the introduction of strains of bacteria goes back more than 100 years (Eaglesham, 1989). The first inoculum produced went under the name of Nitragin, a trade name that still covers one of the main commercial sources of inoculum in North America today. The main rationale for commercial production of inoculum at that point was to enable introduction of legume species into areas where they had not previously been grown – a strategy that is likely to meet with success in all cases where none of the indigenous bacteria are capable of nodulating the introduced species.

The countries that produce the largest amounts of rhizobial inoculants in the world are the USA, Brazil and Argentina – the three main producers of soybean (Saint Macary *et al.*, 1992). Virtually all of the inoculants produced in these or other countries

are for use with soybean which, as discussed in Chapter 8, has been widely introduced into new regions in recent years. The high-yielding varieties that are generally used are highly specific in nodulation and compatible rhizobia are rarely present. The only real exception to this rule is in Australia, where inoculants are commonly used for pasture legumes, as many of the legumes were introduced from other continents.

Promotion of inoculation as a technology has in some cases meant that research and improvement of N_2-fixation in legumes has been equated solely with inoculation and has led to the abandonment of other aspects of N_2-fixation research as a main discipline in the improvement of tropical grain legume crops. In many cases where rhizobial inoculation is said to have failed, this occurred because inoculation was recommended for crops that do not respond with the inoculant strains. If farmers are sold products that do not work, they are unlikely to adopt the technology. Another major reason for the failure of inoculation derives from the almost random selection of strains tested and this is discussed in detail in the subsequent section.

Assessing the need for inoculation

Three situations can be identified when introduction of rhizobia is necessary to establish nodulation and effective N_2-fixation in legumes: (i) where compatible rhizobia are absent; (ii) where the population of compatible rhizobia is too small to give sufficiently rapid nodulation; and (iii) where the indigenous rhizobia are ineffective or less effective in N_2-fixation with the host legume of interest than selected inoculant strains. It is important to realize that the simple observation of 'poor' nodulation on a field-grown legume is not clear evidence that any of these conditions apply, due to the enormous number of environmental constraints that can interfere with nodule formation (Chapter 13), and due to difficulties in observing or recovering nodules on deeper roots.

Potential benefits from inoculation are best assessed by conducting need-to-inoculate trials in the field, in which uninoculated plots, inoculated plots and plots fertilized with substantial amounts of N are included (Vincent, 1970; Date, 1977; Sylvester-Bradley, 1984). If growth of the legume is not improved by N-fertilizer then it is likely that other factors are limiting and inoculation is unlikely to result in improvements in yield. Most workers recommend either that adequate amounts of other nutrients are added to the soil in such need-to-inoculate experiments to ensure that benefits from inoculation are not limited by soil fertility, or that plots both with and without addition of other nutrients are included. This is an important point in guiding decisions as to the practical benefits that are likely to accrue from inoculation – they may depend on the availability and cost of other fertilizers.

The likelihood of responses to inoculation can also be assessed by counting the population of rhizobia in the soil using an appropriate trap host (Thompson and Vincent, 1967; Woomer *et al.*, 1990). If there is a small population of effective rhizobia (less than 20–50 cells g^{-1} soil) then it is likely that a yield response to inoculation will be found (Singleton and Tavares, 1986; Thies *et al.*, 1991a). A simple model was developed to predict the likelihood of inoculation responses based

on the initial N status of the soil and initial number of indigenous rhizobia (Thies *et al*., 1991b). However, although this method can demonstrate where responses to inoculation are likely, the presence of a large indigenous population of compatible rhizobia certainly does not preclude the possibility that responses to inoculation can be obtained if competitive and highly effective strains are introduced in high-quality inoculants. If compatible rhizobia are absent, nodulation and N_2-fixation in the crop are likely to increase in proportion to the number of rhizobia applied in the inoculum (Brockwell *et al*., 1985, 1989).

A useful flow diagram as to the decisions to be taken in the adoption of inoculant technology is given by Saint Macary *et al*. (1992) (Fig. 14.1) based on experience in a wide variety of tropical countries. The main point to note in this diagram is that if responses to inoculation are not found it is important to ascertain whether the reason crucial to the success of inoculation in the field is the prior choice of appropriate strains. As discussed, if rhizobia compatible with the legume of interest are not present in the soil then virtually any effective strain is likely to improve yield. But if inoculants are to be used in soils that contain effective rhizobia, or if environmental stresses are particularly great, then much more care must be taken in the choice of the inoculant strains. Methods for selection of strains of rhizobia are considered further below.

Inoculum technology

Methods of inoculation

Introduction of soil from an area where plants are well nodulated can work well as an inoculant of rhizobia when other methods are not practical. The most widely used form of inocula, however, is pure cultures of rhizobial strains impregnated into a solid substrate such as finely ground peat (Williams, 1984). The peat serves as a carrier to allow the inoculant to be coated on to the surface of the seed, and also serves to protect the bacteria against dessication. Peat-based inoculants are coated on to seed with a sticking agent, such as a solution of methyl cellulose, that increases the amount of peat that adheres, and then the inoculant is dried on to the seed. Synthetic polymers have also been developed that promote the adherence of dry peat inoculants on to seed without the need for wet sticking agents.

Much research has been done in different localities to find suitable alternatives where peat is not available (Williams, 1984; Stephens and Rask, 2000). In many regions the by-products of sugarcane refineries, bagasse and filter mud, can provide useful carriers. The main criteria for the selection of a suitable solid carrier for inoculants are a high water-holding capacity, an ability to support the growth of rhizobia and an ability to favour survival of rhizobia. Application of inoculant in liquid form to the soil is also effective (Hynes *et al*., 1995) but liquid inoculants have a more limited shelf life and need to be stored at cooler temperatures, which adds to the cost of their use (Stephens and Rask, 2000).

Methods of inoculation other than seed coating have been proposed, but the only other methods that have achieved widespread success involve the use of

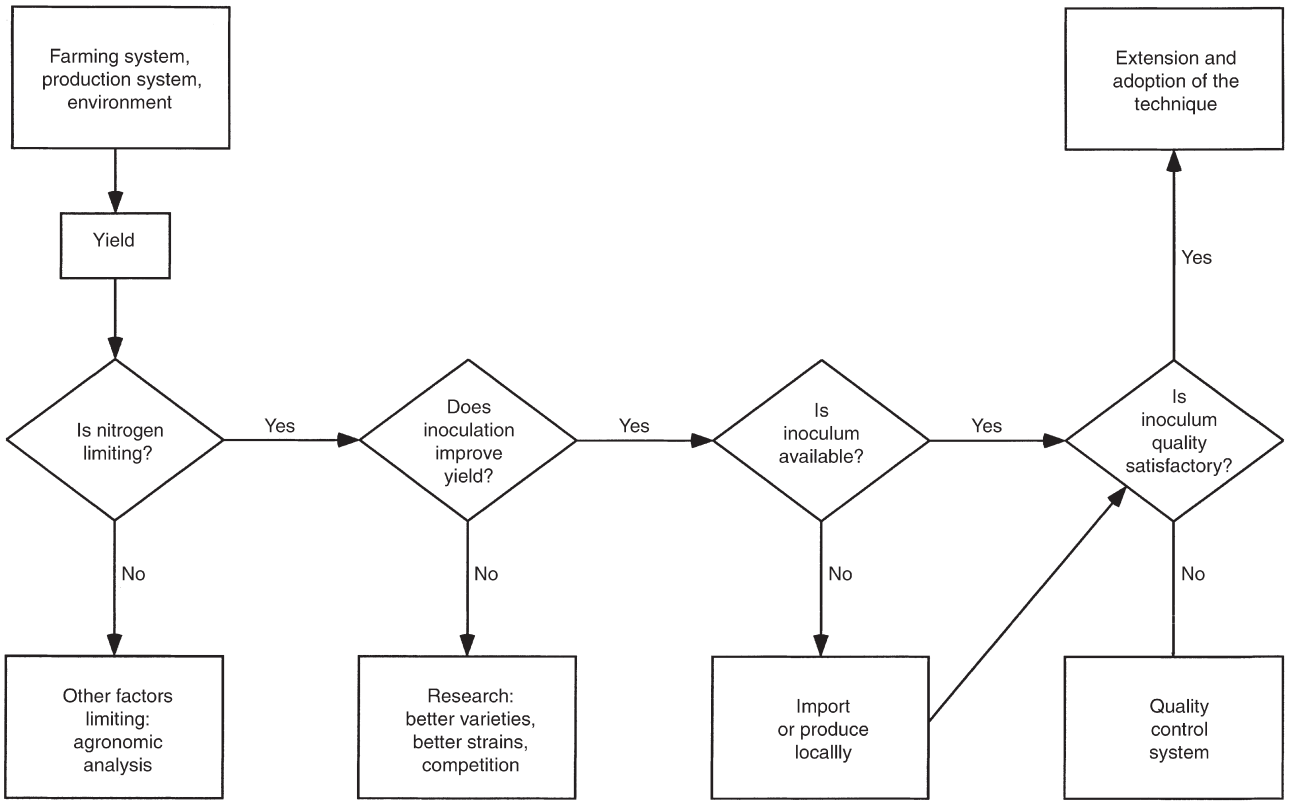

Fig. 14.1. A flow diagram demonstrating the decisions that need to be taken in deciding whether it is necessary to recommend inoculation for a legume. (After Saint Macary *et al.,* 1992.)

seed-pelleting or granular inoculants. Pelleting of seed in lime or rock phosphate was developed in Australia as a method of introducing legumes into acid soils (Norris, 1967) and peat inoculants can be readily incorporated into the pellet. Molybdenum and other micronutrients can also be added to the pellets (De Polli and Döbereiner, 1974). Comparisons of pelleted inoculants with conventional peat-based inoculants suggest that there is no advantage to be gained from pelleting (Sylvester-Bradley *et al.*, 1990). Granular inoculants also involve the use of a solid carrier, such as peat or clay, but the inoculant is prepared as small, dry granules that flow readily and can thus be easily applied into the furrow below the seed, by hand or machine (Stephens and Rask, 2000). Higher numbers of bacteria can be applied using granular inoculants than is possible with seed coating. Granular inoculants are also of value if seed is to be dressed with insecticides or fungicides that are toxic to rhizobia or where the seed is too fragile for seed inoculation, such as with groundnut (Nambiar *et al.*, 1984). Where sufficient water is available, peat-based inocula can be diluted and applied as a liquid below the seed (Nambiar *et al.*, 1984).

Production of inoculants

The production of rhizobial inoculants is simple and requires only modest technology. Pure cultures of the required strain are grown in broth in a fermenter. Several types of fermenter have been designed for small production units in the tropics (Somasegaran and Hoben, 1985; Saint Macary *et al.*, 1986).

The culture is then inoculated into a solid carrier, which can be used to apply the inoculant to seed as described above. Most unmodified peats are too acid for good survival of rhizobia and must be adjusted to neutral pH by addition of lime. As the peat must in any case be ground, the lime is usually added to the dried peat before grinding to ensure that the lime and peat are thoroughly mixed together (Williams, 1984). To reduce competition from other microorganisms, and thus to ensure a high population of the inoculant strain, sterile peat should be used (Roughley and Vincent, 1967). Generally the peat carrier is packaged into suitably sized polyethylene bags before being sterilized by autoclaving, irradiation with gamma rays, or more recently by electron acceleration, which is effected in seconds (Stephens and Rask, 2000). After sterilization, sufficient rhizobial suspension to moisten the peat is inoculated into the bag, usually by injecting the suspension with a sterile syringe. The hole is then resealed. Cultures can be diluted up to 1000-fold before inoculation as the bacteria will then multiply in the peat (Somasegaran, 1985). Thus, with this added step of multiplication in the peat, small fermenters can be used to produce large quantities of inoculants. Polyethylene is sufficiently permeable to oxygen to allow growth of the rhizobia once the bag is sealed, and inoculated bags are usually kept at 25°C for 2 weeks to encourage rhizobial growth (a process known as curing of the inoculant) before storage.

Quality control

An effective method of quality control of inoculants – that is, ensuring that the inoculants contain the desired strain of rhizobia in sufficient numbers – is of paramount importance for their success (Vincent, 1977; Thompson, 1991). Legislative

standards for inoculant quality vary enormously between countries: in some, quality control is a legal requirement for all inoculants to be marketed; in others, there are no regulations at all (Marufu *et al.*, 1995; Lupwayi *et al.*, 2000). Where quality control is compulsory, the basis of the regulations is a minimum number of viable rhizobia contained in each gram of inoculant, and some countries also stipulate a maximum allowed number of contaminating bacteria (Williams, 1984). The quality control should ideally be carried out by an independent organization and not solely by the manufacturer, but this is often not the case (Thompson, 1991; Saint Macary *et al.*, 1992).

There are examples, which are not usually widely publicized, where inoculants have failed due simply to poor quality – the inoculant has not contained sufficient effective, compatible rhizobia for the inoculated legume. Published reports indicate that less than 20% of the inoculants sold in India are of acceptable quality – despite the existence of regulatory protocols – because there is no effective monitoring agency (Rupela and Hegde, 1997). Production of inoculants of consistently high quality requires checking of cultures for contamination by other microbes at all stages of production (Lupwayi *et al.*, 2000). It is also essential that the parent cultures are checked frequently for effectiveness in N_2-fixation on the target, legume, as inadvertent substitution of cultures, contamination, or genetic instability in the inoculant strain can lead to loss of inoculum effectiveness (Vincent, 1977).

Storage of inoculants

The optimum conditions for storage differ between carriers and strains. Survival of rhizobia is frequently better if the inoculants are stored at 4–5°C, but storage in clay pots in the shade is also effective (Mabika and Mariga, 1996). It is generally recommended that peat inoculants should not be stored for more than 6 months before use, even when it is possible to keep them refrigerated. This means that the inoculants must be prepared fresh each season, which is not a great problem where a single type (often a single strain) is being produced in large quantities, but is much more problematic if a diverse range of inoculants is required, as is the case with tropical pasture legumes. Alternative methods of inoculant production that can ensure a much longer shelf-life are required so that inoculants for each individual legume do not have to be produced close to the time of use. Freeze-dried cultures are one possible solution, as viable cell numbers can remain high for several years when the cultures are stored under vacuum. Such inoculants can be produced using existing technology for the production of vaccines, which is available in nearly all countries, but further research is required to solve problems of survival of the rhizobia when they are removed from the vacuum and inoculated on to seed.

Is reinoculation necessary each season?

Whether or not it is necessary to inoculate in successive seasons will depend on the survival of the strain in the soil. Experiments with inoculation of soybeans in Congo indicated that there was no need to reinoculate in the second or third seasons, as good nodulation was maintained in previously inoculated plots (Saint Macary *et al.*, 1992). Similar experiments conducted in Tanzania, which gave similar results, led

other workers to conclude that repeated inoculation was necessary (Chowdhury *et al*., 1983). In both the experiments uninoculated plants formed few nodules in the first season but were as well nodulated as plants from some of the inoculated plots in subsequent seasons. Certainly in the experiments in Congo this was not due to movement of the inoculant strains (which were detected using antibiotic resistance markers) into the uninoculated plots and may have been due to multiplication of effective rhizobia which were initially present in the soil in small numbers. Rhizobia are well adapted to life in the free-living state in soil and can survive for over 30 years even in the complete absence of legumes (Mårtensson and Witter, 1990).

The stability of symbiotic effectiveness of inoculated strains is an important issue. Loss of symbiotic effectiveness has been demonstrated in the laboratory (Labandera and Vincent, 1975; Weaver and Wright, 1987) and contrasting results have been found in the field. The stability of symbiotic effectiveness of a *B. japonicum* strain was shown not to vary in the 'short' term in an experiment where it was reisolated 9 years after it was first introduced (Gibson *et al*., 1990). On the other hand, strains of *R. leguminosarum* bv. *trifolii* reisolated from the field after several years differed significantly in effectiveness when compared with the parent culture (Gibson *et al*., 1990). Genetic instability has been looked at in particular detail in some of the rhizobia that nodulate *P. vulgaris*. Substantial genetic rearrangement takes place in the symbiotic plasmid of the *phaseoli* biovars of *R. leguminosarum* and *R. etli* and is a possible cause for frequent loss of effectiveness (Martínez *et al*., 1990; Brom *et al*., 1991).

It is thus difficult to generalize as to whether repeated inoculation is necessary, as this will vary between different environments (particularly if the soil environment is not conducive to rhizobial survival) and between strains of rhizobia and the need must therefore be established in each situation.

Some inoculation success stories

Grain legumes

It is no coincidence that all the success stories that will be reported here are with soybean, for which (as described previously) compatible rhizobia are rarely present when the crop is introduced into new regions. In contrast to the approach of breeding soybeans to nodulate with indigenous rhizobia as suggested in Nigeria (Chapter 8), in Brazil the approach to solving the problems of soybean nodulation has been to recommend inoculation with rhizobia (Döbereiner, 1974). Initial problems with imported inoculants in the acid cerrado soils were overcome by selection of strains adapted to the local conditions (Scotti *et al*., 1982). In 1980 it was estimated that inoculation saved more than US$800 million in production costs (Döbereiner and Duque, 1982) and soybeans are now routinely grown with inoculation in Brazil (Hungria *et al*., 2000).

In Rwanda the extension of the production area of soybeans (largely at the expense of *P. vulgaris*) was dependent on the development of local production of inoculants (Saint Macary *et al*., 1986). Inoculum production rose from 100 packets

of 40 g (each enough to inoculate 200 m^2) in 1983 to 60,000 such packets in 1990 (Scaglia and Hakizimana, 1992). The small packet size was appropriate for use by farmers in Rwanda where farms are generally less than 1 ha in area. A careful programme of experiments on research stations, followed by widespread testing of the need for inoculation in farmers' fields, ensured that the inoculants generally gave benefits under realistic conditions. Inoculation alone generally gave substantial increases in yield, indicating that N is one of the most important limiting factors. Tragically the scientist responsible, Athanase Hakizimana, died with his family during the *interahamwe* and the inoculant production facility was destroyed. Scientists from Makerere University in Uganda later assisted in supplying 'Inoculants for Hope' during a 'Seeds for Hope' programme to assist farmers but it seems that the inoculant production facility has not been fully restored.

In Zimbabwe, a recent initiative has assisted a large number of smallholders to grow soybean with rhizobial inoculants and has exploded a long-held belief that soybean was an inappropriate crop for smallholders. Success of the programme has depended on a range of issues, but a major one has been careful attention to education of farmers in the use of inoculants that are produced mainly for the commercial agriculture sector (Marufu *et al.*, 1995). This example is considered further below when discussing selection for promiscuity in soybean.

In both of these cases the researchers have been closely involved with farmer evaluation of inoculants, and in active promotion and education in the appropriate use of inoculants with both extension staff and farmers. The popularity and demand for inoculants among smallholder farmers that has been generated makes a stark contrast to the rather negative farmer response to inoculation technology reported in Thailand by Hall and Clark (1995).

Promising uses of inoculants

Perhaps pride of place for development of an appropriate inoculation technology for crop production in an extremely hostile environment still goes to the work of Duong and colleagues on soybean production in acid sulphate soils of the Mekong Delta in Vietnam. The soils are extremely acid (pH 4.5–5.0), largely as a result of the oxidation of reduced sulphur to sulphate on drying (Chapter 1). Inoculation with an imported granular inoculant increased yields from less than 500 kg ha^{-1} to nearly 2700 kg ha^{-1} (Duong *et al.*, 1984a). In further trials, yields of inoculated soybeans (> 2800 kg ha^{-1}) were ten times as high as those without either inoculation or nitrogen fertilizer and over twice as high as uninoculated plants that received 80 kg N ha^{-1} as urea (Duong *et al.*, 1984b) (Table 14.1).

This remarkable yield increase was achieved by planting of inoculated soybeans in holes together with ash from burning of rice straw (Duong and Diep, 1986). Ash caused an increase in pH and improved germination, early growth and nodulation of the soybeans. The importance of the ash treatment is illustrated by the observation that nodulation was virtually restricted to the holes to which ash had been added, presumably due to the reduced acidity and also to the calcium supplied in the ash (Fig. 14.2). Mulching also gave significant improvements in nodulation when ash was not used, as nodules formed at the interface between the mulch and the soil

Table 14.1. Response of soybean to N fertilizer as urea or inoculation with rhizobium in acid sulphate soils of the Mekong Delta. (From Duong *et al.*, 1984b.)

Treatment (kg N ha^{-1})	N in harvested grain (kg ha^{-1})	N in plant remains at maturity (kg ha^{-1})	Grain protein (%)	Grain yield (kg ha^{-1})
0	17	1	36	290
20	19	–	31	385
40	29	8	26	680
60	24	8	28	870
80	32	14	28	1140
Inoculated + 0N	185	15	40	2870

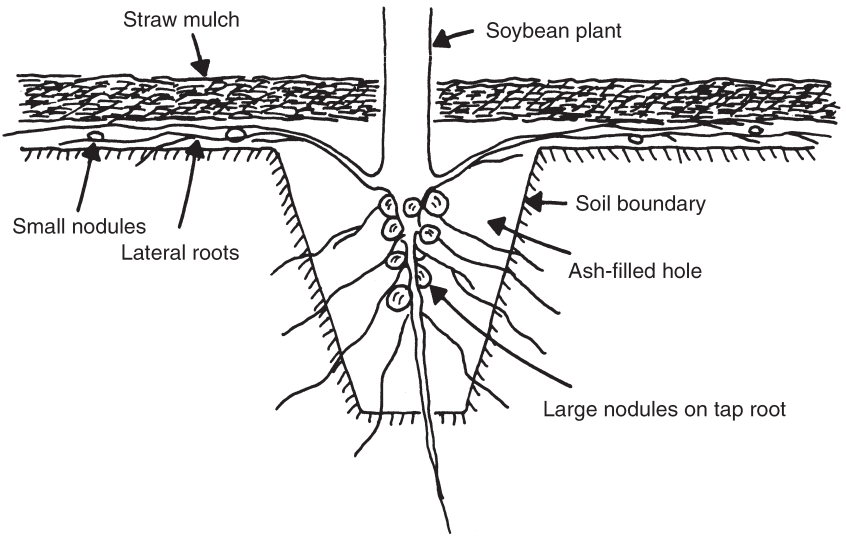

Fig. 14.2. Nodulation of inoculated soybean plants grown in ash-filled holes in an acid sulphate soil. (After Duong and Diep, 1986.)

surface later during the growth period of the crop. Given the abundance of rice straw in Southeast Asia which is often simply burned in the fields, this is a superb example of an appropriate technology for use by smallholders.

Selection of *Rhizobium* Strains

Researchers working with legumes would dearly love to be able to obtain spectacular increases in yield by inoculation in crops other than soybean but this has rarely been achieved. In many ways this is good for farmers in the tropics as they therefore get the benefits possible from N_2-fixation without needing to worry about inoculation.

This is why emphasis has been placed on selection and breeding for promiscuous nodulation in soybean (Chapter 8), which is discussed further below. If it is hoped to gain benefits from inoculation into soils that already contain compatible rhizobia, the criteria for selection must be examined more carefully. Obviously if the inoculant strains are not better at fixing N_2 than the strains in the soil, or if the inoculant strains are unable to form many of the nodules on the plant, then a response in plant growth is unlikely. In the case of legumes that have not been intensively studied, if strains are desired for a particular legume it is common practice to write to one of the well-established culture collections and request a 'recommended' strain. However, in many cases the strain supplied will simply represent an isolate collected by an itinerant rhizobiologist on a chance visit to the field. Most such strains will not have been subjected to any rigorous screening for their ability to give inoculation responses in the field against other rhizobia. This approach has been a great disservice to the scientific study of rhizobia. There is an acute danger of frightening off farmers (and funders) by promising too much without sufficient preliminary research.

The term 'selection' of strains is used here simply to mean choice of suitable strains and not in the genetic sense of selecting variants with a genetic change. Three main approaches have been taken to identify good strains for use in inocula: 'Leonard jar' assays; screening for specific traits in laboratory media; and screening in cores soil. The differences between these methods warrant detailed discussion.

Screening of strains in Leonard jars

Most strains of rhizobia have been selected for use in inoculants by screening single strains for their ability to support growth of plants in sterile media under controlled conditions, often in Leonard jars (Fig. 14.3a) (Leonard, 1943). The total N content of plants grown under such conditions is an accurate reflection of the amount of N_2 fixed by the strain. This method of screening is similar to selecting pasture legumes on the basis of their growth in nutrient solutions in controlled environments and then expecting them to survive competition from grasses, grazing, nutrient stresses, drought and all of the other stresses likely to be encountered in the field. Is it therefore surprising that rhizobial strains tested only in this manner often do not work well in the field? With soybean it has been possible to get away with such sloppy methods because native rhizobia that can nodulate soybean are often absent. But for other legumes it is necessary to select strains that are clearly more effective in N_2-fixation than the indigenous strains and that can form sufficient of the nodules to have a beneficial effect.

Competitive ability of rhizobial strains

Before going on to discuss further methods of screening and manipulation that have been applied in an attempt to obtain more suitable inoculum strains, a few aspects of rhizobial competition will be reviewed briefly.

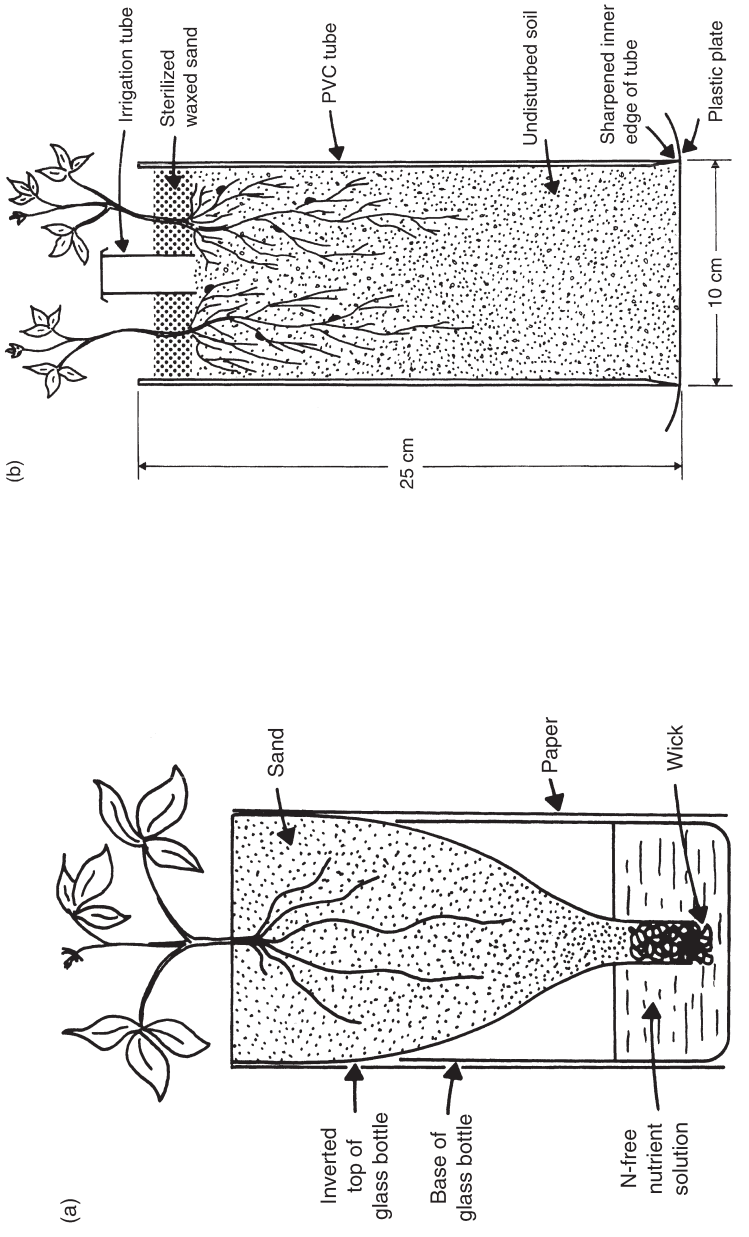

Fig. 14.3. (a) A Leonard jar assembly for screening single rhizobial strains for effectiveness in N_2-fixation with legumes. (b) A soil core used for screening rhizobial strains for ability to stimulate growth of the legume when indigenous rhizobia are present in the soil. (After Sylvester-Bradley and Kipe-Nolt, 1990.)

'Competition' is a word that is much bandied about, and encompasses an enormous range of phenomena. In broad terms it refers to interactions between two or more organisms endeavouring to gain a limited resource (see Chapter 5 for further discussion in relation to intercrops). It should be distinguished from *adaptation* to environmental conditions, e.g. a tolerance to high salinity, although in practice it is often difficult to distinguish the two parameters, as any adaptation that increases the fitness of an organism will obviously contribute to its competitive success.

In the case of rhizobia, the term competition is most commonly used to refer to competition for nodule occupancy. This is for two reasons: primarily, because this is the competitive phenomenon of greatest importance to rhizobiologists; secondly, it is the easiest aspect of rhizobial competition to study. However, aspects of the adaptation and competitive ability of free-living rhizobia in soil are also important in considerations of the need for repeated inoculation, as well as in the extent to which they affect competition for nodule occupancy.

Rhizobial competition has been the focus of a large number of studies and several reviews are available (e.g. Bottomley, 1992; Sadowsky, 2000; Scupham *et al.*, 2000). This profuse literature illustrates the fact that different rhizobial strains show different abilities to compete for nodule occupancy, and that the relative success in achieving nodule occupancy is affected by environmental factors, by host-plant species and cultivar, by initial population size, by distribution in the soil and by competition from other organisms. A table in Bottomley (1992) summarizes 19 different factors that may affect competition for nodule occupancy. Here only a few points will be selected for further discussion.

The first is that there seems to be only one example where the ability of a rhizobial strain to outcompete other strains that are capable of nodulating the same cultivar of the host species has been attributed to a single factor. This is in the case of *R. leguminosarum* bv. *trifolii* strain T24. The superior competitive ability of this strain was shown to be due to production of an antibiotic, named 'trifolitoxin', which exerts a bacteriostatic effect on almost all *R. leguminosarum* bv. *trifolii*, bv. *viciae* and bv. *phaseoli* strains tested. This effect was virtually specific to the *R. leguminosarum* group. Among other bacterial species tested for sensitivity to trifolitoxin, three *S. fredii* strains were also sensitive, but almost all *S. meliloti*, *Bradyrhizobium* and strains of diverse plant pathogenic bacteria examined were found to be resistant (see review by Scupham *et al.*, 2000). In all other cases, no single attribute can be pinned down to account for the superior (or inferior) competitive ability of a particular strain.

Another point of relevance to inoculum technology is the simple influence of numbers on competitive success. Unfortunately the results of different experiments, as usual, do not fit into any neat pattern. Several authors have demonstrated that there is little chance of inoculated strains forming many nodules if there is a significant indigenous population of compatible rhizobia. Singleton and Tavares (1986) found that a substantial rate of nodule occupancy by an inoculant strain could only be predicted if the indigenous population was less than 100 cells g^{-1} soil. Weaver and Frederick (1974) likewise concluded that a 1000-fold excess of inoculant cells over indigenous rhizobia was needed for successful inoculum establishment. On the other hand, no significant differences could be detected in the sizes of indigenous

populations of different serogroups of *B. japonicum* in bulk soil, and in the rhizosphere of host and non-host plants, even though strains of serogroup USDA123 were always most successful in competing for nodule occupancy (Moawad *et al.*, 1984). Moreover, it has recently proved possible to isolate rhizobial strains that cause an inoculation response in the tropical pasture legume kudzu (*Pueraria phaseoloides*) even when inoculated with as few as 5×10^3 cells per seed into soils containing indigenous populations of 3.5×10^3 compatible rhizobia g^{-1} soil (Sylvester-Bradley *et al.*, 1991).

Data on rhizobial population sizes are hard to evaluate, not least because of the extreme variability. Indigenous populations have been measured ranging from < 10 to 10^7 g^{-1} soil (Bottomley, 1992) and they can range from 10^2 to 10^7 g^{-1} soil within a single field (Wollum and Cassel, 1984). Perhaps greatest attention should be paid to sampling procedures in methods to assess rhizobial populations. Another important factor overlooked by many of these surveys is the diversity of strains. Population sizes are usually estimated using a plant infection count in which the presence of rhizobia able to induce nodules on a particular trap host is assessed at a series of dilutions, and is used to back-calculate the population of compatible rhizobia in the soil – the most probable number or MPN method (Thompson and Vincent, 1967). This technique is unable to discriminate directly between strains, yet application of other techniques such as multi-locus enzyme electrophoresis have shown that at least 10–20 different strains capable of nodulating a particular host plant can occur at a particular location (e.g. Pinero *et al.*, 1988). The true diversity of rhizobial strains within a single field is probably much higher.

Clearly it is important to know not just how large the population is, but also where it is in relation to the root. Bacteria are not highly mobile in soil, only showing significant migration in soil that is nearly saturated with water (Hambdi, 1971; Wong and Griffin, 1976). Moreover, their motility is substantially lower in true soil than in the artificial materials often used in experiments to assess bacterial motility and rhizobial competition, presumably due to strong adherence to components of soil (Wong and Griffin, 1976). Consistent with these observations, a *B. japonicum* non-motile mutant was shown not to be significantly affected in competition ability when tested in soil (Liu *et al.*, 1989). In other work, nodules were found to form only on zones of the soybean root that grew through areas where a *B. japonicum* inoculum had been placed initially, in a carefully controlled experiment in which pots were always watered from below to reduce passive movement of rhizobia due to water flow (Hardarson *et al.*, 1989). Since it has also been demonstrated that regions of the legume root are only transiently susceptible to nodulation (Bhuvaneswari *et al.*, 1981), the distribution of rhizobia in relation to infectable zones of the root is clearly more important than their overall numbers.

Finally, there are a couple of points about the interactions between strains that are important to bear in mind. One is the phenomenon of nodulation blocking, whereby a strain that was unable to nodulate a particular cultivar of pea was shown to suppress nodulation completely by an otherwise nodulation-competent strain (Winarno and Lie, 1979). Thus, even 'non-symbiotic' strains of 'rhizobia' that may be abundant in soil (Segovia *et al.*, 1991) may be able to exert a very specific effect on

competitive outcomes. A second point is that, although occupancy of nodules by more than one strain is well documented (Lindemann *et al.*, 1974; Johnston and Beringer, 1976), assays typically used to assess nodule occupancy only give a positive identification of a single strain and would therefore not be able to detect this phenomenon.

Screening for adaptation to stress

To take some account of the need to have rhizobial strains that are adapted to local environments, procedures have been developed for selection in the laboratory of strains that are tolerant of particular stresses. Strains have been screened for tolerance to low pH in acid media, and likewise for tolerance to high aluminium, low phosphate, or high salt concentrations, all in laboratory media (e.g. Karanja and Wood, 1988; Bottomley, 1992).

Such a reductionist approach of screening organisms by application of single stresses is unlikely to have much relevance to the field for reasons discussed above. For example, the idea that strains able to tolerate low pH in laboratory-based media would compete better in acid soils has not been borne out (Date and Halliday, 1979; Keyser and Munns, 1979a,b). Little correlation was found for *S. meliloti* between ability to grow on acid media in the laboratory and acid tolerance expressed in the field (Howieson *et al.*, 1988). Even when rhizobia indigenous to soils with a specific property, such as acidity, are examined in the laboratory to see whether a high proportion are specifically adapted to this stress, there is rarely any strong correlation (e.g. Vargas and Graham, 1988; Woomer *et al.*, 1988; Richardson and Simpson, 1989). In addition, the tolerance of a symbiosis to stress is likely to be markedly different from the tolerance of the free-living symbionts. For example, the relative ability of rhizobia to grow well on saline agar media did not correlate with tolerance to salinity of the comparable symbioses formed with pigeonpea, even when tested in the glasshouse where other stresses were absent (Subbarao *et al.*, 1990b).

What such laboratory-based screening occasionally can do is to provide possible explanations for the performance of individual strains that have been found to give inoculation responses in the field under specific conditions. For instance, the *R. tropici* strain CIAT 899 is a good inoculant strain for *Phaseolus* beans on acid soils (Sylvester-Bradley and Kipe-Nolt, 1990) and was indeed found to be tolerant of acid solutions and moderate concentrations of aluminium (Karanja and Wood, 1988). This strain appears to be the most acid-tolerant among rhizobia studied to date (Graham *et al.*, 1994).

Strain selection in soil

An alternative method of screening rhizobia directly in soil for their ability to improve growth of tropical pasture legumes has been remarkably successful (Sylvester-Bradley *et al.*, 1983, 1988a). In this approach the rhizobia are screened directly on plants growing in soil that contains indigenous rhizobia. For the initial screening, cores of

soil are taken from the field (in the wet season) by hammering 25 cm lengths of 10 cm wide PVC piping into the ground and then digging out the pipe full of soil. Soil cores sown with the test legume are then inoculated with strains of rhizobia and the ability to support N_2-fixation is assessed by measuring the total N accumulated by the plants after a period of growth. Uninoculated controls (to allow comparison of the nodulation and amounts of N_2 fixed by the indigenous rhizobia) and treatments in which N fertilizer was added (to demonstrate whether N was limiting plant growth) are included in all experiments. Strains that give good legume growth are then included in further screening tests in soil cores (e.g. Fig. 14.3b) before the best strains are selected for testing in inoculation trials in the field. The beauty of this method is that any strain which is successful in promoting plant growth must: (i) be adapted to conditions in the soil; (ii) be effective in N_2-fixation with the test legume; and (iii) be able to establish itself against the indigenous population of rhizobial strains (Fig. 14.4).

This method has been used to select rhizobia for many tropical pasture legumes in acid Oxisol soils with a high aluminium toxicity (Sylvester-Bradley *et al.*, 1983, 1988a, 1990, 1991). Strains of rhizobia have been selected for use in inoculants with each of several forage legumes and these inoculant strains consistently cause increased N production in pasture legumes growing in soil that contains large numbers of compatible rhizobia (Sylvester-Bradley *et al.*, 1988a, 1990, 1991). Experiments indicated that the strains were highly competitive and formed up to 70% of the nodules on inoculated legumes growing in soil that contained as many as 10^6 compatible indigenous rhizobia per gram of soil. In contrast, strains previously selected in Leonard jars for the same pasture legumes gave no responses in the field (Sylvester-Bradley, 1984). All of the strain screening was carried out on soils from a single location in the Colombian llanos, and it might be thought that this would lead to selection of strains that are site-specific. In fact the opposite was true – the selected strains gave inoculation responses over a wide range of locations throughout Central and South America (Sylvester-Bradley and Kipe-Nolt, 1990).

The undisturbed cores of soil are used in this screening method because it was found that the disturbance caused by digging up soil and mixing it for pot experiments stimulated such a release of soil N that nitrogen no longer limited growth of the legumes. In the field, pasture legumes are often introduced into pre-established grass swards by cutting slots in the pasture, or are sown in mixtures with grasses. Thus the undisturbed soil cores realistically mimic the situation in the field. In contrast, grain legumes are usually sown in soil that has recently been cultivated. A modification of this 'undisturbed soil core' method has been used to select rhizobia for use with grain legumes. The legume is grown in pots of soil collected from representative fields where the legume is commonly grown. Organic matter rich in carbon is added to the soil in order to stimulate microbial immobilization of soil N and thus reduce the amount of N available for plant growth. Pots of this soil are then used to screen strains against the indigenous population of rhizobia for their ability to stimulate growth of the grain legume. This method has been used to select strains for *P. vulgaris* which have given promising results when tested for their ability to increase the growth of *Phaseolus* in the field (Pineda and Kipe-Nolt, 1990).

The undisturbed soil core method is a remarkably simple and pragmatic approach to the problem of selecting strains that are highly effective in N_2-fixation, and are able to achieve this in the field in the presence of large numbers of other

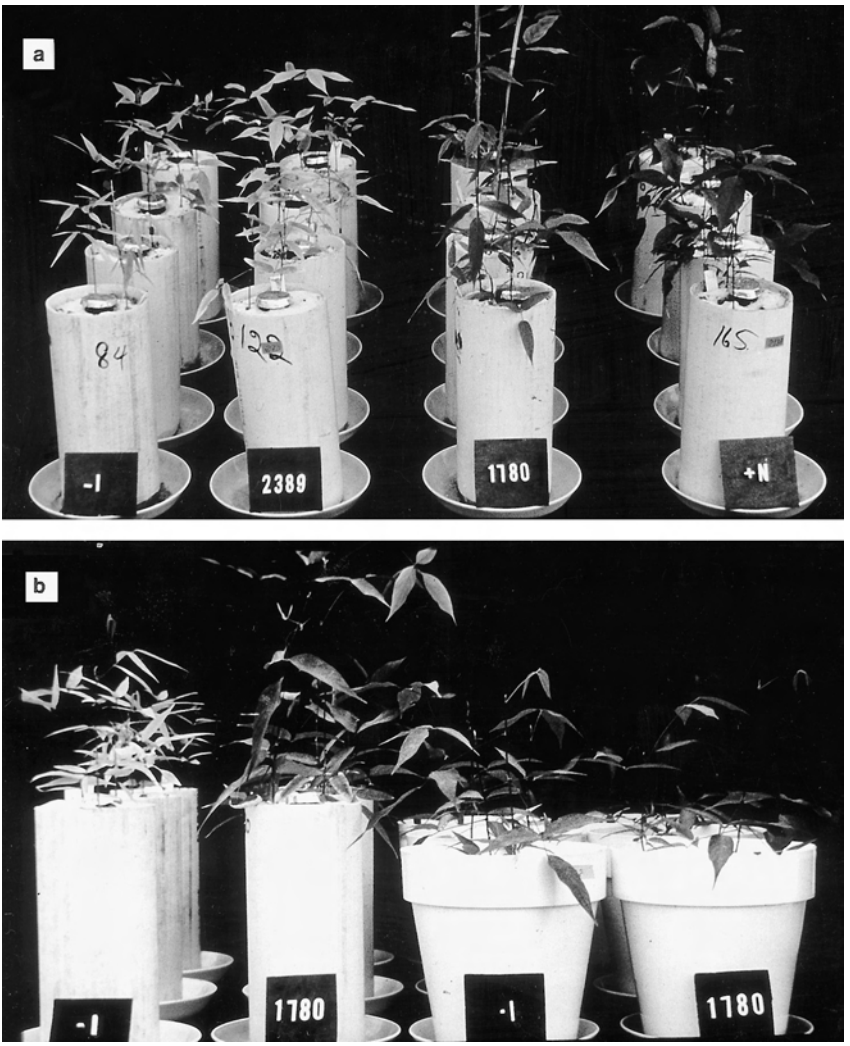

Fig. 14.4. Screening of rhizobia for *Centrosema macrocarpum* in soil cores. (a) Treatments are: uninoculated plants of *C. macrocarpum* (–I); plants inoculated with CIAT strain 2389, which nodulates and fixes N_2 with *C. macrocarpum* but gives no inoculation response; and plants inoculated with CIAT strain 1780, which gives a dramatic increase in growth and N yield of *C. macrocarpum* similar to that with a large dose of added fertilizer N (+N). (b) If the soil is excavated, mixed and put into pots (on right) before sowing and inoculating *C. macrocarpum*, a large amount of N is released by mineralization and no responses to inoculation are seen; treatments as in (a).

compatible rhizobia. It is surprising how little attention has been given to this method for screening strains, as it could yield equally exciting results in the future for other legumes.

Other roles for rhizobia?

It is important to remember that the beneficial role of rhizobia is not necessarily limited to N_2-fixation. For example, it is known that certain strains of rhizobia produce plant growth factors (e.g. Phillips and Torrey, 1970; Evensen and Blevins, 1981) and can thereby (or otherwise) influence plant growth (see also Chapter 6 for discussion of plant hormone production by *Azospirillum*). Inoculation has even increased growth of rice through hormonal effects (Biswas *et al.*, 2000).

There is evidence that certain strains of rhizobia can enhance the partitioning of fixed nitrogen to seeds, and thereby lead to increased yield (Neves *et al.*, 1985), though this effect was not reproduced (Herridge and Peoples, 1990). At least one strain of *Rhizobium* has been identified that produces an anti-rhizobial toxin (see above), and it is possible that other strains could have similar effects on soil micro-organisms that could be deleterious for crop growth. The possibility of factors other than N_2-fixation producing changes on rhizobial inoculation should be considered.

Manipulating the bacterium

The previous section discussed simple screening of different strains of rhizobia for properties that might lead to them forming successful inoculum strains in the field. Another approach is to try to manipulate the rhizobia directly so that they acquire a specific property that may improve their quality as inoculum strains. This can be attempted by application of recombinant DNA techniques that allow engineering of a precise genetic change in the rhizobia.

Construction of new rhizobial strains

There are already several examples in the literature where molecular biology has been used to alter strain characters, with the stated aim of obtaining improved strains for use in the field. For example, carbon supply to bacteroids is believed to be one possible factor limiting the rate of symbiotic N_2-fixation. Additional genes encoding the proteins necessary for transport of dicarboxylic acids were transferred to *B. japonicum* with the aim of alleviating this possible constraint to N_2-fixation (Birkenhead *et al.*, 1988) and the engineered strains were found to have increased nitrogenase activity in the free-living state. Genes encoding the rhizobial-specific trifolitoxin (see above) have been introduced into symbiotically effective strains for lucerne, clover and bean which consistently outcompete strains lacking trifolitoxin, even in the field (Robleto *et al.*, 1998; Scupham *et al.*, 2000). A symbiotic plasmid

from an acid-tolerant *R. leguminosarum* bv. *trifolii* strain was transferred to an acid-sensitive but more effective strain, and the resulting strain possessed both desirable properties (Chen *et al.*, 1991a).

Perhaps the most interesting example is the use of a mixture of conventional genetics and molecular biology in the construction of a pea cultivar/*R. leguminosarum* bv. *viciae* combination where the cultivar can only nodulate with the introduced inoculant strain and not with indigenous rhizobial strains in North America. This is based on the observation described in Chapter 2 that wild pea cultivars from Afghanistan and the Middle East fail to form nodules with rhizobial strains present in western European and in North American soils. This was shown to be due to a plant gene, called *sym-2*, that conferred resistance to nodulation by European rhizobial strains on the Afghanistan pea cultivars. However, the local rhizobia in the centre of diversity of the Afghanistan cultivars possess a gene called *nodX* that overcomes this resistance by altering the structure of the Nod factor (Firmin *et al.*, 1993). The *sym-2* gene has now been bred into a commercial pea cultivar, 'Trapper', and the *nodX* gene has been engineered into a North American strain of *R. leguminosarum* bv. *viciae*, with the result that the new cultivar failed to nodulate except when the engineered rhizobial strain carrying the *nodX* gene was inoculated (Fobert *et al.*, 1991). In this way, it is possible to manipulate a desired host/symbiont combination so that there is absolute specificity of recognition, and the problem of competition by indigenous strains is virtually eliminated. This approach is perhaps more far-sighted than the related approach of trying to breed for exclusion of particular strains, as has been done with soybean and *B. japonicum* serogroup USDA123 (Chapter 8).

As yet, no such attempts at manipulation have produced a strain that has been demonstrated consistently to increase legume yields in the field. Although field trials in the USA demonstrated that genetically engineered *S. meliloti* could give increased yields of lucerne (Bosworth *et al.*, 1994), this was shown to be due to the locus into which the genes were inserted, and not to the introduced genes (Scupham *et al.*, 1996). In fact, most engineered strains have not been tested in the field, due in part to the restrictions on the release of recombinant microorganisms in the environment. However, one might predict that many of these strains, which have been engineered in one trait only, would be no better equipped to face the multitude of stresses in the field environment than their parent strains.

The role of molecular biology

As well as the role of recombinant DNA technology in directly manipulating the bacteria (so-called genetic engineering) there are at least three other ways in which molecular biology can have an impact on N_2-fixation research.

1. Recombinant DNA technology has led to many of the most significant recent advances in our understanding of the mechanism of the rhizobial/legume symbiosis. For example, the discovery of the role of flavonoid compounds in root exudates in stimulating rhizobial *nod* gene activity (Chapter 2) came about through the use of molecular biological techniques. This finding has led to new ideas about how the symbiosis might be improved.

2. Molecular genetics makes it possible to test ideas about the importance of certain attributes with great precision. For example, the role of uptake hydrogenase in enhancing the efficiency of the symbiosis could be tested much more rigorously once it became possible to construct isogenic rhizobial strains that differed only in presence or absence of hydrogenase activity (Chapter 3), or isogenic strains that differ only in trifolitoxin production (see above).
3. Recombinant DNA technology can provide tools that greatly facilitate and/or refine the execution of some very routine types of experiments. For example, as discussed in Chapter 2, DNA sequence data is being used for the identification of bacteria and determination of their evolutionary relationships. Another example is the use of marker genes to develop much simpler screens for rhizobial competitive ability (Wilson *et al.*, 1991, 1995). The GUS marker gene has been shown to be a particularly powerful tool for studying competition between rhizobial strains, as it allows large numbers of root nodules to be screened very rapidly (Wilson *et al.*, 1999). This method is now being applied widely in competition studies (e.g. Streit *et al.*, 1992; Anyango *et al.*, 1998; Khan *et al.*, 1999; Bloem and Law, 2001).

Thus molecular biology can be a powerful tool in aiding our understanding, even if the final product contains no element of genetic engineering. In fact, it is perhaps more likely that, in the short term, molecular biology will make a contribution to the practical application of N_2-fixation in this role, than through engineering of novel bacterial strains or host plants.

Improvement of Legumes for N_2-fixation

There have been few concerted efforts to improve N_2-fixation in legumes for use in agriculture. Perhaps the closest has been to select legumes for use as cover plants, as green manures or as pasture legumes under conditions in the field where N is severely limiting. In virtually all successful cases, selection of legumes for use in tropical pastures or as cover plants has been achieved through screening newly collected new germplasm in the field for well-adapted ecotypes rather than by breeding. The rationale here is similar to that of selection of strains of rhizobia by screening in soil. By screening plants in acid soils, in the environment in which they are likely to be used, plants with a broad adaptation to the multiple stresses of acid soils are more likely to be identified.

The situation is particularly complicated for pasture legumes. Early attempts to select legumes for use in tropical pastures compared biomass production in small plots under clipping, but this led to selection of varieties that performed very poorly and were rapidly lost from the sward when subjected to the additional dangers imposed by the grazing animal. A realization of this problem, which had led to a 'loss of credibility' with farmers regarding the use of legumes in tropical pastures, allowed development of a new strategy for selection of pasture legumes in which the new accessions are subjected to the rigours of grazing at an early stage.

Breeding legumes for increased N_2-fixation

It can be argued that the methods that breeders have employed in crop improvement of grain legumes have tended to select against the ability to fix large amounts of N_2. Most breeding programmes try to reduce environmental variability so that genetic variability is more readily quantified, and this has meant that large amounts of fertilizers (including N) have been applied in the field. One can question the logic of applying rates of fertilizer as high as 200 kg N ha^{-1} to fields in which tropical grain legumes are selected for use in subsistence agriculture, but this was certainly a common practice in the past.

The simplest way to increase the fixation of N_2 is to select promising materials under conditions in which the supply of combined N is limited. Plants thus selected will be dependent on fixation for their N supply, as long as precautions are taken to ensure that the plants are adequately nodulated. The extent to which attempts have been made to increase the amounts of N_2-fixation in different grain legume species is considered in Chapter 8 and the discussion here will be limited to the main strategies that can be used.

In cases where breeding for enhanced N_2-fixation has been attempted, various approaches have been taken to ensure that the plant genotypes are effectively nodulated. These range from selection of host genotypes in soils with large populations of rhizobia to the application of multi-strain inoculants, or in some cases the deliberate selection of highly specific combinations of host genotypes with a single rhizobial strain. An added complication derives from 'host–strain interactions', reports of which flood the literature on N_2-fixation. The common observation is that some host genotypes fix well in symbiosis with particular strains whilst other genotypes fix best with different strains.

The importance of such interactions in selection of legumes for N_2-fixation has been emphasized by Mytton (1984) and Mytton *et al.* (1988), who proposed use of a joint regression analysis, such as that used by breeders to quantify genotype by environment interactions, to help to separate the variability due to the host, the strain and the environment. Mytton *et al.* (1988) further suggested two methods to develop legumes that can be useful in N_2-fixation when faced with a diverse array of rhizobial strains: (i) 'genetic buffering' in which all individuals in a population have a broad spectrum effectiveness (i.e. they can fix well with a range of strains); and (ii) 'population buffering' in which a diverse population is developed to contain genotypes which can fix well with particular rhizobial strains. The applicability of these two approaches will depend to some extent on the breeding system of the legume in question; in a species that outcrosses readily, neither strategy may be possible to regulate closely. If a species is to be selected in soils where there is an abundance of rhizobia with which it can nodulate, then it will be more difficult to select for N_2-fixation with a specific strain (unless a strain that is highly competitive in those soils is used), whilst if the soil is devoid of rhizobia either strategy can be employed. It is important that a wide variety of rhizobial types are included if genotypes with a broad spectrum of effectiveness are to be selected.

Other researchers have advocated a completely different approach and suggested that breeding for increased specificity between strains of rhizobia and plant genotypes will allow greater improvements in N_2-fixation by preventing strains that are ineffective or less effective in N_2-fixation from nodulating the specific host genotype (see Chapter 8 and above).

There are few examples where a rigorous breeding programme has been carried out with the major aim of improving N_2-fixation in a legume in the tropics. The breeding programme for enhanced N_2-fixation in *P. vulgaris* conducted at CIAT has met with limited success, but there most of the field selections have been carried out in soils that are comparatively rich in N (Chapter 8). The case of 'promiscuously' nodulating soybeans has been discussed in some detail (Chapter 8). This breeding programme achieved its objectives of combining the ability to nodulate with strains of rhizobia indigenous to African soils, derived from soybean genotypes of Asian origin, with the high yield potential of varieties bred in North America. However, it did not wholly solve the problem of ensuring good nodulation and N_2-fixation in the field and so inoculation with rhizobia may still be required in many cases.

Despite these limitations, the introduction of new larger-seeded varieties has led to replacement of the older 'Malayan' variety in Benue State, the main soybean-producing area of Nigeria (Sanginga *et al.*, 1999). Presumably the spread of these varieties depended to a large extent on a gradual build-up of compatible rhizobial populations, as inoculants were not freely available. Identification and release of high-yielding promiscuous varieties from the breeding programme at IITA had led to their widespread use by farmers. Uptake of the new promiscuous varieties was initially slow but gained rapid momentum as they became more widely known to farmers and the new varieties were being grown by 75% of male farmers and 62% of women farmers by 1996 (Sanginga *et al.*, 1999). Varieties developed more recently are even more promiscuous and are likely to be widely used by smallholders in future (Sanginga *et al.*, 2001). The success of soybean in Nigeria was undoubtedly linked to training in household utilization of the beans (to overcome the 'off-flavour' if they are not rapidly boiled), which reached more than 47,000 people (Sanginga *et al.*, 1999). The other major factor was development of a secure market in local urban centres, due to the processing of soybeans for a type of 'tofu' that has become widely popular. Soybean has had a major impact on the general welfare of smallholder farmers in Nigeria through the income generated, on household food security, and on the nutritional status of children.

In Zambia and Zimbabwe, specifically nodulating soybean varieties are an important crop on large-scale commercial farms and rhizobial inoculants are used each year (Carr *et al.*, 1998; Mpepereki *et al.*, 2000). The promiscuous soybean varieties 'Magoye' and 'Hernon 147' were strongly promoted after their release in 1981 in Zambia. By 1986 roughly 5000 smallholder farmers were growing promiscuous soybeans (Javaheri and Joshi, 1986), but collapse of a parastatal agency that had supported the programme led to a sudden decline in the crop (Javaheri and Joshi, 1986). By contrast, in Zimbabwe, soybean was promoted as a smallholder crop in the 1980s using specifically nodulating varieties with inoculation. When project support ended,

it failed to become a major crop for smallholders, largely due to problems for smallholders in accessing seed and inoculants (Mpepereki *et al.*, 2000). A recent programme linking smallholder producers to markets has led to rapid expansion of soybean in smallholder agriculture, from 50 farmers in the 1996/7 season to an estimated 10,000 farmers 3 years later. Although the initial aim of the scientists was to promote the promiscuously nodulating 'Magoye', the programme has largely relied on assisting farmers to access seed of specifically nodulating varieties, together with careful extension in the use of inoculants. This was necessary because there is simply no system for seed production of the promiscuous varieties to meet the rapid increase in farmers' demand. Farmers are keen to grow both the specifically nodulating varieties, because of their greater yield potential as a cash crop, and the 'promiscuous' varieties that they regard as more robust, as their production does not depend on the farmers being able to obtain inoculants (see below). Farmers also recognize the greater potential of the promiscuous varieties for fodder and soil fertility improvement (Mpepereki *et al.*, 2000).

In neighbouring Malawi, where there is very limited capacity to manufacture and supply inoculants, promiscuous soybeans are the only varieties likely to bring benefits to farmers. In the 1998/99 season, 'Magoye' grown without supplemental fertilization yielded over 1.5 t ha^{-1} whereas unfertilized maize produced less than 0.5 t ha^{-1}. Smallholder production of soybean rose steadily after 'Magoye' was introduced by a local NGO to peak at more than 90,000 ha in 1995, when there was a large demand for soybean from the World Food Programme in Malawi. Prices for soybean collapsed when this demand ceased the following year and the area under the crop declined. Most regrettably, major development aid to Malawi, which was targeted to assist smallholder farmers throughout the country to diversify from maize production, has distributed seed of specifically nodulating soybean varieties to smallholder farmers *without* inoculants.

Smallholder farmers in many regions of the tropics face enormous problems in gaining timely access to inputs of fertilizers and other agrochemicals, and these are particularly stark in parts of Africa. Given these problems, which are further magnified in the case of products for 'niche' markets such as rhizobial inoculants, promiscuously nodulating legumes are by far the best approach to ensuring effective nodulation and N_2-fixation of grain legumes. There is great potential to combine the highly promiscuous characters of 'Magoye' with the greater yield potential of commercially available varieties – a goal that has recently been taken up in Vietnam (D.F. Herridge, 1999, personal communication).

The ability to nodulate in the presence of large concentrations of available N has been emphasized as a character important for enhancement of N_2-fixation in legumes in developed countries (Chapter 8). Mutants of grain legumes that can nodulate in the presence of high concentrations of nitrate (Carroll *et al.*, 1985) are undoubtedly of interest scientifically – particularly in the study of regulation of nodulation in legumes – but, not surprisingly, do not give enhanced yields or N_2-fixation in the field (Song *et al.*, 1995). It can be argued that N_2-fixation in the presence of nitrate is of little practical relevance for improvement of N_2-fixation in legumes for tropical agriculture. There may be little advantage in fixing N_2 in the presence of nitrate if the

N spared is lost from the cropping system by leaching or denitrification. In very many tropical cropping systems legumes are grown as intercrops and the content of available N in the soil is limited. A legume capable of efficient N_2-fixation when N is limiting but able to respond to N when present in the soil is perhaps a better model, and one to which many grain legumes already fit.

Promising directions for future breeding

The relative importance of different environmental factors in determining the amount of N_2 fixed by a legume varies with the sensitivity of the different species (or genotypes of those species) to particular stresses. Given the unpredictability of climatic conditions in many parts of the tropics, it is essential that promising genotypes are tested for adaptation under the full range of environmental conditions that they are likely to encounter in the field. A useful approach to breeding for adverse environments has been suggested by Lawn and Imrie (1991).

Whilst the ability to fix N_2 may be an important factor in determining the production and usefulness of legumes in tropical agriculture, it is but one of the many plant characters that must be considered in crop improvement programmes. Work on breeding legumes for enhanced N_2-fixation without consideration of all the other important characteristics, such as disease and pest resistance and environmental adaptation, is unlikely to yield results of practical use. This is particularly important in *P. vulgaris*, which has an amazing array of seed types and sizes, for which there are marked local preferences. Given the enormous variability that exists in most legume species, one must remain optimistic about the possibility of improvement of N_2-fixation by plant breeding but, given the complexity of blending all of the characteristics required in any single grain legume variety, this is beyond the individual role of researchers on N_2-fixation. The obvious conclusion is that the most rapid advances in breeding for enhanced N_2-fixation will come from legume breeders persuaded to conduct their selection programmes in N-limited soils (Herridge and Rose, 2000). This approach has been taken to improve the adaptation and yield of *P. vulgaris* on soils of poor fertility for smallholder agriculture in Africa (Wortmann *et al.*, 1995). Emphasis has been placed on obtaining varieties that can yield well without added fertilizers and are efficient in use of phosphorus, but part of the gains that have already been realized are undoubtedly due to enhanced capacity to fix N_2 under the conditions that prevail in smallholder agriculture.

Finding New Niches for Legumes in Tropical Cropping Systems

Analysis of the majority of cropping systems in the tropics reveals that the actual amount of land sown to legume crops is generally small. This point is often overlooked in considerations of improving the inputs from N_2-fixation but, as argued elsewhere (Giller and Cadisch, 1995), the fastest way to boost N_2-fixation inputs is simply to grow more legumes. A corollary to this argument is the enormous impact that significant amounts of N_2-fixation in the major cereal crops would bring, but

this is unlikely to be realized in the foreseeable future. Whilst not everyone may wish to become strict vegetarians, and cereal and tuber crops will certainly continue to be the major staple foods, in most cropping systems there are significant niches into which legumes may fit. The following sections explore some of the ways in which the productivity and area sown to grain legumes has been increased, or where new legumes have been successfully incorporated into cropping systems.

Multi-purpose food legumes

Although the term 'grain legume' implies that these species are grown solely for their seed, the different species have a wide variety of uses (as discussed in Chapter 8). It is possible to grow a legume for its cash value, to harvest its leaves and pods as vegetables, to harvest the grain and to feed it as fodder to livestock – though of course there are direct trade-offs between each of these uses. Some grain legumes are grown for primarily different reasons in contrasting environments, which is a testament to the long history of plant selection that has been made in the past. For example, cowpea is grown primarily as a vegetable and grain in Southeast Asia but primarily for fodder in some parts of West Africa.

As noted in the case of soybean described above, smallholder farmers may rapidly adopt a new legume crop, or varieties of a legume crop with which they are already familiar, if it fits a different niche. Introduction of climbing varieties of *P. vulgaris* in the highland environments of Rwanda and Burundi led to rapid uptake by smallholder farmers, from cultivation on small plots by only 5% of farmers in 1986 to over 40% of bean-producing households, numbering some 500,000 farmers, in 1994 (Sperling and Loevinsohn, 1993; Sperling *et al.*, 1994). Another interesting example is the recent uptake of a multi-purpose variety of cowpea by farmers in Nigeria. A farmer visiting a field day in Kano State, northern Nigeria, to view trials evaluating new cowpea varieties took just 200 g of seed of a grain-and-fodder or multi-purpose cowpea from border rows of the plots (Inaizumi *et al.*, 1999). Only 4 years later this variety was grown by 1500 farmers – representing more than 90% of the farmers in two villages and more than 60% in a third. The popularity of the variety stems from its capacity to grow in the dry season so that it yields when market prices are high – filling a niche identified by research scientists at IITA – and also provides N-rich fodder at a time when it is scarce.

Smallholder farmers in Malawi and Mozambique have rapidly adopted a new variety of pigeonpea from ICRISAT. This genotype has the right seed quality to be sold as 'organically produced' for high prices in Europe (R.B. Jones, 2000, personal communication), a product label that most crops in southern Malawi achieve by default. The case of adoption of soybeans in Nigeria and southern Africa has already been discussed at some length, both here and in Chapter 8. In each of the different cases described above where soybean production has been successfully taken up by farmers, a strong emphasis has been placed on training for local consumption. This has been a key factor in driving the expansion of soybean as a smallholder crop, but continued production by smallholder farmers on a substantial proportion of their

land depends on access to viable markets for sale of surplus produce (discussed in Chapter 15). If a secure market is not available, soybean tends to take its place alongside other minor food legumes and is cultivated on a small area of the farm. Although farmers who are wealthier tend to be the first to experiment with a new crop such as soybean, it has a small investment cost compared with other crops (Rusike *et al*., 1999). This is due in part to the ability of soybean to fix its own N, and means that the poorer farmers in communities are likely to be those who become more dependent on this crop for earning cash in future.

Multi-purpose fodder legumes

Many traditional systems of livestock production in the tropics make good use of legumes for browse and fodder – for example, *Faidherbia albida* and other species of *Acacia* in Africa. There are fewer examples where farmers have invested in cultivating their own fodder. *Calliandra calothyrsus*, which is widely naturalized in Java and used by many farmers to 'cut and carry' for their cows, has been mentioned (Fig. 12.1). Legume fodder trees find a niche elsewhere in the tropics where population densities are high and cattle are prevented from roaming freely, or where milk production or livestock fattening schemes are profitable. Uptake of *Stylosanthes guianensis* fodder banks in West Africa was initially slow but is now gaining rapid momentum (Elbasha *et al*., 1999). In this region, cowpeas are an established component of the traditional system but are restricted by conflicting demands for labour early in the cropping season, as farmers are unprepared to forego yield of cereals (Bartholomew *et al*., 1992). Even here, the example of the new dry season multi-purpose variety demonstrates that new opportunities to integrate legumes into the farming systems can be found.

Constraints on use of legumes for fodder or pasture improvement that are most commonly cited include the lack of labour and the costs of fencing to prevent overgrazing of the legume, or losing the investment in forage production to others in the community (e.g. Thomas and Sumberg, 1995). The exception to poor uptake and utilization of legumes for fodder is tropical Australia, where there is a huge area of improved pastures and leys – 200 t of *Stylosanthes* seed is produced annually which, at a rough calculation, is some 9×10^{10} seeds (Pengelly and Staples, 1997).

Multi-purpose legumes for soil fertility replenishment

Experience from across the tropics shows that green manuring with legumes, whether using herbaceous legumes such as mucuna and *Crotalaria*, or fast-growing legume trees, is rarely adopted by farmers solely for the soil fertility benefits that might accrue. Simple economic analyses of green manuring for lowland rice (Becker *et al*., 1995b; Ali, 1999) or for upland cropping (Whitmore *et al*., 2000) in Asia demonstrate that the relative costs of mineral fertilizer do not justify the investment of time and effort. There is currently a strong research emphasis on green manures for uplands (Chapter 9), as there was 10 years ago for green manures in lowland rice,

and it is worth spending some time discussing the potential of legume-based soil-improving technologies.

A useful 'rule of thumb' is that the yield of the crop after a green manure or improved fallow should be doubled to make it worthwhile. This is very rarely achieved. Economic analyses suggest that only small yield increases, as low as 0.2 t maize ha^{-1}, may be required to make a system profitable in purely economic terms, largely because much less labour is invested in an improved fallow than in maize cultivation (Swinkels *et al.*, 1997). But this depends very much on the relative prices of labour and fertilizer, other opportunities for farmers to earn income elsewhere, and how the farmer perceives the benefits, and the subsequent yield benefits are often not sufficient to justify foregoing a crop, particularly where rainfall is less (Carsky *et al.*, 1999).

Two examples where mucuna has been widely used by farmers, in Central America and Benin, have been discussed at length in Chapter 9, and in both cases the primary reason for farmers using the legume was to control pernicious weeds and therefore reduce the labour required to restore or maintain land in cultivation. History teaches that, apart from traditional uses in maintaining soil fertility before the advent of mineral fertilizers, cover crops and green manures have been used most widely on large farms. The oil palm and rubber plantations of South and Southeast Asia are the prime example where cover crops cover millions of hectares, largely to control soil erosion, and the widespread use of green manures in southern Africa and the southern states of the USA was discussed in Chapter 9. A more recent example is the rapid development of zero-tillage systems on (very) large farms in southern Brazil, which are estimated to cover some 10 million ha, and where legume and non-legume cover crops are used extensively.

This tends to indicate that green manures and improved tree fallows will find more acceptance and interest from farmers who are relatively better endowed with land and other resources. Even in the case of Atlantic Honduras, where many farmers spontaneously adopted mucuna, it was used more on larger farms (Buckles and Triomphe, 1999). Poorer farmers often have less incentive or capacity to use soil-improving, N_2-fixing legume-based approaches due to a variety of constraints, including lack of land and because they often must sell their labour to others. Evaluation of approaches such as green manuring under 'real' farmers' conditions or 'farmer managed' conditions is difficult to achieve, as in practice farmers tend either to give special attention to a field or to completely ignore it because they identify that it does not belong to them.

In eastern Zambia, the recent spread of improved fallows of *Sesbania sesban*, from 200 to more than 3000 farmers in 3 years (Kwesiga *et al.*, 1999) is an exciting example of the interest that farmers can have in new approaches. On an optimistic note, although there was a strong association between improved fallows and wealth among farmers evaluating improved fallows, 22% of the poor and 16% of a 'very poor' group were using them (Phiri *et al.*, 2001). But even here there are dangers of artificially overestimating the potential of interventions, simply by having a large research presence or by buying back seed of green manures – seed of *Tephrosia vogelii*,

a legume with great promise for undersowing with maize, was more valuable than most grain legumes in the markets in Malawi in recent years.

To summarize, in very many cases growing a legume purely to improve the fertility of the soil is 'just not worth the effort'. This is not new – early researchers in Africa and elsewhere produced the same conclusion long ago (Whyte *et al.*, 1953; Vine, 1968). As Brown (1958) stated, green manures 'suffer the handicap of occupying the land unproductively for a whole year'. However, farmer interest can change in response to altered circumstances. The widespread use of mucuna by farmers in Benin to tackle an increasing problem with *Imperata* was only possible because the idea was introduced to them by researchers, even though it was an approach that had been initially developed, but was not widely employed, by the 1930s. Farmers recognize the role of mucuna in soil improvement, but when the *Imperata* problem is under control there is little incentive to sow green manure again.

Mucuna has been shown to be particularly useful in control of *I. cylindrica* and could play a major role in the rehabilitation of the anthropic savannah that covers vast areas of Southeast Asia, but this is unlikely to happen without major external investment (von Uexküll and Mutert, 1994). Even then, if funds for such an investment are available it may be more sensible to grow other legumes that also have a direct benefit, such as groundnut.

The main conclusion from past experience is that for legumes to play a greater role in soil fertility improvement, they must bring additional advantages to farmers, whether as fodder, fuelwood or food. This has stimulated researchers to embark on the development of less toxic varieties of mucuna so that it can be grown for grain, although we must question whether farmers might not prefer other grain legumes. There is little sense in researchers continuing to seek new legumes as green manures unless they will be used, which emphasizes the need for such research to be done in close collaboration with farmers (e.g. Buckles, 1993; Fischler and Wortmann, 1999). All systems of agriculture are dynamic and researchers must be responsive to assist in fitting N_2-fixing legumes into cropping patterns as the opportunties arise – and fade. Enthusiasm for N_2-fixation and dedication to promoting legumes are not enough. All legumes for soil fertility improvement must have multiple uses.

Conclusions

On a global scale, the greatest success of N_2-fixation research is probably the development of the technology for rhizobial inoculation that can consistently result in successful nodulation, and thus increased yields, of soybeans when they are first introduced into new regions. This has allowed the rapid introduction of soybeans into cropping systems in many parts of the tropics. The limitation in the application of this technology to other legumes is the difficulty of identifying rhizobial strains that are both efficient in symbiotic N_2-fixation and able to compete successfully with indigenous soil strains for nodule occupancy. One highly promising method to achieve this is the use of undisturbed soil cores for strain screening. With this

approach, several promising strains have been identified for inoculating pasture legumes in South America, and these are now at the stage of needing widespread on-farm testing prior to their recommendation for extension.

Little emphasis has been put on N_2-fixation in legume breeding programmes in the past, and thus there are good possibilities of increasing N_2-fixation through plant breeding, particularly in developing varieties for the rather hostile environment on many smallholder farms. There are a number of exciting examples where development of new varieties of N_2-fixing grain and fodder legumes has led to widespread adoption by farmers. Legumes are unlikely to be widely adopted purely for their benefits in soil improvement, but need to bring additional benefits in labour-saving due to weed control, or other products such as food and fodder.

Chapter 15

Future Impacts of N_2-fixation in Tropical Agriculture

To return to the starting point, tropical environments could be among the most productive in the world, yielding several food crops each year. Maximal rates of N_2-fixation recorded in the lowland tropics reach an astonishing 5 kg N ha^{-1} day^{-1} with *Sesbania rostrata* (Chapter 9). However, the poor growth and nodulation of grain legumes often observed on smallholder farms means that less than 5 kg N ha^{-1} is fixed each year, and when the small amount of legumes sown is taken into account – often less than 10% of land area – the amount of N_2 fixed is insignificant. Putting political problems that hinder food production and distribution aside, what can be done in the future to increase N_2-fixation, and productivity in general in the tropics?

A foundation of any successful agricultural system is that it must be sustainable – that is, the outputs of nutrients in both agricultural products and unwanted losses must be balanced by inputs. In this way, productivity can be maintained at a constant level, providing food, income and security to the farmer. Such thinking led to the concept of 'low external-input agriculture', the idea being that a system that does not rely on external inputs can be readily sustained. Another view is that this is a romantic dream, exported from countries with plenty. In many systems this equates to 'recycling poverty' (Dudal and Deckers, 1993), as the agricultural productivity generated is insufficient to provide for the needs of the community, or improve their quality of life.

In western countries, agricultural production is 'sustained' by mineral fertilizers, despite the growing realization that environmental problems arise from their overuse. There is now a general consensus that acceptable rates of productivity cannot be achieved in the tropics without the use of at least moderate rates of fertilizers. This is against a backdrop where structural adjustment of economies has resulted in a distinctly unlevel playing field where smallholder farmers may pay substantially more for fertilizers. Carr (1997) estimated that smallholders in Malawi paid more than 20

times the price for N compared with farm-gate prices for farmers in North America. This means that approaches relying purely on external inputs (Quiñones *et al.*, 1997) are also not feasible. A consensus has emerged that systems of 'integrated nutrient management' are the only way forward, and it is in this context that we must consider the inputs from biological N_2-fixation.

The challenge, then, in the tropics is to develop agricultural systems that meet the present and future needs of rapidly growing populations. This is where N_2-fixation comes in. Atmospheric dinitrogen is one of the most abundant natural resources that is freely available in all cropping environments – a truly renewable resource. It should be self-evident, then, that N_2-fixation will play a central role in any sustainable agricultural system. The questions addressed in this book have been:

- What is N_2-fixation and how can its role be assessed (Part I)?
- Of what nature and magnitude is its role, and what niches do N_2-fixing plants fill in tropical cropping systems (Part II)?
- What can be done to enhance inputs from N_2-fixation (Part III)?

The Role of N_2-fixation in Tropical Agriculture

N_2-fixation contributes to agricultural production in three ways: (i) in crops where the N_2 fixed goes directly into the harvested product, be it grain, foliage or fuelwood; (ii) where the fixed N goes into fodder used in animal production; and (iii) by contributing to the maintenance and restoration of soil fertility.

Direct contributions to crop production

Under experimental conditions in the field, grain legumes can fix a large proportion (often more than 60%) of their N. Occasionally it is possible to find well-nodulated plants in farmers' fields and these may sometimes achieve such rates of N_2-fixation (Chapter 8). However, more commonly there are only a few nodules on the root systems of grain legumes growing on tropical smallholdings, generally due to one or more of the environmental stresses described in Chapter 13. As discussed, some of these stresses can readily be overcome – for instance, deficiencies of certain nutrients can be corrected by fertilizer addition – but only if the inputs are available and within the economic reach of the farmer. Others, such as the lack of soil moisture at critical periods of crop growth, are more intractable. Thus the actual contribution of N_2-fixation, even to legume crop production in the tropics, may in many cases be small.

Despite the current excitement concerning endophytic N_2-fixing bacteria in grasses and cereals (Chapter 6), convincing evidence is still lacking for their role in agriculture. The available evidence suggests that the amounts of N_2 fixed by heterotrophic bacteria in the rhizospheres of cereal crops and grasses, or as endophytes, are probably too small to make a substantial contribution to the growth of

crops or to fodder production, except perhaps in the case of sugarcane (Chapter 6). If the capacity to fix N_2 is engineered into cereals in the future, the new varieties must be sufficiently robust to withstand environmental stresses if they are to be of use for smallholder farmers – a constraint that has not been considered fully in research on N_2-fixation in cereals to date.

N_2-fixation in animal production

Legumes can provide an important part of the diet of livestock, particularly by improving fodder in terms of protein content (Chapter 10). Provision of useful sources of animal feed is not restricted to legumes in sown pastures. Many legume trees have been used traditionally for fodder in 'cut-and-carry' systems or are browsed by animals on rangelands, and crop residues of grain legumes are often fed to livestock. The few estimates that are available for N_2-fixation by legumes in grazed pastures, or by trees, suggest that a high proportion of the plant's N is derived from N_2-fixation. However, deliberate use of legumes for increased animal production has been limited to their introduction into only a small proportion of the improved pastures and other potential niches in farming systems of the tropics.

N_2-fixation in the maintenance of soil fertility

The amounts of N_2 fixed by free-living bacteria both in the soil and specifically in the rhizospheres of plants, while contributing little directly to crop production, are useful in maintaining the long-term fertility of soil. Greater amounts of N are fixed by photoautotrophic cyanobacteria than heterotrophic bacteria, particularly in water-logged paddy fields. The aquatic fern *Azolla* has traditionally been used for maintaining soil fertility as a green manure (Chapter 7). Green manures can have substantial beneficial effects in contributing fixed N to the soil and subsequent crops. Apart from some local examples in Central America, Benin and Asia, green manures are not widely used by smallholder farmers, and their use has decreased globally since mineral fertilizers became widely available.

Legumes contribute to increased productivity of other crops when incorporated into cropping systems as intercrops or in crop rotations. The legume contributes a direct agricultural product, and contributes to maintenance and restoration of soil fertility by fixing a large proportion of its own N. The resulting increase in soil N available to, say, a cereal intercrop is more due to the sparing effect than to any direct transfer of N from the legume (Chapters 5 and 8), although N may be contributed to the soil as plant parts (fallen leaves, nodules and roots) decay. In crop rotations, grain legumes may give a residual benefit of fixed N to crops grown subsequently, although this may again be largely due to a sparing effect. In some cases more soil N is removed in the grain legume crop than is returned as fixed N in the crop residues. When grain legume residues are returned to the soil the residual benefits of growing grain legumes may be striking, often doubling yields of cereal crops on poor soils in farmers' fields

and sometimes exceeding the benefit that can be ascribed to N_2-fixation alone. Forage legumes in ley/arable rotations have great potential to contribute to soil fertility for crop production (Chapter 10), but such systems are rare in the tropics outside Australia. The N_2 fixed by forage legumes is also recycled to crops in manure from livestock, albeit somewhat inefficiently.

One of the greatest successes for the use of N_2-fixation in agriculture is in the rubber and oil palm plantations of South and Southeast Asia, where the use of legume cover crops has become a widespread practice (Chapter 11). The legumes contribute significant amounts of N to the system and reduce the requirements for fertilizers considerably, such that the use of cover crops gives long-term economic benefits.

There are several examples of traditional use of legume trees for improvement of soil fertility in agricultural systems (Chapter 12), particularly in drier regions (e.g. *Faidherbia albida*, *Prosopis cineraria*). The upsurge of interest in the use of N_2-fixing trees has occurred in response to the potential benefits that have been indicated by agricultural research, rather than to their actual use by farmers, though there are promising examples where agroforestry may gain acceptance and these are discussed below.

Impact of Research into N_2-fixation

There has been an enormous research effort on the subject of N_2-fixation and yet this has done little to improve production for the tropical farmer. That is not to say that the research is of no importance – much of the fascinating story of the process of recognition and nodulation of legume roots by rhizobia and knowledge of the diversity, and sometimes surprising similarities, of N_2-fixing bacteria has come from such research. Nevertheless, the practical application of this research in agriculture is more distant than we would wish. A view of likely impact of future research on N_2-fixation is described in Fig. 15.1, the main point being that the amount of N_2-fixation in tropical agriculture could be increased enormously if current understanding was put to more effective use. Beyond this the most rapid gains are likely to come from adapting legumes to different niches in cropping systems, and other approaches are likely to take much longer to yield benefits.

Unfortunately, much of the agronomic research that has been conducted on field stations may bear little relation to the situation in farmers' fields. For example, most of the measurements of N_2-fixation quoted in diverse N_2-fixing organisms or symbioses have been made on research stations where adequate water and nutrients other than N were supplied, and the amounts of N_2 fixed in farmers' fields are likely to be much smaller. Even when experiments are conducted on farmers' fields to explore the potential of N_2-fixation under more realistic conditions, amounts of N_2-fixation are likely to overestimate the benefits likely to be realized by smallholder farmers. This research has, of course, served to increase understanding of the *potential* benefits from N_2-fixation enormously.

The reasons for the lack of research impact are complex. The professional expectations, reward structures and interests of scientists often strongly conflict with

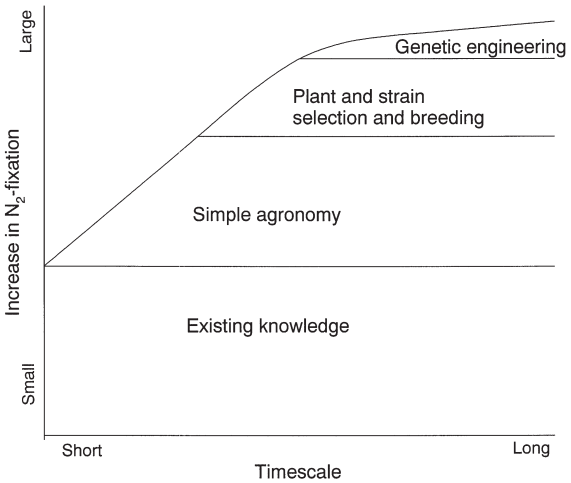

Fig. 15.1. Likely impact from investment in effort on various research approaches in relation to the time required. Note that massive impact could be made by applying existing knowledge. (After Giller and Cadisch, 1995.)

the direct goal of agricultural improvement (Giller and Cadisch, 1995; Hall and Clark, 1995). Multi-disciplinary research is even more problematic, as it seldom attracts funding and the results are difficult to publish. This often leaves a large gap between knowledge held within institutes of learning and the extension agencies, NGOs and others who are more directly involved with development programmes at the community or farm scale. There are now, however, examples where results of research on N_2-fixation have been implemented, and some of these have been very successful (Chapter 14).

Holistic or ecological approaches?

Perhaps one of the main lessons that has been learned from the failures and successes in the application of research into N_2-fixation to date is that N_2-fixation, or components of N_2-fixation, cannot be considered in isolation. For example, rhizobial strains screened purely for single characters such as acid tolerance in laboratory tests have failed to perform well in the field (Chapter 14). This section discusses the role of N_2-fixation in possible future directions for tropical agriculture. However, it is acknowledged that N_2-fixation may have a greater or lesser role to play in different systems, and that N_2-fixation must be considered as one of a suite of options within the system.

Ameliorating environmental constraints

The overwhelming problem for agricultural production in the tropics is the limitation of yields imposed by environmental constraints. If yields in farmers' fields could be raised to those commonly achieved on experimental stations, production would be more than doubled.

Environmental constraints, and problems and prospects for alleviating them, were discussed extensively in Chapter 13. In some cases environmental constraints

continue to worsen. For example, in many areas served by large irrigation schemes, water tables have gradually risen and salinity problems are resulting where irrigation has not been well managed. In addition, problems of soil erosion and deforestation are widely acknowledged to be of mounting severity. If environmental conditions cannot readily be altered, then it is necessary to maximize exploitation of the ecological adaptation of different components of an agricultural system. In the case of N_2-fixation, this means capitalizing on the enormous variability of grain, pasture or tree legumes and their rhizobia in tolerance of many climatic and edaphic stresses. In Chapter 14 promising methods were discussed for the improvement of N_2-fixation by direct selection of rhizobia in soil and by strategies of plant breeding to ensure that genotypes are constantly selected for their adaptation to difficult environments as well as for increased N_2-fixation ability.

The constraint that appears to override all others in most of tropical agriculture is the availability of phosphorus. There is clear potential to exploit variability in plants to access phosphorus from less available pools in the soil, but this must not be viewed as an 'alternative to fertilization', as has been suggested (Radin and Lynch, 1994). We cannot escape the fact that all nutrients exported in produce, apart from N, will have to be replaced if productivity is to be sustained in the long term.

Development, testing and extension of promising technologies

Lessons learned?

Perhaps one of the most ambitious schemes for increasing production of legumes in the tropics was the groundnut scheme in eastern Africa (Wood, 1950). The scheme failed in its objective of turning large tracts of uncultivated land in East Africa over to mechanized production of edible oil. While this scheme was not carried out with the small farmer in mind – its aim was to use colonial land to produce a cash crop to bolster the economy of post-war Britain – an important lesson of tropical environments was learnt, which is still very relevant today. Another example was the 'loss of credibility' that occurred with the initial introduction of tropical pasture legumes in the savannahs of Latin America. This can be attributed partly to the premature introduction of species that had proved successful in Australia, where aluminium toxicity, the main constraint for plant growth in Latin America, is not a major problem. A reason for the failure of many of the early pasture legumes was that they had been selected for biomass production under cutting regimes that bore little relation to the pressures encountered by the plants under grazing. Rhizobial inoculants have suffered a similar fate where they have been supplied for use by farmers for crops that do not respond to inoculation with sufficient regularity to justify using the technology. More recently alley cropping has been consigned by many to the list of romantic visions of agricultural scientists which have no future in the real world, though there may be certain conditions where it may find a role.

Despite the enthusiasm and yearning to make impacts, a 'promising' technology should never be recommended for wide-scale adoption by farmers until it has been rigorously evaluated in the field under realistic conditions. Thorough evaluation

must involve farmers, who can often enormously increase the speed with which progress towards an appropriate technology is made through their own innovation, and must be done for long enough to see how variability of climates from year to year can affect the success.

The need for multi-disciplinary teams

Several workers have emphasized the need for agronomists and plant breeders to work in close conjunction with microbiologists in the development of better legume/rhizobial combinations or other N_2-fixing systems (e.g. Sylvester-Bradley *et al.*, 1988a; Vincent, 1988; James, 2000). This recognizes the need to get all biological components right at the same time for crop production. Molecular biology would also be able to make a much more realistic contribution if there was closer collaboration with scientists in other disciplines, particularly in the identification of problems that are worthy of attention.

No doubt such an approach does allow substantially more rapid advances in understanding, but the view of the term 'multi-disciplinary' must be widened substantially if the results of research are to make an impact in agriculture. It can also be argued that individual understanding must be broadened if contributions from others, including those of farmers, are to be embraced fully.

The farmer's role

The participatory or 'Farmer First' approach to agricultural research was proposed for the development of appropriate, low-input technologies more than 10 years ago (e.g. Chambers *et al.*, 1989). According to this view, an informed and perceptive understanding of individual cropping systems, and of the constraints to production, must first be developed through close collaboration with farmers before new interventions are sought. Development research is currently dominated by 'participatory' approaches, but much 'participation' is simply an extractive process, used to justify research and to analyse farmers' needs, but leaving farmers confused as little is offered in return by the scientist, and the farmers' expectations are not met. Many of the current generation of scientists working in agricultural research in the tropics come from families of smallholder farmers and have much better perceptions of the cropping systems than they are credited with. As the previous chapters testify, science has a depth of understanding of the potential of a diverse array of N_2-fixing systems to contribute to improving agricultural productivity. It seems that the pendulum of fashion has swung so far that the wealth of accumulated knowledge is often ignored.

This suggestion is not an argument for a return to purely 'top-down' approaches, but science clearly has an important role to play. Farmer insights may be gained at the expense of firm data (Buckles and Perales, 1993), but promising research avenues will undoubtedly be identified more rapidly together with farmers. Farmer experimentation will also provide a useful adjunct to more detailed experiments to understand the mechanisms and processes underlying potential improvements. Thus 'top-down' and 'bottom-up' approaches should not be considered as substitutes for each other, but should be combined (in what Meine van Noordwijk has termed the 'ostrich model') in a cyclical, reiterative process.

New models for agricultural research and development?

Even when improved plant cultivars, bacterial strains, or cropping systems have been identified, there are often major barriers restricting their adoption by farmers. A new crop may be readily adopted if it can be integrated into an existing cropping cycle. Examples are the rapid spread of climbing bean varieties among farmers in the highland environments of Rwanda and Burundi and the recent uptake by farmers of a dry-season grain-and-fodder variety of cowpea in Nigeria (Chapter 14).

By contrast, adoption of other technologies may be more problematic for farmers particularly those targeted at improving soil fertility which require substantial investment of time and labour, and yet the improvements in yield might be realized only after several years. The case of alley cropping is a clear example where agronomic research ran ahead without a full understanding of the constraints facing the farmers for whom it was targeted (Scherr and Muller, 1990; Dvorák, 1996). It has been reported that improved fallows of fast-growing trees have 'come of age in the tropics' (Sanchez, 1999). Tree fallows can have major effects on soil fertility and crop yields, and there is strong farmer interest in using legume fallows, but we are only starting to understand fully under what conditions they are appropriate.

A cash crop such as soybean may provide an immediate source of income, but production will only occur on a small scale if there is no market for the produce or if prices are poor. This was clearly the case with the rapid initial expansion and subsequent decline in smallholder production of soybeans in Zambia and Malawi (Chapter 14). The key to the success of all of the approaches discussed above is crystallized by Dorward *et al.* (1998) (Fig. 15.2). If technological development outpaces development of all the other 'institutions' required for a technology to be successful then it will be doomed to failure (point B in Fig. 15.2). To take the example of soybeans, if rhizobial inoculants cannot be produced and distributed in

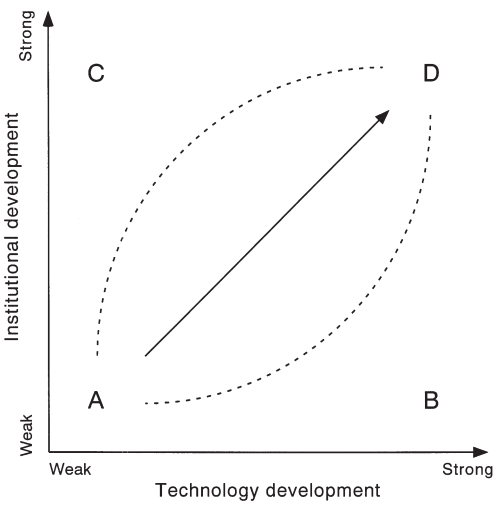

Fig. 15.2. The linkage between development of technology and the development of institutions, used here in a broad sense to include all aspects of extension, input supply and marketing. See text for further explanation. (Adapted from Dorward *et al.*, 1998.)

sufficient quality and quantity, and if no mechanisms are available for marketing the produce, then farmers are unlikely to grow it in any quantity. Conversely, as is the current situation with soybean in Zambia and Malawi, if there is a strong market demand for a legume crop but well-adapted varieties for the farmers' conditions are not available, it cannot be produced (point C in Fig. 15.2). Only when all of the necessary components are in place to guarantee both good productivity and strong markets for the produce will a highly productive technology be achieved on a wide scale (point D in Fig. 15.2). The rapid uptake of soybeans by smallholder farmers in Nigeria and Zimbabwe exemplifies this case.

Even having developed a technology that works, and for which the market linkages exist, it is not possible simply to sit back and be self-congratulatory with an 'I've played my part, now it's up to others to implement my findings'. There is a major role for researchers to become more involved in education of farmers and those involved in agricultural extension – NGOs or formal government agencies – whether through 'farmer field schools', participatory learning approaches or the popular media. It is depressing to realize the very long time-lag between publication of information in the scientific literature and its inclusion in course materials, even within universities. Uptake of technologies may require key policy decisions to be taken by governments which must involve different avenues of communication.

A final word of caution: we should not be too ready to write off a new technology. It can take a considerable time before there is widespread testing and diffusion of new innovations among farmers, as seen with *Stylosanthes* fodder banks in West Africa (Elbasha *et al*., 1999). And we must remain alert for new opportunities for older 'off-the-shelf' approaches as social and economic circumstances change.

Appropriate technologies for smallholder agriculture

The adoption of soybeans by smallholders in Africa provides a clear lesson in targeting the results of our research for different farmers groups. Farmers with ready access to inputs such as basal fertilizers and inoculants, representing the large-scale commercial farms in Zambia and Zimbabwe, grow specifically nodulating soybean varieties and regularly inoculate with rhizobia. More progressive and well-endowed smallholder farmers in Zimbabwe have been able to mimic this approach, and there is evidence that as the crop becomes more established it is more widely adopted by poorer farmers and may help to lift them out of the 'recycling poverty' trap. For those farmers in more remote areas of these countries, or farmers in neighbouring Malawi, who grow soybean solely for local consumption, the promiscuous varieties are a more appropriate option.

Though we may wish it to be otherwise, the poorer households in most communities are also those who can least afford to invest in new technologies. They often lack even the basic capital to invest in external inputs and their labour is often tied up working on someone else's farm. Where green manure legumes have been successfully adopted by farmers, they have been used in such a way that the overall requirements for labour have been decreased by reducing the need for weeding (Bunch, 1990; Versteeg *et al*., 1998). The history of use of green manure legumes indicates that they have gained most widespread use on large farms in intensive

agriculture, both in the case of mucuna in arable cropping (Chapter 9) and with cover crops in plantations (Chapter 11). Even among smallholders in Honduras, mucuna is used more on larger farms (Buckles and Triomphe, 1999).

Although it might be presumed that the poorest farmers are those most likely to benefit from approaches to enhance the 'free inputs' from N_2-fixation, this is often not the case. Although responses throughout this book have been discussed in terms of yields of grain or N per hectare, most smallholder farmers are more interested in maximizing the production they can achieve from their whole farm as a unit. This can often mean trade-offs between investing labour and other inputs in one or other crop. Grain legumes are often women's crops, which are grown largely for subsistence rather than to earn income, and are afforded little attention when it comes to allocation of scarce inputs (Mapfumo *et al.*, 2001). This highlights the need for grain legume varieties that will yield well in poor-fertility soils, a goal targeted by the CIAT-coordinated breeding programme on *Phaseolus vulgaris*, a staple food for the poorest in many countries, which has recently been scaled down in Africa due to lack of donor funding. Allocation of inputs must also be considered at a whole-farm scale where legumes may only benefit from the residual fertilizer after other crops in rotations.

Conclusions

The biological fixation of atmospheric nitrogen, particularly through legume/rhizobial symbiosis, has a central role to play in a productive and sustainable agriculture in the tropics. Whilst research has indicated many promising avenues for introduction of N_2-fixing plants into cropping systems and for enhancement of the contributions from N_2-fixation, to date few of these technologies have been adopted by farmers. In the earlier edition of this book and elsewhere (Giller and Cadisch, 1995), we argued for an ecological approach to agriculture, needing to consider all of the system components within the context of their environment. This alone is not enough as our research must be targeted to the needs of farmers, not to the scientists' need for new invention, if we are to make real advances in the implementation of N_2-fixation in the tropics.

An important and optimistic note is that most of the technologies that are likely to lead most rapidly to improvements of N_2-fixation in tropical cropping systems (described in Chapter 14) are well within the reach of research programmes in developing countries. The most rapid benefits for agricultural production are likely to be seen through alleviation of environmental constraints on N_2-fixation by the judicious use of small amounts of lime and fertilizers (especially phosphorus and potassium), and through exploitation of the enormous genetic variability that is available for bacterial strain and host genotype selection.

One of the most important lessons that has been learnt at all scales is that there are no global solutions. Plant and bacterial genotypes, and agricultural systems, that are adapted to local variations in the physical and socio-economic environment are required if they are to be successful.

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Appendix: List of Common Names

For further details of common names and authorities for scientific names used in the text see http://www.ildis.org/

Adzuki bean *Vigna angularis*
alang-alang *Imperata cylindrica*
alfalfa *Medicago sativa*
arhar dhal *Cajanus cajan*
asparagus pea *Psophocarpus tetragonolobus*
babul *Acacia nilotica*
Bambara groundnut *Vigna subterranea*
bean *Phaseolus vulgaris*
Bengal gram *Cicer arietinum*
black gram *Vigna mungo*
black wattle *Acacia mearnsii*
blue vetchling *Lathyrus sativus*
Brazilian stylo *Stylosanthes guianensis*
broad bean *Vicia faba*
bush bean *Phaseolus vulgaris*
butter bean *Phaseolus lunatus*
cacao *Theobroma cacao*
calopo *Calopogonium mucunoides*
carob *Ceratonia siliqua*
catjang *Vigna unguiculata* ssp. *cylindrica*
chickling pea *Lathyrus sativus*
chickpea *Cicer arietinum*
clusterbean *Cyamopsis tetragonoloba*
cocoa *Theobroma cacao*
coconut *Cocos nucifera*
common bean *Phaseolus vulgaris*
common indigo *Indigofera tinctoria*
cowpea *Vigna unguiculata*
crimson clover *Trifolium incarnatum*
daincha *Sesbania cannabina*
elephant grass *Pennisetum purpureum*
European yellow lupin *Lupinus luteus*
faba bean *Vicia faba*
fenugreek *Trigonella foenum-graecum*
field bean *Phaseolus vulgaris* or *Vicia faba*
field pea *Pisum sativum*
fishbean or fish-poison bean *Tephrosia vogelii*
garbanzo *Cicer arietinum*
garden pea *Pisum sativum*
grasspea *Lathyrus sativus*
green gram *Vigna radiata*
greenleaf *Desmodium intortum*
groundbean *Vigna subterranea*
groundnut *Arachis hypogaea*
guar *Cyamopsis tetragonolobus*

gum arabic *Acacia senegal*
hairy indigo *Indigofera hirsuta*
haricot bean *Phaseolus vulgaris*
Hausa groundnut *Macrotyloma geocarpum*
horse bean *Vicia faba* or *Canavalia ensiformis*
horse gram *Macrotyloma uniflorum*
hyacinth bean *Lablab purpureus*
Indian hemp *Crotalaria juncea*
jackbean *Canavalia ensiformis*
Japan pea *Glycine max*
Kallar grass *Diplachne fusca*
Kersting's groundnut *Macrotyloma geocarpum*
khesari dhal *Lathyrus sativus*
kudzu *Pueraria phaseoloides*
lentil *Lens culinaris*
Lima bean *Phaseolus lunatus*
lucerne *Medicago sativa*
mung bean *Vigna radiata*
navy bean *Phaseolus vulgaris*
oil palm *Elaeis guineensis*
oriental bean *Vigna umbellata*
pea *Pisum sativum*
peanut *Arachis hypogaea*
pigeonpea *Cajanus cajan*
puero *Pueraria phaseoloides*
quinine *Cinchona* spp.
red clover *Trifolium pratense*
red gram *Cajanus cajan*
red kidney bean *Phaseolus vulgaris*
rubber *Hevea brasiliensis*
runner bean *Phaseolus coccineus*
rice bean *Vigna umbellata*
scarlet clover *Trifolium incarnatum*
scarlet runner bean *Phaseolus coccineus*
shittim wood *Acacia seyal*
showy crotalaria *Crotalaria spectabilis*
snap bean *Phaseolus vulgaris*
soybean *Glycine max*
sword bean *Canavalia gladiata*
star grass *Imperata cylindrica*
stylo *Stylosanthes* spp.
subclover *Trifolium subterraneum*
subterranean clover *T. subterraneum*
sunnhemp *Crotalaria juncea*
tamarind *Tamarindus indica*
tepary bean *Phaseolus acutifolius*
tarwi *Lupinus mutabilis*
tick bean *Vicia faba*
Townsville stylo *Stylosanthes humilis*
tropical kudzu *Pueraria phaseoloides*
tur dhal *Cajanus cajan*
urd dhal *Vigna mungo*
velvet bean *Mucuna* spp.
white clover *Trifolium repens*
white melilot *Melilotus alba*
winged bean *Psophocarpus tetragonolobus*
yam bean *Pachyrhizus* spp.
yardlong bean *Vigna unguiculata* ssp. *sesquipedalis*

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