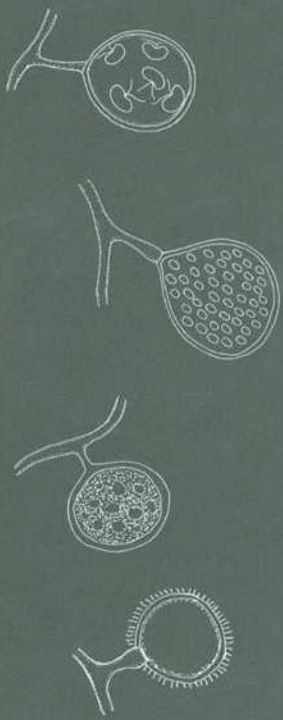
Plant Pathogen Detection And Disease Diagnosis



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Marcel Dekker, Inc. New York · Basel · Hong Kong

ISBN: 0-8247-0040-6

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016
http://www.dekker.com

Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

Preface

There are many kinds of plant pathogens ranging from ultramicroscopic entities to well-defined multicellular organisms, with wide variations in pathogenic potential. They are known to be the principal causes of destructive diseases of many economically important crops cultivated all over the world. Many of them are widely distributed and survive in varied habitats. It is well recognized that development of effective crop disease management depends on the rapid detection and precise identification of the pathogen(s) causing the disease in question. In this context, knowledge of the different methods available for the detection and identification of pathogens is a basic requirement for the successful management of disease(s) affecting the various crops in any location. This volume provides this vital information on currently applied methods of detection and diagnosis.

During the past decade, many sensitive methods of detecting microbial plant pathogens have been developed as a result of intensive research efforts undertaken in different laboratories. In this book, both conventional and modern molecular methods of detecting plant pathogens have been described in an easily understandable manner to enable researchers both in the laboratory and the field, who may not be very familiar with molecular techniques, to understand and follow these methods in their investigations. Many examples for the detection, identification, differentiation, and quantification of different kinds of microbial pathogens are included, with protocols in appendices of the appropriate chapters. The usefulness and limitations of the different methods are indicated. Researchers and personnel of disease diagnostic centers, plant protection, plant quarantine, and seed certification services will find a wide choice of techniques to suit the requirements and facilities available. It is hoped that this book will serve as a valuable reference in this aspect of plant pathology.

I am very happy to express my sincere thanks to all my colleagues and students who helped me in preparing the manuscript and illustrations for this

book. Dr. K. Umamaheswaran and Dr. T. Ganapathy need special mention. Photographs and figures provided by scientists are individually acknowledged in the text. I wish to thank the editors and publishers for permission to reproduce figures and photographs included in this volume.

I thank Mr. M. Anifa and Mrs. K. Mangayarkarasi for typing the manuscript.

P. Narayanasamy

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1
Introduction

The incidence of plant diseases even in the prehistoric period can be inferred from fossils about 250,000 years old, and it has been estimated that about 80,000 diseases may affect various crops, resulting in losses as high as U.S. $60 billion throughout the world annually (Klausner, 1987; Agrios, 1969; Chu et al., 1989). Crop diseases are caused by distinct groups of organisms, predominantly fungi, bacteria, mollicutes, viruses, and viroids. Nematodes and protozoa are able to cause disease in some crops. Effective management of crop diseases depends essentially on rapid detection and accurate identification of the pathogens causing them.

Pathogen detection and disease diagnosis may be required for several purposes: a) to determine the presence and quantity of the pathogen(s) in a crop in order to take plant protection measures; b) to assess the effectiveness of application of cultural, physical, chemical, or biological methods of containing the pathogens; c) to certify seeds and planting materials for plant quarantine and certification programs; d) to determine the extent of disease incidence and consequent yield loss; e) to assess pathogen infection in plant materials in breeding programs; f) to detect and identify new pathogens rapidly to prevent further spread; g) to study taxonomic and evolutionary relationships of plant pathogens; h) to resolve the components of complex diseases incited by two or more pathogens; and i) to study pathogenesis and gene functions.

In this book discussion is confined to microbial plant pathogens; the information useful for the diagnosis of crop diseases incited by microbial pathogens is presented in the following chapters.

1.1 NATURE AND CAUSES OF PLANT DISEASES

All plant species are affected by one or more diseases. Cultivated plants are infected by thousands of diseases all over the world. Each kind of crop may

be affected by 100 or more diseases, causing different magnitudes of losses. Crops may suffer as a result of diseases induced by pathogenic (biotic) or physiogenic (abiotic) causes. Identification of the cause of the disease is the basic and primary requirement of disease diagnosis. Many plant pathogens induce characteristic symptoms in susceptible plant species, whereas some pathogens, especially viruses, may produce general systemic symptoms similar to those caused by environmental factors such as nutritional disorders.

Pathogenic causes of plant diseases may be microbes, such as fungi, bacteria, viruses, viroids, and mycoplasma-like organisms, and others, such as nematodes and parasitic phanerogams. The physiogenic (nonpathogenic) causes may be nutrient deficiencies, toxicity due to excess of minerals, lack or excess of soil moisture, temperature extremes, light, oxygen, air pollution, variations in soil pH, etc. Pathogenic diseases can be transmitted from infected plants to healthy plants by using appropriate inoculation methods. On the other hand, physiogenic diseases are nontransmissible, and the affected plant may recover from the disease if the adverse condition is removed. The pathogenic diseases are far more numerous and varied than the physiogenic diseases, and hence the diagnosis of such diseases becomes more difficult. Such a complex situation is revealed by the tomato, which is reported to be infected by 80 species of fungi, 11 species of bacteria, 16 viruses, and several nematodes, whereas the apple and potato are infected by about 200 pathogenic diseases (Agrios, 1969).

The nature of the pathogen can be established by isolating it from infected plants and inducing the disease by inoculating healthy plants with the organism isolated. Different steps in Koch's postulates have to be followed; then the organism isolated can be considered the pathogen causing the disease. Frequently such a straightforward approach has not been found to be possible, and the use of reliable methods for correct identification of the pathogen has become necessary in the case of microbial plant pathogens. Apart from classification of the pathogens based on taxonomic characteristics, development of serological techniques and nucleic acid hybridization methods have been employed for rapid detection, identification, and assay of microbial pathogens in the recent years.

SUMMARY

The nature and causes of different diseases affecting various crops are described briefly to produce a basic understanding of the pathogens inducing such diseases. The need for the detection of pathogens in plants and other habitats is emphasized. Application of diagnostic methods for studying different aspects of host plant–pathogen interaction and disease management is indicated.

2
Characteristics of Pathogenic Microbes

Plant pathogenic microbes such as fungi, bacteria, and viruses belong to the same groups as the pathogens infecting human beings and animals (Fig. 2.1). None of the plant pathogens can infect humans or animals. However, the insect-transmitted propagative plant viruses can cause diseases both in plants and in their vectors. The plant pathogens are characterized by their ability to grow and multiply in the affected plants, resulting in characteristic symptoms, and to spread from diseased plants to healthy ones, causing new infections.

The plant pathogens become intimately associated with the host plant, drawing nutrients and water from the host, their parasitic nature leads to reduced efficiency in normal growth and reproduction of the host. Parasitism and pathogenicity are intimately associated, as the ability of the parasite to invade and become established in the host plant results in different types of disease symptoms, depending on the host–microbe combination. Some pathogens, including downy mildews, powdery mildews, rusts, and the entire group of viruses, require the presence of living hosts; they are known as obligate parasites. Others can live on living or dead hosts or host tissues; they are called nonobligate parasites. Nonobligate parasites include facultative saprophytes, which live most of the time or most of their life cycles as parasites but are capable of existing as saprophytes if required, and facultative parasites, which live most of the time on dead organic matter but can become parasitic under certain conditions. There frequently appears to be no correlation between the degree of parasitism of a pathogen and the severity of disease, since some weak pathogens may cause more damage to plants than more virulent pathogens. Pathogens may produce different kinds of enzymes, toxins, and other metabolites which may be responsible for structural and physiological changes which lead to specific symptoms of diagnostic value.

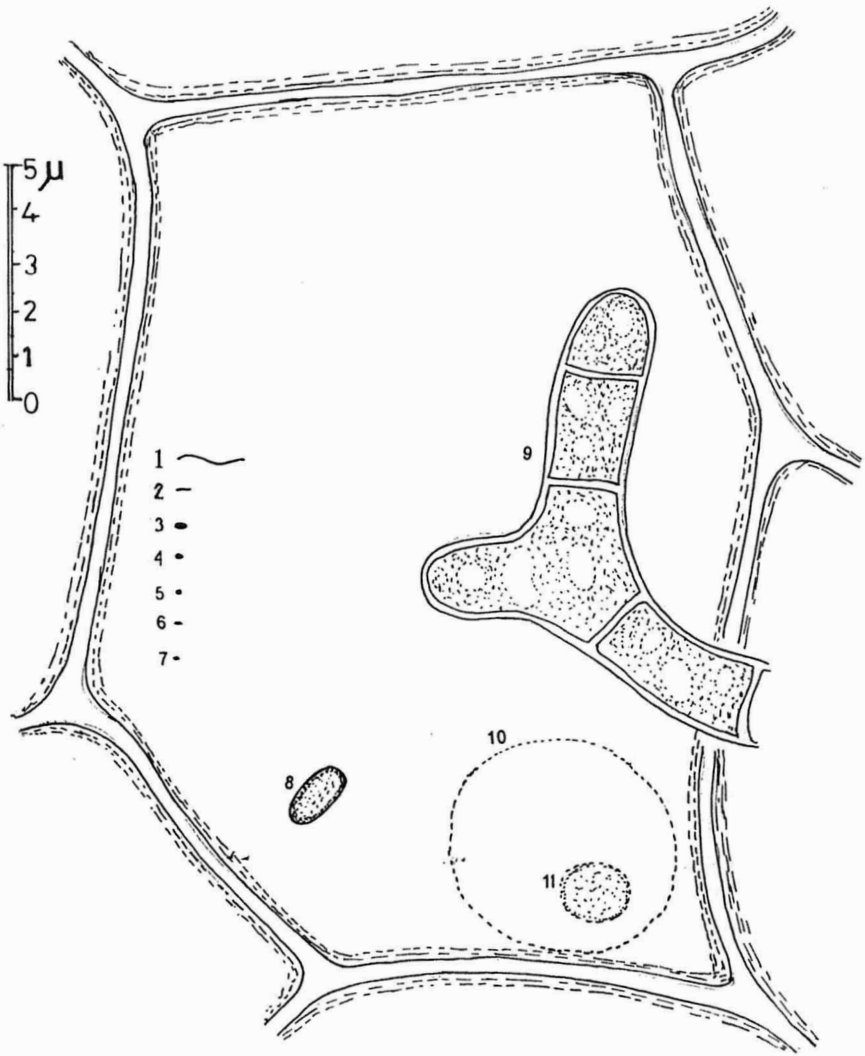


Figure 2.1 Microbial plant pathogens in a plant cell to indicate their relative sizes and shapes: 1, Beet yellows virus; 2, tobacco mosaic virus; 3, wheat striate mosaic virus; 4, wound tumor virus; 5, apple mosaic virus; 6, tobacco necrosis virus; 7, hemoglobin molecule; 8, bacterial cell; 9, fungus; 10, nucleus; 11, nucleolus. Academic Press Inc., USA. (Adapted from Agrios, 1969.)

The characteristics of plant pathogenic microbes can be studied by using different traditional methods involving light microscopy in the case of fungi and bacteria, whereas electron microscopy is required for studying the characteristics of viruses and mycoplasma-like organisms (phytoplasmas) (Chapter 7). For bacterial pathogen identification, in addition to determining morphological characteristics by light microscopy, it is essential to carry out many biochemical and physiological tests (Chapter 6).

2.1 LIGHT MICROSCOPY

Light microscopes can be used to examine the different vegetative/sexual reproductive structures formed by the fungi either in infected plant tissues or in cultures. Many fungal pathogens may be identified up to generic level and in some cases even up to species level by studying characters by means of light microscopes, leading to rapid diagnosis of the diseases caused by them.

2.1.1 Rapid Examination of Pathogens

The fungal pathogens present on the seeds and other plant parts can be directly examined by a stereomicroscope, which is useful for observing fungal spore-bearing structures such as acervuli, pycnidia, or sclerotia produced on infected plant organs and seeds and for rapidly assessing the extent (or percentage) of infection of seeds. The fungal structures produced outside the plant tissues can be scraped by a sharp scalpel or razor blade and examined under a compound microscope. Different models of compound microscopes are manufactured by various companies. The resolving power of these microscopes varies widely to suit different purposes. The compound microscope, irrespective of model, essentially consists of objective and ocular lenses, light source, base, condenser, stage, arm, and body (Fig. 2.2) and of other accessories that allow it to be used as a phase contrast/fluorescent microscope for certain specific studies. The description of various features of the microscope is provided by the manufacturer.

The magnification of a microscope depends on the magnification of the objective and ocular lenses used, and the total magnification of an object can be determined by multiplying the magnification of the objective by that of the ocular lens. The resolving power of the microscope depends on the wavelength of the light source and the optical quality of the lenses. By substituting an ultraviolet light source for white (normal) light, the resolving power of the microscope can be increased. Proper illumination is required for achieving optimal resolution. The iris diaphragm regulates the amount of light passing

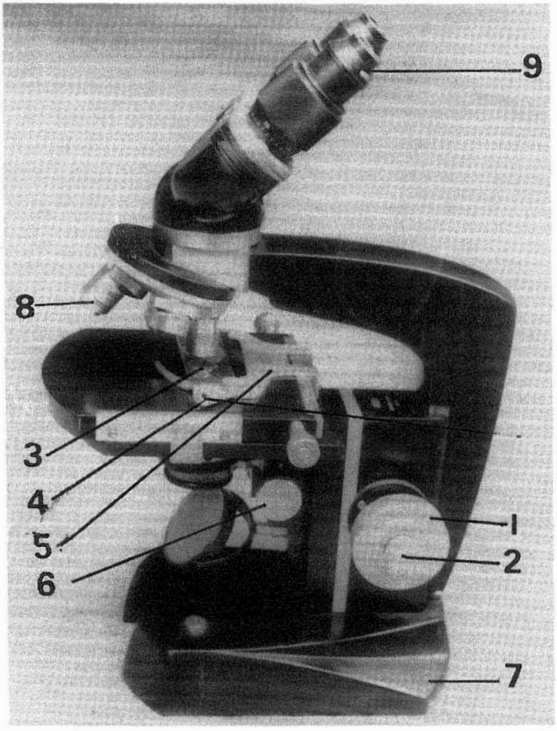


Figure 2.2 Light microscope: 1, coarse adjustment; 2, fine adjustment; 3, microscope slide; 4, slide holder; 5, mechanical stage; 6, condenser adjustment knob; 7, base; 8, objective lens; 9, ocular lens.

through the condenser, and excessive light may obscure the specimen as a result of lack of contrast.

The upper end of the body tube supports the ocular lens (eyepiece) with a magnification of 10×–12.5× for viewing the image of the object (specimen). The monocular microscope has only one ocular lens, whereas the binocular microscope has two ocular lenses, with a provision for adjusting the distance between the two lenses for better viewing. The lower end of the body is fitted with a rotating nosepiece to which objective lenses of varying magnification, usually designated as low-power, high-power, and oil immersion lenses, are attached. More powerful microscopes are provided with additional ocular and objective lenses. The microscope may have a fixed stage with two clips to hold the slides or a mechanical stage with a slide holder, and the assembly can be moved by means of adjustment knobs. The substage attached to the stage consists of a condenser, an iris diaphragm, and a mirror. The condenser, consist-

ing of several lenses, causes light to converge on the object, whereas the iris diaphragm regulates the angle and amount of light required. The light from the source is reflected by the concave side of the mirror via the condenser. Improved models have a built-in light source, which is easily focused. The images of objects are viewed directly when low- and high-power objectives are used, whereas a drop of immersion oil is placed over the slide and then the oil immersion objective is put into position and then focused for viewing. All objective and ocular lenses should be thoroughly cleaned with lens paper moistened with xylol.

2.1.1.1 Induction of Sporulation

Fungal spores with characteristic features may not be present on the infected plant tissues. Incubation of such tissues in moist chambers maintaining high humidity may induce production of spores which can be examined under the microscope to allow the pathogen to be identified. Seed lots are incubated on moist blotters in a humid atmosphere to induce the production of identifiable structures or to produce symptoms of infection. Usually seeds of temperate crops are incubated at 14°–20°C, whereas seeds of tropical crops are incubated at 28°C. In cultures of certain pathogenic fungi sporulating structures are not formed, unless they are exposed to certain treatments. Exposure of fungal cultures to wavelengths of near ultraviolet (NUV) region light is the most effective means of inducing sporulation in pathogens such as *Alternaria solani* and *Septoria lycopersici*, cool white daylight fluorescent lamps emitting an appreciable amount of NUV radiation are suitable for this purpose. A cycle of 12 h of ultraviolet radiation alternating with 12 h of darkness is effective in the case of many fungi. However, the requirement of the individual fungal pathogen has to be determined. Production of sexual spores by many *Ascomycotina* pathogens is favored by very low temperatures. By storing plant tissues infected by powdery mildew pathogens at 0°–5°C for several weeks, formation of cleistothecia may be induced.

When seeds are incubated in several layers of filter paper, they usually germinate and symptoms of infection may appear or intensify. *Leptosphaeria maculans* forms pycnidia on the seed coats of *Brassica* spp., and dark brown or black streaks appear on the coleoptile. Using a steromicroscope, such infected seeds can be easily recognised. *Alternaria brassicae* and *A. brassicola* can be differentiated by the presence of conidia and absence of pycnidia, though *L. maculans* and *Alternaria* spp. produce similar symptoms of infection. Some pathogens produce some specific identifiable product(s), when incubated under NUV light. *Leptosphaeria nodorum* infection of wheat seeds leaves fluorescent products in the blotter (Kietreiber, 1980). The roll-towel or paper dolly method of germination testing may also be useful to detect pathogen infection in seeds

of cereals, legumes, and peanuts during the long period of incubation (Neergaard, 1977).

2.1.1.2 Preparation of Temporary and Semipermanent Mounts

The spores or sporulating structures formed on plant tissues as such or after incubation may be removed by using a scalpel or needles and placed into a drop of mounting fluid kept on a glass slide and stained. The mounting fluids or media and stains commonly used are presented in Appendix 2(i). After applying enough stain, a cover slip is placed over the mounting medium, carefully preventing entry of an air bubble, and is examined under the microscope for the presence of spores and sporulating structures. For taking free-hand sections, rectangular pieces of infected leaf tissues are usually placed in the partially split end of the pith cylinder taken from cassava stem or carrot. Then, using sharp razor blades, thin sections are cut either to observe the pathogenic structures in deep-seated tissues or to study the histopathological characteristics of the infected tissues. The sections are transferred to water kept in watch glasses or petri plates. Thin sections are selected and transferred to drops of mounting medium placed on glass slides by needles and stained and examined under the microscope. These slides can be preserved for some time by sealing the edges of the cover slip with a sealant (such as euparal or glyceel).

Using a strip of transparent cellophane tape, a temporary mount of fungal pathogen can be prepared. A drop of lactophenol cotton blue or aniline blue stain is placed in the center of a clean glass slide. A strip of transparent cellophane tape, about 10 cm in length, is held between the thumb and forefinger, and the sticky side of the tape is firmly pressed onto the surface of a sporulating fungal colony grown over a suitable medium in the petri dish. The tape is then gently removed and the sticky surface is placed over the drop of stain solution kept on the glass slide lengthwise and pressed onto the slide. The extended ends of the tape, if it is longer than the slide, may be folded over the ends of the slide. The spores and sporulating structures produced by the fungal pathogens adhering to the tape can be examined by this simple and rapid technique.

2.1.3 Preparation of Permanent Slides

The host–parasite relationships may be studied in detail by fixing the plant tissues infected by pathogens in different fixatives and embedding them in paraffin then cutting sections by using the microtome. Details of various steps have been excellently described by Johansen (1940). The different steps in the preparation of permanent slides are described briefly. The fixatives and the staining procedures used widely are presented in Appendices 2(ii) and (iii), respectively.

The usefulness of the permanent preparations for rapid identification of plant pathogens is, however, limited.

2.1.3.1 Microtome Sectioning

a. Fixation of plant tissues. Preparation of permanent slides involves killing and fixation of infected plant tissues in appropriate fixatives. Fixation aims at preservation of all cellular and structural elements in the natural living condition as far as possible. It may also allow ready recognition of structures which are obscured or become invisible when observed in the living conditions and help harden soft structures which may otherwise be damaged during subsequent treatments. The optimal time required for fixation depends on the plant materials and fixation fluids. Formalin–acetic acid–alcohol (FAA) mixture, Carnoy's fluid, chrom-acetic acid at different concentrations, and Navashin fluid have generally been used.

b. Washing. It is necessary to wash the plant materials thoroughly, after allowing enough time for fixation, prior to dehydration and infiltration. When formalin–acetic acid–alcohol (FAA) mixture is used for fixation and tertiary butyl alcohol for dehydration, washing out killing fluid is not necessary. But washing in two changes of 50% ethyl alcohol may be needed in other methods. Woody materials have to be washed for 2 days in running tap water and then softened for a period of 3–6 weeks in a 50% aqueous solution of hydrofluoric acid. If Carnoy's fluid is used for fixation, washing in two changes of 95% ethyl alcohol, followed immediately by paraffin infiltration, is required. Materials fixed with chrom-acetic acid or Navashin fluid should be washed free of killing fluids in either running tap water or several changes of water.

c. Dehydration. All traces of water should be removed by using absolute alcohol before substituting it with the clearing agent or solvent of paraffin. Dehydration with tertiary butyl alcohol is satisfactory in most cases. The dehydration process begins with immersion in ethyl alcohol, the concentration of which increases from 5% to 11%, 18%, and 30%. The material is left in each solution for 2 hours. A series of solutions of water, ethyl, and tertiary butyl alcohols is used for further dehydration as recommended by Johansen (1940). Finally dry erythrosin dye is added to the last solution to give a red tinge to the material. This is useful for easy orientation of the material during embedding and microtoming. The material is placed into three changes of pure tertiary butyl alcohol; in one change it has to be immersed overnight.

d. Infiltration. The transfer of the dehydrated material in butyl alcohol to paraffin has to be done gradually. The material is transferred to a mixture of equal parts of paraffin oil and tertiary butyl alcohol and allowed to remain for

1 h or more. Then it is placed on the top of the solidified but not cold paraffin (Parowax) kept in a vial and covered with a butyl alcohol–paraffin mixture. The vial, with its contents, is immediately placed into the paraffin oven. As the paraffin melts and butyl alcohol is evaporated, the material will slowly sink through the melting paraffin (Parowax), until it settles on the bottom of the vial. The paraffin oil prevents damage to the tissues due to heat and also permits the wax to diffuse in gradually. After about an hour, the contents of the vial are poured off and replaced with melted pure Parowax. The material is transferred to two changes of Parowax during the next 6 h. Finally the material is transferred to a good-quality paraffin that will melt at temperatures around 56°C. It can be embedded after about 30 min.

e. Embedding. Embedding plant tissues in paraffin is the most common method. The paraffin infiltration procedures used by early workers required the use of clearing agents such as bergamot oil, cedar oil, and xylol (xylene). Later on, tertiary butyl alcohol was found to be a good substitute for the clearing agents mentioned and helped to reduce the time needed, select suitable materials, and eliminate excessive hardening of tissues.

The embedding process involves pouring of the contents of the vial into suitable receptacles, aligning the material in proper order, and rapidly cooling the entire mass. Simple folded paper trays or ready-made trays are used. The vial containing the material is placed over a gas flame, and, as the wax melts, the contents are quickly transferred to the tray. If necessary, melted paraffin may be poured just to cover the material. By using a needle heated slightly in the flame, pieces of material are arranged in the desired order, leaving enough space between specimens. After arranging the material, the paraffin should be cooled as quickly as possible. In warm weather conditions, ice water may be used for this purpose. The blocks of paraffin in which the plant materials are embedded are stored in small boxes.

f. Microtoming. Microtomes are of two types, the sliding and rotary. The rotary microtome is generally used for sectioning materials embedded in paraffin, whereas the sliding microtome is employed for cutting sections of materials processed by other methods. In some models, the forward movement is directly related to the up-and-down movement (Minot microtome), whereas in later models (Spencer No. 820) the horizontal and vertical movements are entirely independent, providing greater stability and precision. Microtome knives are interchangeable, and use of different knives by different individuals is desirable.

With a sharp scalpel a straight furrow is cut across the paraffin block in which the plant material is embedded. A piece of tissue is then separated by

holding the block firmly in the hands and breaking it apart along the cut. Paraffin is trimmed down the piece of tissue until it is enclosed in a thin shell of paraffin measuring about 3 mm at the top and 5 mm at the bottom. Square or rectangular hardwood blocks are dipped into melted paraffin to a depth of about 2 mm, and a mound of paraffin is formed on top of the wooden block when it is cooled. A scalpel or a similar flat spatula is heated and touched first to the paraffin on the wooden block and then to the bottom of the embedded piece, placing the two together while the paraffin is still more or less melted. Gently and carefully touching the two paraffins with the hot scalpel fuses them to form a single mass on the wooden block. The wooden block with the embedded plant tissue is placed into cold water to cool the paraffin. Excess paraffin is trimmed away using a sharp straight scalpel till the face of the block is either a square or a rectangle so that a straight ribbon of paraffin is formed when the block is cut by the knife of the microtome.

The wooden holder is inserted into the clamp and fixed firmly, taking care to see that the part of the wooden block projecting from the clamp is not more than 3 mm in length. The micrometer scale in the microtome is set to create sections of the desired thickness. The wheel is moved with a steady and even stroke. A ribbon is formed as sections are cut one by one. A straight ribbon is the most desirable.

The ribbon is placed on a piece of black cardboard or thick paper and cut into small sections of the required length (about 5 cm) by a sharp scalpel. The glass slides are smeared with Haupt's adhesive to form a very thin film and flooded with 3% formalin immediately. A section of ribbon is transferred to the slide, which is then placed on a warm plate at 43°C to flatten out the wrinkles in the paraffin. Then the slides are cooled and sections are positioned properly before the excess water is drained off. Absorbent paper can be used to remove as much water as possible. The slides are put aside in a dust-free place to dry completely and then stored until they are stained.

The paraffin has to be removed before staining with xylol (xylene). The slides are placed either individually or in groups in a jar containing xylol for 5 min or more. They are then slowly withdrawn from xylol and transferred to a jar containing a mixture of absolute alcohol and xylol (1:1). After 5 min, the slides are exposed to a mixture of absolute alcohol and ether (anesthetic ether) (1:1) and about 1% celloidin for 5–10 min. The slides are air dried and immersed in ethyl alcohol at 95% or 70% for 5 min and then in 35% ethyl alcohol for another 5 min. If an alcohol solution of stain is to be used, immersion in 35% alcohol can be omitted. Various stains used to stain the sections of tissues infected by fungal pathogens are indicated in Section 2.1.3.2. After staining of the sections, they are dehydrated before mounting them in Canada

balsam. If aqueous stains are used, the sections are immersed in 35% alcohol and then in 70% and 95% alcohol. Immersion in 35% and 70% alcohol is not required if alcoholic stains are used, and they can be placed in 95% alcohol directly for dehydration.

The slides are then placed in a differentiator consisting of USP clove oil (1 part) and a 1:1 mixture of absolute alcohol and xylol (1 part) and moved slowly back and forth for about 10 sec. They are transferred to a jar of xylol to which a trace of absolute alcohol is already added to remove the moisture, if any. The slides are moved back and forth for a few seconds and then placed into a jar of pure xylol.

Before mounting, the slides are taken out and the lower surfaces are wiped with a clean cloth. A small drop of thin balsam is placed on top of the sections. A clean cover slip is dipped into a mixture of equal parts of 95% alcohol and xylol, excess fluid is removed by touching to a paper towel, and, after quickly passing through a clean alcohol flame, the cover slip is carefully placed over the balsam, taking care not to allow any air bubble into the balsam. The slides are then dried on a warming table or in an incubator at 60°C for 1–2 days. They are then sealed with a cellulose-based sealant (glyceel) or a mixture of camsal, sandarac, eucalyptol, and paraldehyde (euparal).

2.1.3.2 Stains

Various stains have been used to stain plant tissues to study the characteristics of cellular organization and organelles. Some of the more commonly used stains are described. For staining procedures refer to Appendix 2(ii) A–C.

a. Hematoxylin. Hematoxylin is a natural dye derived from the logwood *Hematoxylin campechianum*. The stain is always prepared in combination with different metallic salts, such as iron (always the ferric form), aluminum, and copper, since the dye as such has little affinity for tissues. Iron or aluminum acts as a mordant. Harris's or Delafield's hematoxylin solution is frequently used, with good results.

1. *Harris's hematoxylin.*

Hematoxylin crystals	5.0 g
Aluminum ammonium sulfate	3.0 g
50% Ethyl alcohol	1000 ml

The dye is dissolved along with the salt by heating. Mercuric oxide (6 g) is then added, boiled for 30 min, and filtered. Alcohol (50%) is added to return the material to the original volume and acidified by adding hydrochloric acid at the rate of 1 ml/100 ml solution.

2. *Delafield's hematoxylin.*

Hematoxylin crystals	4.0 g
95% Ethyl alcohol	25.0 ml
Ammonium aluminium sulfate (saturated solution)	400.0 ml

To the saturated ammonium aluminium sulfate, hematoxylin dissolved in alcohol is added drop by drop, and the mixture is exposed to light and air for 4 days. Then glycerol (10 ml) and methyl alcohol (100 ml) are added and allowed to stand for about 2 months for ripening.

3. *Heidenhain's iron hematoxylin.* A 10% solution of iron hematoxylin in absolute alcohol is prepared. This solution is diluted with distilled water to a 0.5% concentration, when the stain is to be used.

b. Fast green FCF. The dye fast green FCF belongs to the acidic diaminotriphenyl methane group. It stains tissues rapidly and does not fade even after long periods. It is prepared as 1% aqueous or 0.1% alcohol solution. Better results are obtained by using stain prepared by adding sufficient dry dye to a mixture of methyl cellosolve, absolute alcohol, and clove oil (1:1:1) to produce a dark green 0.5% solution.

c. Safranin. The stain safranin is frequently used in morphological and cytological investigations. Safranin solution is prepared by dissolving the dye (4.0 g) in methyl cellosolve (200 ml), adding alcohol (100 ml) and distilled water (100 ml) to this solution, then adding sodium acetate (4.0 g) and formalin (8.0 ml). Sodium acetate intensifies the color, while formalin acts as a mordant. As safranin generally overstains the sections, differentiation is necessary. This is achieved by adding picric acid to the dehydrating alcohol (95%). Excess stain has to be washed with distilled water.

2.1.3.3 Processing plant tissues infected by fungal pathogens

The plant tissues infected by fungal pathogens are fixed in various fixatives and stained to study their morphological characteristics and the effect of infection on host tissues/organelles. Portions of potato tubers infected by *Spongospora subterranea*, which causes powdery scab disease, may be fixed in a chrom-acetic acid medium and stained in iron hematoxylin and fast green. Roots infected by *Plasmodiophora brassicae*, which causes cabbage club root disease, may be fixed in formalin–acetic acid–alcohol, followed by tertiary butyl alcohol dehydration. Medium chrom-osmo-acetic fluid or Navashin's fluid is suitable for fixing the tissues. Iron hematoxylin and fast green stains are useful.

The sporangiophores and sporangia in the pustules formed by white rust pathogen *Albugo* spp. can be studied by fixing the tissues in a chrom-acetic fluid or formalin-aceto-alcohol medium followed by staining with iron hematoxylin and counterstaining with orange G.

Leaves infected by powdery mildew fungi such as *Erysiphe cichoracearum* and *Sphaerotheca pannosa* may be fixed either in FAA or Navashin's fluid, then stained with iron hematoxylin. Leaves infected by *Taphrina deformans*, which causes peach leafcurl disease, are fixed in formalin–propiono–alcohol and then stained with either safranin and fast green or iron hematoxylin. Wheat leaves or stem infected by rust pathogens are fixed in formalin–aceto–alcohol and stained with a quadruple combination.

2.1.4 Preparation of Ultrathin Sections for Light Microscopy

Excellent preparations for light microscopy can be obtained by glutaraldehyde–osmium fixation, though this procedure is generally followed for electron microscopy (Chapter 7).

2.1.4.1 Fixation

The plant tissue is cut into small pieces by using a sharp razor blade in a pool of 3% glutaraldehyde in phosphate buffer, pH 7.0–7.4. The tissue is then transferred to a vial containing 3% buffered glutaraldehyde at 4°C. The fixative is changed after 2–3 hr and incubated for 12–16 hr in a refrigerator. The tissue should be washed free of glutaraldehyde by using several changes of cold buffer at 20 min intervals. The tissue is postfixed in 1% buffered osmium tetroxide for 1–2 hr at 4°C and washed with buffer thrice at 10 to 30 minute intervals.

2.1.4.2 Embedding

Epoxy resins are used as embedding matrix. Epon 812 is commonly employed, after dehydration of fixed plant tissues in a graded series of alcohol, as in paraffin embedding. Embedding methods that employ nonsolvents and solvents of plastic have been used. As a nosolvent method, propylene oxide,which is miscible with alcohol and plastic in different proportions, can be used. The plastic mixture with the following components gives good results:

Epon 812	54.90 g
DDSA (dodecenyl succinic anhydride)	29.10 g
NMA (nadic methyl anhydride)	30.75 g
DMP-30 [2, 4, 6-tri (dimethylaminomethyl) phenol]	0.90 g

The following steps may be used:

Propylene oxide (PO)	3 changes at 5 min intervals or 5, 10, and 15 min intervals
3 parts PO + 1 part plastic mixture	15–30 min
1 part PO + 1 part plastic mixture	30–60 min
1 part PO + 3 parts plastic mixture	60 min to overnight
Plastic mixture alone	12–24 hr

The tissue pieces may be embedded in plastic bottle covers, gelatin capsules, or aluminium foil boats and hardened for about 48 hr at 60°C.

2.1.4.3 Staining

After trimming the block to a pyramid under a binocular stereoscopic microscope, the block is mounted in an ultrmicrotome. Sections that display green interference colors are cut. The floating sections from the trough are collected and mounted on clean glass slides with drops of water. A hair loop or eyelash whisker can be used to transfer the thin sections. The slides are placed in an oven at 60°C or on a warming plate to permit evaporation of water and settling of sections on the slide. Generally no adhesive may be necessary. If required, egg albumin or gelatin adhesive may be used for coating the slides before mounting the sections.

The slides are then removed from the oven or warming plate and cooled to room temperature. The sections are treated with 1% periodic acid for 5 min at room temperature to remove osmium and to prevent rapid fading of the stain. The sections are rinsed in distilled water and slides are wiped by suitable absorbent paper. The required amount of stain is added in drops to the sections, and the slide is placed on either the warming plate or an alcohol (sprit) lamp for different periods, depending on the nature of the plant tissue. The sections are washed with distilled water to remove the excess stain and dried on a warming plate. The slides are then immersed in two changes of xylene and dried by draining. Suitable mounting medium is added to the section and covered. For further details refer to Berlyn and Miksche (1976).

2.2 METHODS OF ISOLATION OF PATHOGENS

Plant pathogens that cause different diseases on various crop plants have to be isolated in pure culture, and their morphological characters and physiological and biochemical activities are studied with a view to using them as bases for their identification and differentiation from closely related species. Facultative

parasites and facultative saprophytes which have the ability to grow in cell-free media can be isolated and maintained on appropriate media for characterization of these pathogens. But obligate fungal pathogens, viruses, viroids, and most of the mycoplasma-like organisms (phytoplasmas) have to be maintained on live host plant species by adopting different methods of transmission (Chapter 4).

2.2.1 Preparation of Media

The growth and sporulation of microbial pathogens are favored to different extents on the basis of the nature of the media used and cultural conditions provided. Both solid and liquid media have been used to cultivate pathogens, depending on the objectives of the experiments. Agar is the basic component of all media used in all solid media. Media rich in carbohydrates and slightly acidic (pH 6–6.5) in nature favor the growth of fungi, whereas bacteria prefer media with neutral or slightly alkaline pH. The media have to be sterilized at the required temperature and pressure. Many proprietory media are available for ready use, and addition of water alone is required for such media. The compositions of various media used for cultivation of fungal and bacterial pathogens are presented in Appendix 2(iv).

2.2.2 Isolation and Identification of Fungal Pathogens

The fungal pathogens can infect different plant parts, causing visible symptoms of the disease after the completion of the incubation period. The pathogens may be isolated from infected plant tissues such as leaves, stems, fruits, and roots by following different techniques for studying characters through which they can be identified.

2.2.2.1 Isolation from Leaves and Other Plant Parts

The infected leaves are thoroughly washed in sterile water. Then the infected tissues along with adjacent small unaffected tissues are cut into small pieces of 2–5 mm squares and transferred by using flame-sterilized forceps to sterile petri dishes containing 0.1% mercuric chloride solution. The tissue pieces are surface-sterilized in this solution for 30–60 sec and washed in sterile water two or three times. Clorox (10%), sodium hypochlorite (1%), or hydrogen peroxide (50%) may also be used to sterilize the surface. The tissue pieces are aseptically transferred to petri dishes containing a nutrient medium (such as potato dextrose medium) supplemented with streptomycin sulfate at the rate of three to five pieces per plate. The plates are incubated at room temperature (25°–27°C). The fungal mycelium growing on the nutrient medium is then transferred to agar slants kept in tubes.

The fungal pathogens from stems, roots, or fruits in which they may be present in deep-seated tissues have to be isolated by culturing pieces of internal tissues. The infected tissues are thoroughly washed in sterile water and then swabbed with cotton wool dipped into 80% ethanol, followed by exposure to an alcohol flame for a few seconds. The outer layer of tissues are quickly removed by a flame-sterilized scalpel. Small pieces from the central core of tissues in the area of the advancing margin of infection are removed by a sterilized scalpel or scissors and sterilized by dipping into 90% alcohol then flaming for a few seconds. The tissues, thus sterilized, are transferred to nutrient agar kept in petri dishes and incubated. The fungal mycelium growing from the infected tissues is transferred to agar slants kept in tubes.

2.2.2.2 Purification of Fungal Cultures

The cultures of fungal pathogens growing in agar slants have to be purified by either the single hyphal tip method or single spore isolation for precise identification. A small bit of agar medium containing fungal growth is transferred to the center of petri dishes containing nutrient medium, using a flame-sterilized inoculation needle, and incubated at room temperature for a few days. As the fungus grows, the advancing edge of the fungal growth will have well separated hyphal tips which are marked by a glass marking pencil by observing the bottom petri dish under the low power of the microscope. The bits of agar bearing a single hyphal tip marked earlier are carefully removed by a flame-sterilized inoculation needle and individually transferred to agar slants in tubes in which the hyphal tips will grow into a pure colony.

The spores of the fungal culture growing in the agar slant are suspended in sterile water by transferring the fungal growth to sterile water kept in a sterilized test tube then vigorously shaking the tube for a few minutes. This spore suspension is serially diluted by transferring 1 ml aliquots to a series of tubes containing 9 ml of sterile water. After attaining optimal dilution, 1 ml aliquots of the spore suspensions are mixed with melted nutrient agar at about 45°C and poured into sterile petri dishes, and the medium is spread to cover the entire surface by tilting the dishes suitably. The petri dishes are incubated at room temperature and examined at intervals of a few hours under the low power of the microscope. Individual germinating spores are marked by using a glass marking pencil as in the single hyphal tip method. The germinating spores along with the medium in the marked area are individually transferred to agar slants. The spores will grow into pure colonies.

The characteristics of asexual and sexual spores and spore-bearing structures of fungal pathogens are studied and used as bases of identification and

differentiation of various species of fungal pathogens. The general outline for the classification of fungi up to the level of subclass is presented in Chapter 3.

2.2.3 Isolation and Identification of Bacterial Pathogens

The bacterial pathogens infecting leaves and other plant parts are generally isolated by preparing a bacterial suspension. Infected leaves showing clear symptoms of infection are cut into small bits 3 mm in diameter, surface-sterilized with 70% ethyl alcohol or 0.1% mercuric chloride solution for 30–60 sec and washed repeatedly in several changes of sterile water. The bacterial suspension may be prepared in two ways: The surface-sterilized infected tissue bits may be immersed in sterile water in a test tube and incubated for 6 h at room temperature, as in the case of *Xanthomonas oryzae* pv. *oryzae*, which causes bacterial leaf blight in rice. This suspension is then streaked on potato–peptone–glucose agar (PPGA) medium, using a sterilized inoculation needle.

In the second method, the surface-sterilized infected tissues are crushed in sterile water kept in an aspetic mortar with a pestle to get a suspension of bacteria, as in the case of *Xanthomonas campestris* pv. *malvacearum*, which causes bacterial leaf blight in cotton. This suspension is then streaked on nutrient agar medium kept in petri dishes by a sterile inoculation needle. The plates are incubated at room temperature for 24–48 h. The bacteria in individual colonies are then transferred to agar slants and purified by the serial dilution plate technique.

The serial dilution plate method is based on the principle that when appropriately diluted each viable bacterial cell will develop into a colony, ensuring purity and homogeneity of cells in the bacterial culture. A known volume of bacterial suspension (1 ml) is added to known volume (9 ml) of sterile water blank and agitated to produce a uniform suspension of bacterial cells. Serial dilutions of this suspension 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and above are prepared by pipetting 1 ml aliquots into dilution blanks of 9 ml each. Then 1 ml aliquots of different dilutions are added to separate petri dishes to which sterilized melted nutrient agar medium is added at the rate of 15 ml/dish. The plates are incubated in an inverted position for a few days at 25°C. The bacteria from well-isolated colonies are streaked on nutrient agar medium or special selective media suitable for the bacterial species. Tetrazolium medium (Kelman, 1954) is used for the isolation of *Pseudomonas solanacearum*, which causes bacterial wilt of banana, whereas 523 medium (Kado, 1971) is used for the isolation of *Xanthomonas campestris* pv. *malvacearum*.

The bacterial pathogens are identified on the basis of colony characters, Gram's staining, and the properties determined by various biochemical and physiological tests (Chapter 6). The general outline for the classification of bacteria is presented in Chapter 3.

2.2.4 Isolation and Identification of Viruses

Plant viruses that infect different plant species are obligate parasites that require the presence of living cells/plants. They cannot be isolated and grown on cell-free media as fungi or bacteria, but they have to be maintained on susceptible host plants by artificial inoculation at regular intervals. Sap inoculation and/ inoculation employing appropriate vectors are done, and a plant species that favors rapid multiplication of the given virus is used as the source plant. If a virus is found along with another virus in the same plant, it has to be separated by inoculating on a diagnostic host which either forms local lesions or characteristic systemic symptoms which are distinct from those caused by the contaminating virus. An inoculum from a single local lesion can be prepared and inoculated on healthy plants, and the culture of virus can be obtained in pure form by such repeated inoculations. Insect vectors also can be used to separate the virus from contaminants on the basis of the specific nature of the virus–vector relationship.

The viruses can be purified, after extraction of the sap from infected source plants, by following appropriate purification procedures, which depend on the host–virus combinations (Matthews, 1991; Narayanasamy and Doraiswamy, 1996). The purified virus preparations are required for studying various properties of the viruses, such as virus particle morphological characteristics, the nature of the viral genome, the molecular weights of capsid protein and viral nucleic acid, amino acid and nucleotide sequences, and serological properties. The host range of viruses, reaction on diagnostic host plants, stability in vitro, and nature of the virus–vector relationship are also studied, and these properties are considered in the classification of plant viruses (Chapter 3).

2.2.5 Isolation and Identification of Mycoplasmas (Phytoplasmas)

The plant-infecting mycoplasma-like organisms (MLOs), currently termed phytoplasmas, were recognized as a distinct group of plant pathogens after the studies on certain yellows types of plant diseases by Doi et al. (1971) and Ishiie et al. (1971). Though intensive efforts were made by researchers for over two decades, isolation and cultivation of these organisms in cell-free media have been not been achieved, except in the case of *Spiroplasma citri* (Markham et al., 1974; Chen and Liao, 1975; Williamson and Whitcomb, 1975). Hence it has not been possible to study the morphological features and other biochemical properties required for their characterization and precise identification. However, with the development of modern molecular methods of detection and identification, the relationships of MLOs can be studied with reasonable reliability and certainty. These methods are described in Chapters 8 and 9.

2.3 FUNGI

The fungi are generally microscopic plants without chlorophyll and conductive tissues. Among about 100,000 fungal species known to exist, more than 8000 species are pathogenic to plants, and about 50 species cause disease in humans and animals.

The fungal body (thallus) consists of numerous filamentous hyphae weaving into a mycelium. The hyphae may or may not have cross-walls (septa). The hyphal walls may be uniform in thickness or irregularly thickened, tapering into thinner or broader portions. The fungal cells delimited by well-defined cell walls contain one or two nuclei per cell. The coenocytic mycelium, as in Phycomycotina, contains many nuclei, and the mycelium becomes one continuous tubular, branched, or unbranched multinucleate cell or multinucleate hyphae, if septa are formed later. Mycelial growth occurs through elongation of hyphal tips. Fungi belonging to Plasmodiophorales lack true mycelium and form naked, amoeboid, multinucleate plasmodia, whereas members of Chytridiales produce a system of strands which are dissimilar and continuously varying in diameter, known as rhizomycelium.

The fungi reproduce efficiently by both asexual and sexual means. Specialized propagative bodies called spores or conidia are formed in the asexual reproductive cycle, whereas the sexual spores, called oospores, zygospores, ascospores, and basidiospores, are formed after a sexual process. In the Phycomycotina, the asexual spores are produced in a saclike sporangium and are later released through an opening of the sporangium or when the sporangial wall ruptures. The spores may be either motile by means of flagella (zoospores) or nonmotile (aplanospores) (Fig. 2.3). Conidia are formed by Ascomycotina and Deuteromycotina fungi by cutting off of terminal or later cells from the specialized hyphae known as conidiophores (Fig. 2.4). In some fungi, individual hyphal cells may be enlarged and develop thick walls. They are separated at maturity and can germinate to form a new colony. These structures are chlamydospores. Sclerotia are formed when a group of vegetative cells are enclosed in thick walls. Aseuxal spores may be formed in specialized reproductive structures, such as pycnidia, acervuli, and sporodochia.

In Phycomycotina, during sexual reproduction, union of two cells (gametes) of equal size and similar appearance results in the production of a zygote called the zygospore in some fungi; in other fungi the oospore is produced when gamates of unequal size unite. The sexual spores in Ascomycotina are formed in the zygote cell (ascus) produced by the union of two unequal-sized gamets. Usually eight ascospores are produced in each ascus, which may or may not be enclosed in the fruiting body called the ascocarp. In some fungi, any one cell of a mycelium may fuse with any cell of another compatible mycelium. Spores (teliospores) are produced from such a mycelium. Sexual

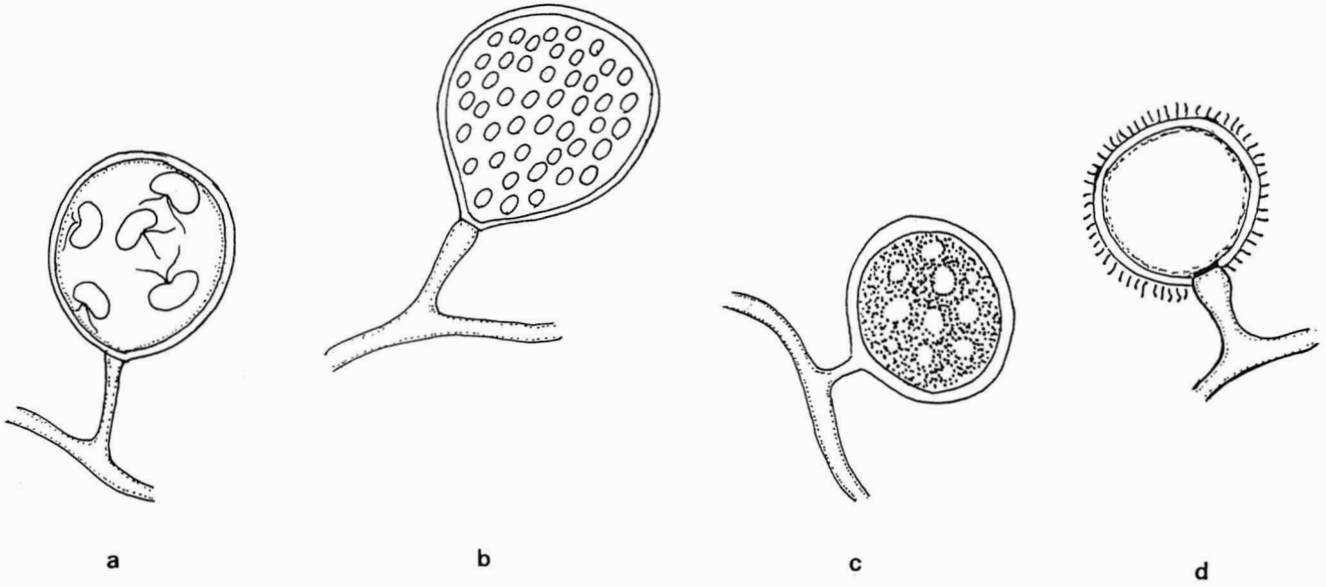
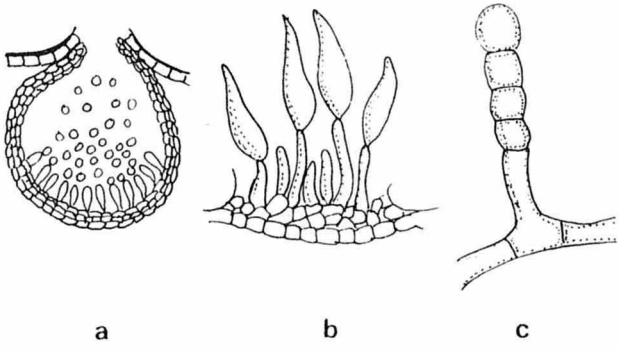


Figure 2.3 Asexual and sexual spores produced by members of Phycomycotina: a, Sporangium with zoospores; b, sporangium with aplanospores, c, oospore; d, zygospore.



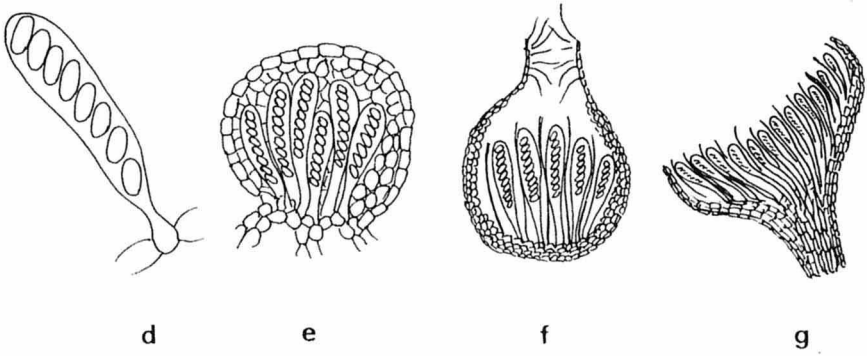


Figure 2.4 Asexual and sexual spores produced by Ascomycotina: a, Pycnidium with conidia; b, conidiophore bearing single conidium; c, conidiophore bearing chain of conidia; d, ascus with ascospores; e, cleistothecium with asci; f, perithecium with asci; g, apothecium with asci.

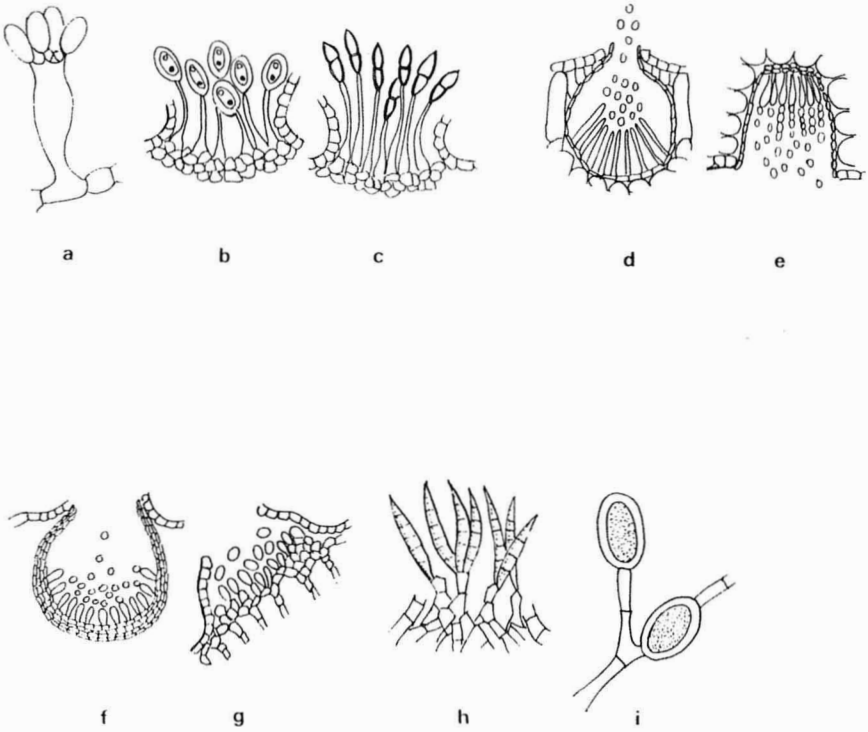


Figure 2.5 Spore-forming structures produced by Basidiomycotina and Deuteromycotina: a, Basidium with basidiospores; b, uredium with uredospores; c, telium with teliospores; d, pycnium with pycniospores; e, aecium with aeciospores; f, pycnidium with conidia; g, acervulus with conidia; h, sporodochium with conidia; i, chlamydospores.

spores formed outside the zygote cell (basidium) are known as basidiospores, as in the case of fungi belonging to the Basidiomycotina (Fig. 2.5). No sexual spores are produced by a large group of fungi (Fungi imperfecti), and they are known to reproduce only asexually by forming various kinds of conidia.

2.4 BACTERIA

About 200 of 1600 bacterial species are known to infect various kinds of plants. Most of the bacteria are strictly saprophytic and help decompose large quantities of organic wastes produced by industrial establishments or by dead plants

and animals. Many bacterial species are beneficial to humans because of their role in building up the nutritional level of the soil. Bacteria are simple in structure; they may be rod-shaped, spherical, ellipsoidal, spiral, comma-shaped, or filamentous. Some are motile by means of flagella; many of them are nonmotile. Some bacterial species transform themselves into spores, whereas spores are formed at the tips of some filamentous forms.

All plant pathogenic bacteria except two species of *Streptomyces* are rod-shaped. *Streptomyces* sp. is filamentous. The rod-shaped bacterial cells are short and cylindrical, measuring 0.6–3.5 μm × 0.3–1.0 μm. Deviations from this rod shape in the form of a club, a Y or V shape, and branched forms may also be observed. Pairs of cells or short chains of cells may also be formed in certain cases. The bacterial cells may be enveloped by a thin or thick slime layer made of viscous, gummy materials. The slime layer may be found as a larger mass around the cell called capsule. Most of the plant pathogenic bacteria are motile, and the flagella may be present either singly or in groups or distributed over the entire cell surface. The cells of *Streptomyces* spp. consist of nonseptate branched threads which usually have a spiral formation and produce conidia in chains on aerial hyphae. The bacterial colonies may vary in size, shape, color, elevation, form of edges, etc., depending on the species, and these properties may be characteristic of some species. The size of colonies may vary from 1 mm to several centimeters in diameter, and they may be circular, oval, or irregular with smooth, wavy, or angular edges. The colonies may have flat, raised, dome-shaped, or wrinkled elevation and are of different colors: white, yellow, red, or gray.

Rod-shaped plant pathogenic bacteria reproduce asexually by the process referred to as fission or binary fission. During this process, the cytoplasmic membrane grows toward the center of the cell, forming a transverse membranous partition dividing the cytoplasm into two approximately equal parts. Between the two layers of cytoplasmic layers, two layers of cell wall material, continuous with the outer wall, are laid down. As the formation of these cell walls is completed, they separate, splitting the two cells apart. The nuclear material is duplicated, as the layers of cell wall are formed in the center and become distributed equally between the two daughter cells formed from the dividing single cell. The bacteria can divide at an astonishingly rapid rate, dividing once in every 20 min under favorable conditions.

The sexual process known as conjugation occurs in some species. During this process, two compatible cells come into contact side by side and a small portion of the deoxyribonucleic acid (DNA) of the male cell (donor) is transferred to the female (receptor) cell. The female cell then multiplies by fission, resulting in daughter cells that contain the characteristics of both donor and receptor cells.

Variations in the genetic constitution of bacteria may be brought about by three different phenomena, viz. mutation, transformation, and transduction, in addition to conjugation. Mutation may occur in a very small percentage of cells, resulting in changes in genetic material leading to permanent changes in certain characteristics of the bacteria. In some bacteria, the genetic material is liberated from one bacterial cell by either secretion or rupture of the cell wall. A portion of this free genetic material (DNA) gains entry into a genetically compatible bacterium of the same or closely related species, making the recipient cell become genetically different. Transduction requires a bacteriophage as a vector for the transfer of genetic material (DNA) from one bacterium to another. The bacteriophage acquires a portion of genetic material of the infected bacterium. When it is liberated and infects another bacterial cell, the genetic material of the first cell becomes integrated with the DNA of the second bacterium. Thus different characters may be transferred from one bacterial cell to another, which shows new or different characters.

The ability of bacteria to multiply very rapidly and reach high populations within a short period makes them an important factor to be considered in any ecosystem in general and as plant pathogens in particular.

2.5 MYCOPLASMA-LIKE ORGANISMS

Mycoplasmas are known to exist as saprophytes in soil and sewage, and some have been found to be pathogens of human beings and other animals, such as cattle. The possibility of mycoplasmas being plants pathogens was indicated by Doi et al. (1967) and Ishiie et al. (1967). The presence of pleomorphic bodies in the phloem cells of infected mulberry plants was observed; these bodies degenerated, and the infected plants showed remission of the disease after application of tetracycline antibiotics. These observations suggested that some yellows plant diseases may be due to mycoplasmas rather than viruses, as believed earlier. Subsequently several other plant diseases were reported to be due to this new group of pathogens, designated mycoplasma-like organisms (MLOs).

Most of the plant mycoplasmas have not been isolated in pure culture, and hence information on their cultural characters is lacking. Because of their morphological similarities to other mycoplasmas as observed in ultra-thin sections using electron microscope, they are named mycoplasma-like organisms.

Plant and animal mycoplasmas are included in the class Mollicutes. Mycoplasmas differ from the bacteria in the absence of cell wall and penicillin-binding sites. They are, therefore, resistant to penicillin to which bacteria are sensitive. Mycoplasmas have a triple-layered plasma membrane. The name Mollicutes is derived from the property of lack of rigidity of cells of myco-

plasma. They are able to pass through pores of even 220 nm diameter, even though the diameter of a viable cell may be more than 300 nm. The mycoplasmas are pleomorphic (varying in shape and size) generally and form characteristic fried egg colonies on solid media. They have an absolute dependence on sterols for growth; bacteria do not require sterols for their growth.

Among the plant mycoplasmas *Sprioplasma citri*, the pathogen that causes citrus stubborn and corn stunt diseases, has been isolated in pure culture, and its pathogenicity has been proved by following Koch's postulates (Davis and Lee, 1982). All attempts to prove other MLOs pathogenic have been unsuccessful.

2.6 VIRUSES

Viruses are submicroscopic pathogens that cause diseases in all known organisms from bacteria to highly evolved human beings. They are obligate parasites, requiring the presence of living cells for their replication and development. They cause diseases such as the common cold, influenza, polio, rabies, and the recently recognized acquired immunodeficiency syndrome (AIDS) in human beings, and cattle suffer of foot and mouth disease, which is of viral origin. Plants are affected by one or more viruses, and frequently losses caused by them are appreciable.

Plant viruses differ distinctly from other pathogens not only in their size, but also in their shape, which varies from rod-shape or bacilliform to spherical or polyhedral. They are very simple in their constitution, primarily possessing a protein coat which encloses either a ribonucleic acid (RNA) or DNA molecule(s) as the genome. Viruses have a unique method of replication which is not seen in any other known organisms. The protein coat and genomic nucleic acid are synthesized separately in different sites in susceptible cells and assembled together at an appropriate time to form progeny virus particles.

Plant viruses require an agency for transmission, though they can spread through leaf contact, infected seeds, and seed materials in certain cases. The viruses have to be introduced into the susceptible cells through the aid of insects, mites, nematodes, fungi, or parasitic dodders, since they cannot enter the plants through intact epidermis or natural openings. Mechanical inoculation or grafting is done for experimental transmission of viruses to healthy plants.

Plants respond differently to different viruses, depending on the level of their resistance to a given virus. Susceptible plants react to viruses by producing either local lesions, when the virus is confined to the initially infected tissues, or characteristic symptoms, in tissues/organs away from the site of infection/inoculation, when the virus becomes systemic. In addition to macroscopic external symptoms, the viruses induce, in certain host plants, the formation of

inclusion bodies with characteristics of diagnostic value. No other plant pathogenic organism is known to induce the production of inclusion bodies in plants. As the plant viruses neither produce any structure outside the infected tissue nor are liberated from the infected cell, different methods have been used to detect their presence in infected plants.

Some viruses, including tobacco mosaic virus and cucumber mosaic virus, are found in the form of many strains which differ in their host range, type of symptoms induced, method of transmission, species of vectors, and physical and chemical properties. Related strains usually interfere with the development of another; this phenomenon is known as cross-protection. A mild strain of a virus can be used to protect cultivars against infection with severe strains of the same virus.

Studies on the molecular biological characteristics of viruses have helped researchers to understand the functions of different cistrons (genes) in the viral genomes and the various strategies adopted by viruses for replication that result in increase in the viral population under optimal conditions. Biotechnological approaches have been made to develop transgenic crop plants with built-in resistance to several virus diseases.

2.7 VIROIDS

Viroids are the simplest among the plant pathogens capable of causing diseases, for example, potato spindle tuber disease, which is the earliest recognized viroid disease (Diener, 1971). Viroids are subviral pathogens that replicate independently and reach sufficient concentrations, when introduced into cells/tissues of susceptible plants, to produce characteristic symptoms of disease.

The viroids are small covalently closed circular RNA molecules with a highly base-paired, rather stiff rodlike native conformation. Potato spindle tuber viroid (PSTVd), which has been studied in detail, possesses a serial arrangement of 26 double-stranded segments interrupted by bulge loops of varying sizes. The extended secondary structure is folded into a more globular tertiary conformation, as the single-stranded loops do not interact. The viroid structure shows both stability and flexibility. The native rodlike structure of viroids is converted, through thermal denaturation, into a hairpin-containing circle in a highly cooperative fashion (Henco et al., 1979).

The viroid nucleic acids have hundreds of nucleotides, varying from 247 in avocado sunblotch viroid to 371 in citrus exocortis viroid (Table 2.1). The molecular weights of viroid RNAs also vary to some extent. The sizes of cucumber pale fruit viroid (CPFVd) and hop stunt viroid (HSVd) are quite similar, and these viroids are biologically indistinguishable (Shikata, 1985).

Table 2.1 Properties of Viroid Nucleic Acids

Viroids	No. of nucleotides	Molecular weight	References
Avocado sunblotch	247	—	Symons, 1981
Chrysanthemum stunt	354	120,000	Goss et al., 1982
Citrus exocortis	371	125,000	Gross et al., 1982
Cucumber pale fruit	330	110,000	Sanger et al., 1976
	320	—	Sano et al., 1983
Hop stunt	315	—	Sano et al., 1983
Potato spindle tuber	359	—	Gross et al., 1978

SUMMARY

The general characteristics of fungi, bacteria, mycoplasma-like organisms (MLOs), viruses, and viroids which cause destructive diseases on economically important crops are described to give the reader basic knowledge of these disease-causing microbes. These microorganisms can be differentiated on the basis of the differences in characteristics of the morphological and structural features.

APPENDIX 2(i): MOUNTING MEDIA AND STAINS FOR FUNGAL PATHOGENS

A. Mounting Media

i. Lactophenol

Phenol (pure crystals)	20.0 g
Lactic acid (SG 1.21)	20.0 g
Glycerol	40.0 g
Water	20.0 ml

ii. Anhydrous lactophenol

Phenol	20.0 g
Lactic acid	20.0 g (16.0 ml)
Glycerol	40.0 g (31.0 ml)

iii. Glycerine jelly

Gelatine	1.0 g
Glycerol	7.0 g
Water	6.0 ml
Phenol to give 1% concentration	

B. Stains

i. Cotton blue (or trypan blue)

Anhydrous lactophenol	67.0 ml
Distilled water	20.0 ml
Cotton blue or trypan blue	0.1 g

ii. Erythrosin

Erythrosin	1.0 g
Ammonia (10%)	100.0 ml

iii. Lacto-fuchsin

Acid fuchsin	0.1 g
Lactic acid	100.0 ml

This solution and Gurr's water mounting medium are mixed in a 1:1 ratio (Carmichael, 1955).

APPENDIX 2(ii): FIXATIVES USED FOR PREPARATION OF PERMANENT SLIDES

A. Formalin–acetic acid–alcohol (FAA) mixture

Ethyl alcohol (50% or 70%)	90.0 ml
Glacial acetic acid	5.0 ml
Formalin	5.0 ml

Alcohol at lower concentration is used for fixing delicate tissues, whereas higher concentration may be required for woody tissues. Fixation time is 18 h or more.

B. Carnoy's fluids

i. Ethyl alcohol (100%)	15.0 ml
Glacial acetic acid	5.0 ml
ii. Ethyl alcohol (100%)	30.0 ml
Glacial acetic acid	5.0 ml
Chloroform	15.0 ml

Fixation time varies from 15 to 60 min.

C. Chamberlain's chrom-osmo-acetic acid mixture

Chromic acid	1.0 g
Glacial acetic acid	3.0 ml
Osmic acid (1% aqueous solution)	1.0 ml
Distilled water	100 ml

This mixture is suitable for filamentous fungi. Chrom–acetic acid fluid has been used at different concentrations also.

D1. Weak chrom-acetic acid

Chromic acid	2.5 ml
Acetic acid	5.0 ml

Distilled water added to make up 100 ml.

D2. Medium chrom-acetic acid

Chromic acid	7.0 ml
Acetic acid	10.0 ml

Distilled water added to make up to 100 ml.
Chromic acid and acetic acid are used as 10% aqueous solution.
Fixation time is 24 h or more.

E. Randolph's modified Navashin fluid

Solution A:	Chromic acid	1.0 g
	Glacial acetic acid	7.0 ml
	Distilled water	92.0 ml
Solution B:	Neutral formalin	30.0 ml
	Distilled water	70.0 ml

Mix solutions A and B in equal proportions before use. Fixation time varies from 12 to 24 h.

APPENDIX 2(iii): STAINING PROCEDURES (JOHANSEN, 1940)

a. Heidenhain's iron hematoxylin

i. Use xylol to remove paraffin; pass through a mixture of xylol and absolute alcohol (1:1) for 10 min, then through a mixture of alcohol and ether (1:1) + 1% celloidin for 3 min; air dry the slides till they become opaque; immerse successively in 70% alcohol for 5 min, 35% alcohol, and finally water, and rinse in distilled water.

ii. Prepare the mordant solution containing ferric ammonium sulfate crystals (15.0 g), glacial acetic acid (5.0 ml), conc. H_2So_4 (0.6 ml), and distilled water (500 ml); place the slides into this solution for 1–2 h; wash thoroughly in running water for 5 min and then rinse in distilled water.

iii. Place the slides into aqueous hematoxylin solution (0.5%) for 1–2 h or more; wash the excess stain with water.

iv. Destain by immersing the slides in ferric ammonium sulfate (2%) or ferric chloride as long as required; wash in running water for 30–60 min.

v. Dehydrate slides by passing them successively through 50%, 70%, and 95% alcohol for 5 min in each concentration.

vi. Pass the slides through a mixture of absolute alcohol and xylol (1:1) for 5 min and then through two changes of xylol for 5 min each and mount in balsam.

B. Iron hematoxylin and safranin

i. Follow steps as in A(i).
ii. Place the slides in 3% aqueous ferric ammonium sulfate solution (used as mordant) for 2 to 3 hr; wash in running water for 5 min.
iii. Stain in hematoxylin for 2 to 3 hr, followed by differentiation in 3% aqueous ferric ammonium sulfate; transfer the slides to water when sections turn colorless and wash in running water for 1 hr or more.
iv. Stain in safranin for 12–15 hr, followed by differentiation using either 70% alcohol acidified with a few drops of HCl or 95% picro alcohol for not more than 10 sec.
v. Follow steps in A(v) and A(vi).

C. Conant's quadruple stain

i. Follow steps as in A(i) up to 70% alcohol step.
ii. Place the slides in 1% safranin in 50% alcohol for 2–24 hr; rinse thoroughly in distilled water.
iii. Transfer the slides to a saturated aqueous solution of crystal violet for about 1 min and rinse in distilled water, followed by dehydration through two changes of absolute alcohol.
iv. Stain the slides by rapidly dipping 5 to 10 times in 1% fast green in absolute alcohol; transfer to a saturated solution of gold orange (or orange G) in clove oil and agitate the slides till the alcohol is completely diffused into the clove oil.
v. Place the slides successively in a series of three more jars of orange clove oil solution for several minutes in each for further differentiation and clearing of the background.
vi. Rinse the slides in xylol and mount the sections in balsam.

APPENDIX 2(iv): MEDIA USED FOR CULTIVATION OF FUNGAL AND BACTERIAL PATHOGENS

A. Media for fungal pathogens

i. Potato dextrose agar

Potato (peeled)	200.0 g
Dextrose	20.0 g
Agar	20.0 g
Water	1000.0 ml

Sterilize for 20 min at 1.06 Kg/cm^2

ii. Potato sucrose agar

Potato extract	500.0 ml
Sucrose	20.0 g
Water	500.0 ml

Potato extract is prepared by placing peeled pieces of potato (1800 g) in muslin cloth, suspending in water (4500 ml), and boiling for 10 min.

iii. Oatmeal agar

Oats	100.0 g
Agar	15.0 g
Water	1000 ml

iv. Yeast extract glucose agar

Yeast extract	10.0 g
Glucose	10.0 g
Agar	15.0 g
Tap water	1000 ml

v. Water agar (plain agar)

Agar	20.0 g
Water	1000 ml

vi. Czapek (Dox) agar

Sodium nitrate	2.0 g
Potassium dihydrogen Phosphate (KH_2PO_4)	1.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g
Potassium chloride (KCl)	0.5 g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	0.01 g
Sucrose	30.0 g
Agar	20.0 g
Water	1000 ml

B. Media for bacterial pathogens

i. Nutrient broth

Bactopeptone	5.0 g
Beef extract	3.0 g
Water	1000 ml

ii. Nutrient glucose agar

Beef extract	3.0 g
Bactopeptone	5.0 g
Glucose	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Tap water	1000 ml

iii. Potato-peptone-glucose-agar medium (PPGA)

Potato extract	500.0 ml
Peptone	5.0 g
Glucose	5.0 g
Sodium chloride	3.0 g
Sodium monohydrogen phosphate (Na_2HPO_4)	3.0 g
Potassium monohydrogen phosphate (K_2HPO_4)	0.5 g
Agar	18.0 g
Water	500 ml

Potato extract is prepared by placing peeled pieces of potato (200 g) in muslin cloth, suspending in water (500 ml), and boiling for 10 min.

iv. Tetrazolium medium (TTC) (Kelman, 1954)

Dextrose	10.0 g
Peptone	10.0 g
Cis amino acids	1.0 g
Agar	18.0 g
Water	1000 ml

The basal medium in 200 ml aliquots is sterilized at 1.06 Kg/cm^2 for 20 min. Prepare tetrazolium chloride solution by dissolving 1.0 g of 2,3,5-triphenyl tetrazolium chloride in 100 ml of distilled water and sterilize at 121°C for 8 min and store in darkness. Add 1 ml of this solution to 200 ml of basal medium to yield 0.05% concentration before pouring the melted medium into the petri dishes.

v. Medium 523 (Kado, 1971)

Sucrose	10.0 g
Casein acid hydrolysate	8.0 g
Yeast extract	4.0 g
Potassium monohydrogen phosphate (K_2HPO_4)	2.0 g
Magnesium sulfate	0.3 g
Agar	15.0 g
Water	1000 ml

vi. Brinkerhoff medium (Brinkerhoff, 1960)

Dextrose	20.0 g
Potassium monohydrogen phosphate (K_2HPO_4)	50.0 g
Calcium carbonate	10.0 g
Agar	15.0 g
Water	1000 ml

vii. Wakimoto's medium (Wakimoto, 1960)

Potato	200.0 g
Sucrose	15.0 g
Peptone	5.0 g
$Na_2HPO_4 \cdot 12\ H_2O$	2.0 g
$Ca\ (NO_3)_2$	0.5 g
Water	1000 ml

viii. Dye's medium (Dye, 1962)

Glucose	10.0 g
K_2HPO_4	2.0 g
Ammonium phosphate	1.0 g
$MgSO_4$	0.2 g
NaCl or KCl	0.2 g
Water	1000 ml
pH	7.0

3
Symptoms of Plant Diseases

3.1 EXTERNAL SYMPTOMS

Plants are attacked by different groups of pathogens individually or sometimes by more than one pathogen-producing complex and more severe disease. The type of external symptoms can, in most cases, indicate the nature of the pathogen responsible for the disease. Fungi, bacteria, viruses, and mycoplasma-like organisms cause distinct types of symptoms in most host–pathogen interactions. However, there are some diseases which show similarity in symptoms, though they are induced by different groups of pathogens. Further careful examination under microscope or by other methods may be necessary to establish the nature of the pathogen(s). Internal symptoms, such as histological and cytological changes caused by viruses, have diagnostic value, facilitating the identification of viruses in certain host–virus combinations.

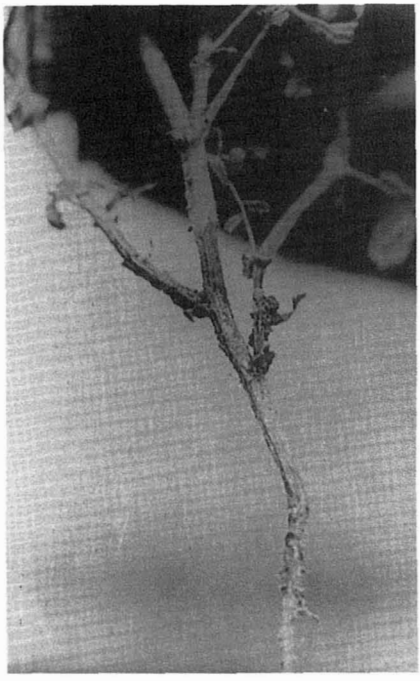
3.1.1 Symptoms Induced by Fungal Pathogens

Fungal pathogens may cause local or systemic symptoms. Fungal infections are generally found to be restricted to infected organs, such as leaves, stem, or flowers. In some cases they cause symptoms on different plant parts, when they reach the vascular tissues. Generally the affected tissues show necrosis or rotting, and in some cases hypoplasia or stunting of plant organs or whole plants or hyperplasia or excessive growth resulting in malformation or modification of organs or the entire plant.

The following are the common symptoms induced by fungal pathogens: root rot, collar rot, stem rot, stem canker, gummosis, club root, galls, warts, blight, blast, leaf spot, shot holes, anthracnose, rusts, powdery mildews, fruit

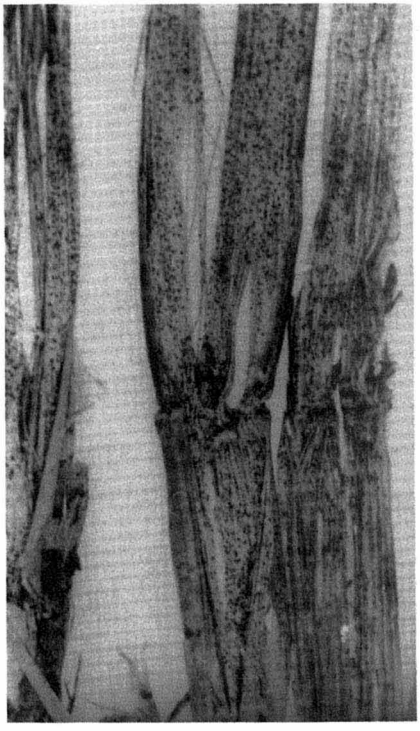
rot, capsule rot, and head rot. These symptoms are generally restricted to the tissues or organs in which infection is initiated. Damping off, wilt, smut, and downy mildew may be due to the systemic infection of plants by pathogens. These symptoms are distinct and indicate that involvement of fungal pathogens (Figs. 3.1–3.4).

The fungi produce specialized asexual spore forms such as sporangia or conidia either on the surface of the infected tissues or within such tissues in spore-forming structures, such as sorus, acervulus, pycnidia, or sporodochia. By using a compound microscope the characteristics of the spores may be studied in detail. At later stages of disease development or in pure cultures of the pathogen, sexual spores may be formed. The characters of sexual and asexual spores are primarily used in the identification and classification of fungal pathogens. For more details on taxonomy and classification of fungi, refer to Bold et al. (1980).

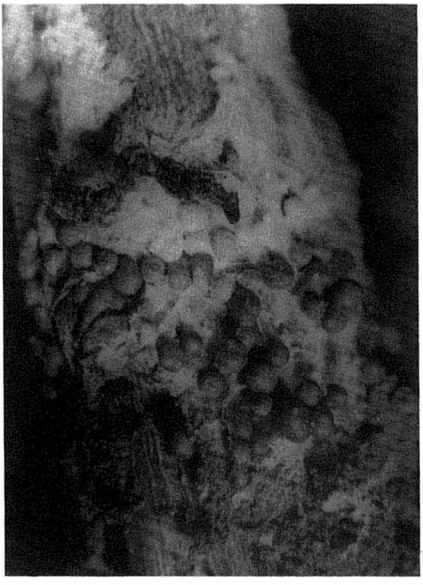


(A)

Figure 3.1 A, Chickpea root rot (*Rhizoctonia solani*) (ICRISAT); B, rice stem rot (*Sclerotium oryzae*) (IRRI); C, pepper (chili) stem rot (*Sclerotium rolfsii*) (AVRDC).



(B)

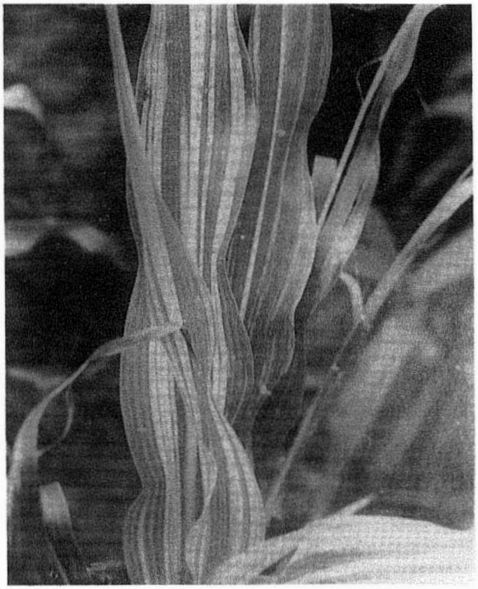


(C)

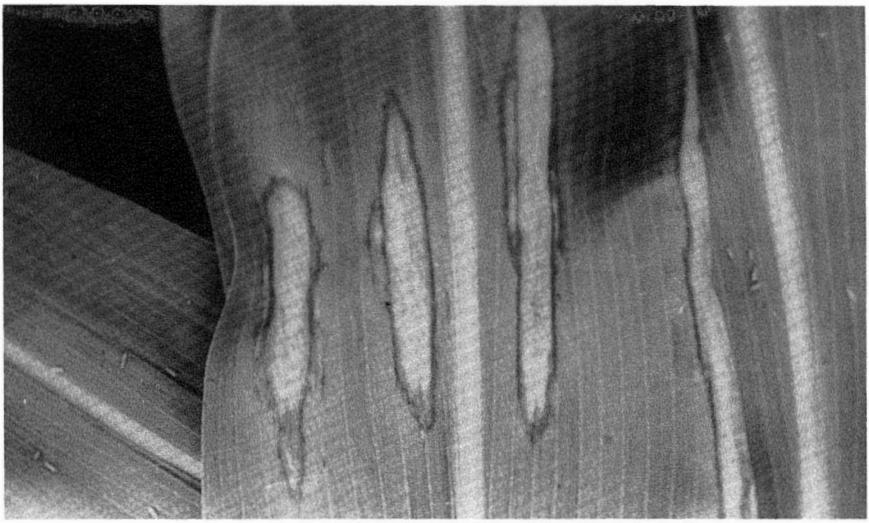
Figure 3.1 Continued

The general outline for the classification of fungi including slime molds up to the level of subclass is as follows (after Bold et al., 1980):

Kingdom Mycetate
Division 1 Gymnomycota
  Subdivision 1 Acrasiogymnomycotina
    Class 1 Acrasiomycetes
  Subdivision 2 Plasmodiogymnomycotina
    Class 1 Protosteliomycetes
    Class 2 Myxomycetes
Division 2 Mastigomycota
  Subdivision 1 Haplomastiogmycotina
    Class 1 Chytridiomycetes
    Class 2 Hyphochytridiomycetes
    Class 3 Plasmodiophoromycetes



(A)



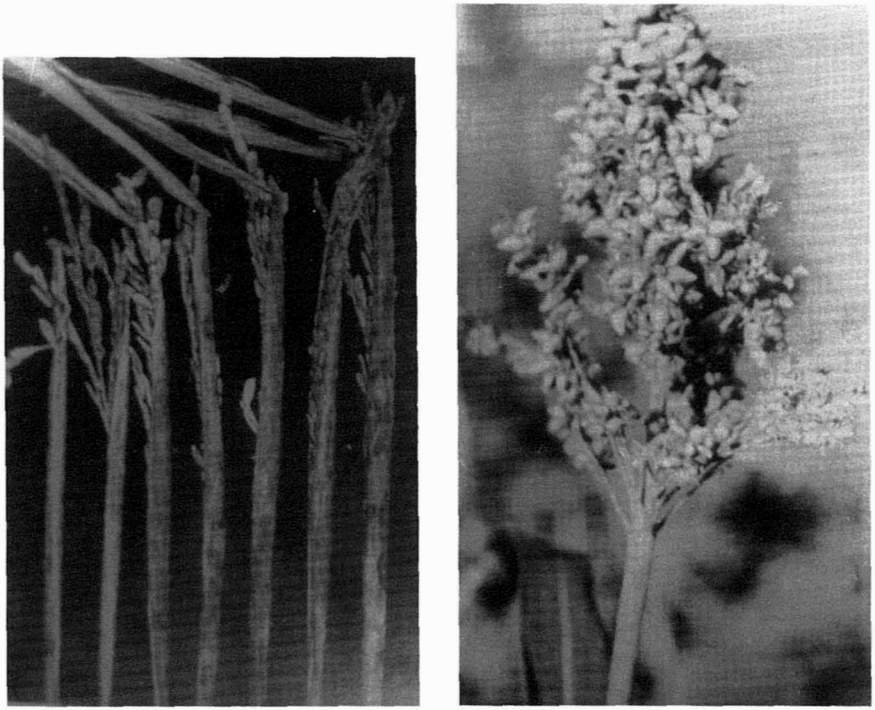
(B)

Figure 3.2 A, sorghum downy mildew (*Sclerospora sorghi*) (ICRISAT); B, sorghum leaf blight (*Exserohilum sorghi*) (ICRISAT).

Subdivision 2 Diplomastigomycotina
Class 1 Oomycetes
Division 3 Amastigomycota
Subdivision 1 Zygomycotina
Class 1 Zygomycetes
Class 2 Trichomycetes
Subdivision 2 Ascomycotina
Class 1 Ascomycetes
Subclass 1 Hemiascomycetidae
Subclass 2 Plectomycetidae
Subclass 3 Hymenoascomycetidae
Subclass 4 Laboulbenimycetidae
Subclass 5 Loculoascomycetidae
Subdivision 3 Basidiomycotina
Class 1 Basidiomycetes
Subclass 1 Holobasidiomycetidae
Subclass 2 Phragmobasidiomycetidae
Subclass 3 Teliomycetidae
Subdivision 4 Deuteromycotina
Form class 1 Deuteromycetes
Form subclass 1 Coelomycetidae
From subclass 2 Hyphomycetidae
Form subclass 3 Agonomycetidae

3.1.2 Symptoms Induced by Bacterial Pathogens

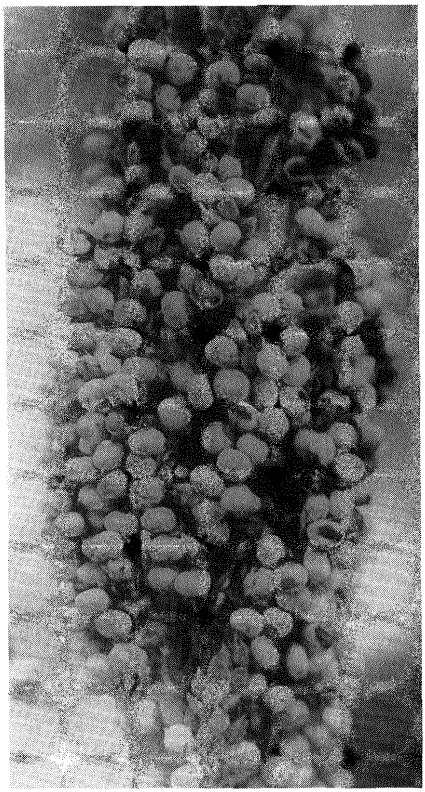
Pathogenic bacteria characteristically induce water-soaked lesions in the infected tissues at the initial stages, and these lesions turn necrotic later. Formation of encrustations or bacterial ooze from infected tissues is another distinguishing feature associated with bacterial diseases. As the infection progresses, leaf spots, blights, scabs, cankers, tumors, wilts, and soft rots of fruits, tubers, and roots may be the prominent types of symptoms caused by bacterial pathogens (Figs. 3.5 and 3.6). Although some symptoms, such as leaf spot and blight, may have similarities with those due to fungal pathogens, microscopic examination will provide a definite indication of the nature of the pathogen. The absence of spores and fungal structures and presence of characteristic bacterial ooze from the cut ends of the tissue under examination may indicate that the disease is likely to be due to bacteria. Isolation of the bacterium using appropriate medium and study of other properties determined by biochemical tests (Chapter 6) are necessary for the identification of the causative bacterium. Detailed information regarding the cultural characteristics and different tests to be done is provided by Schaad (1988). The general outline for the classification of bacteria (Bergey's Manual of Determintive Bacteriology, 1957; see Breed et al., 1957) is as follows:



(A) (B)

Figure 3.3 A, Rice sheath rot (*Sarocladium oryzae*) (IRRI) B, sorghum kernel smut (*Sphacelotheca sorghi*) (ICRISAT); C, sorghum grain molds (*Curvularia lunata*, *Fusarium moniliforme*, *F. semitectum*) (ICRISAT).

Division I	Protophyta Sachs, emend Krassilnikov
Class I	Schizophyceae Cohn
Class II	Schizomycetes von Naegeli (Bacteria and related forms)
Order I	Pseudomonadales Orla-Jensen
Order II	Chlamydobacteriales Buchanan
Order III	Hyphomicrobiales Douglas
Order IV	Eubacteriales Buchanan
Order V	Actinomycetales Buchanan
Order VI	Caryophanales Peshkoff
Order VII	Beggiatoales Buchanan
Order VIII	Myxobacteriales Jahn
Order IX	Spirochaetales Buchanan
Order X	Mycoplasmatales Freundt



(C)

Figure 3.3 Continued

Class III	Microtatobiotes Philip
Order I	Rickettsiales Buchanan and Buchanan emend. Gieszczykiewciz
Order II	Virales Breed, Murray and Hitchens
Division II	Thallophyta Endlicher
Division III	Bryophyta Haeckel
Division IV	Pteriodophyta Haeckel
Division V	Spermatophyta Goebel

Order Pseudomonadales

Suborder I Rhodobacterineae—cells contain red, purple, brown, or green photosynthetic pigments; sometimes granules of free sulfur are also enclosed.

Suborder II Pseudomonadineae—cells do not contain photosynthetic pigments but may produce water-soluble pigments.

(A) (B)

Figure 3.4 A, Pepper (chili) fruit rot (*Colletotrichum gloeosporioides*, *C. capsici*, *C. acutatum*, *C. coccodes*); (AVRDC); B, fruiting structures (acervuli) of *Colletotrichum* spp. in concentric rings (AVRDC).

Suborder Pseudomonadineae

There are 7 families under this suborder.

Family Pseudomonadaceae—cells are not attached to substrate; cells are frequently oxidative, sometimes fermentative, and usually heterotrophic.

Family I Pseudomonadaceae

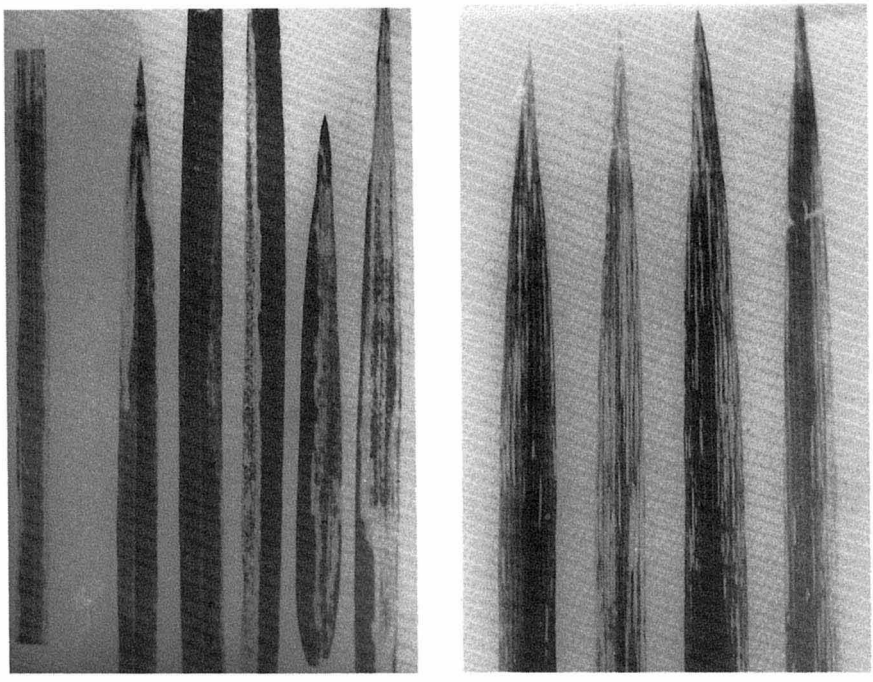
There are 12 genera under this family.

Genus *Xanthomonas*—cultures have a yellow, non-water-soluble pigment. Cells are normally monotrichous, gram-negative; many species cause necrosis.

Genus *Pseudomonas*—cultures may frequently produce water-soluble pigment, which may be differently colored—green, brown, yellow, rose, or lilac; cells monotrichous, lophotrichous, or nonmotile; gram-negative.

Family II Rhizobiaceae

Cells are rod-shaped, sparsely flagellated, with one polar or lateral flagellum or 2–4 peritrichous flagella; usually gram-negative.



(A) (B)

Figure 3.5 A, Rice bacterial leaf blight (*Xanthomonas campestris* pv. *oryzae* (IRRI); B, rice bacterial leaf streak (*Xanthomonas campestris* pv. *oryzae transluscens*) (IRRI).

Genus Agrobacterium
Cells are rod-shaped with 1–4 peritrichous flagella; gram-negative.

Family III Enterobacteriaceae
Cells are straight rods, petritrichous or nonmotile, gram-negative. All species attack glucose-producing acid or acid and visible gas; produce nitrite from nitrates.

Genus *Erwinia*
Cells are rod-shaped; normally do not require organic nitrogenous compounds for growth. In plants, members produce dry necrosis, galls, wilts, and soft rots.

Family IV Corynebacteriaceae
Cells are usually nonmotile, frequently banded or beaded with metachromatic granules; generally gram-positive.

Genus *Corynebacterium*
Cells are straight or slightly curved rods with irregularly stained segments or granules, usually nonmotile, gram-positive.

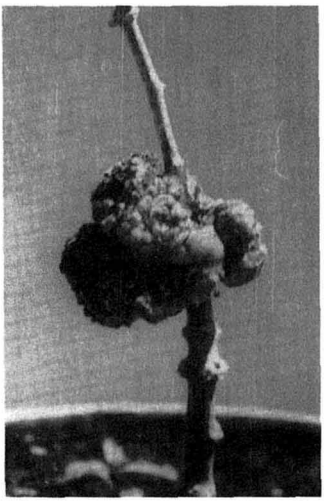


Figure 3.6 Cassava bacterial stem gall (*Agrobacterium tumefaciens*).

Family V Streptomycetaceae
Colony forms vegetative mycelium which does not fragment into individual cells; conidia are formed on sporophores.

Genus *Streptomyces*
Branched mycelium, which is aerial in nature, produces conidiophores bearing conidia in chain.

3.1.3 Symptoms Induced by Mycoplasma-Like Organisms

Although the symptoms induced by MLOs have some similarity to those caused by viruses, there are certain characteristic symptoms that may be useful to differentiate the diseases due to MLOs and those due to viruses. The MLOs cause general stunting or dwarfing of affected plant parts or whole plants. Chlorosis and smalling of leaves are also frequently observed in affected plants. Antholysis of floral parts is the most characteristic feature of MLO diseases, it results from virescence, phyllody, and prolification of floral tissues. Floral parts are transformed into green leaflike structures. Partial or total sterility of infected plants may be commonly noted. These symptoms are observed in plants infected by diseases such as aster yellows, eggplant little leaf, sesamum phyllody, and witches' broom disease of potato, peanut (groundnut), and grain legumes. Proliferation of axillary buds and formation of a large number of thin shoots are observed prominently in eggplant (brinjal) little leaf, rice yellow dwarf, and sugarcane grassy shoot diseases (Fig. 3.7). Reduction in leaf size

(A) (B)

Figure 3.7 A, Yellow dwarf MLO-infected rice plant; B, little leaf MLO-infected eggplant.

and internodal length and a tendency for the leaves to stand out stiffly, giving a spikelike appearance to the infected branches, are the distinguishing symptoms of sandal spike disease. Tomato big bud disease is characterized by hypertrophy of floral parts, leading to upright disposition of flower buds, which remain swollen and unopened. The general outline for the classification of mycoplasmas is as follows:

Taxonomy of Mycoplasmas (Archer and Daniels, 1982)
Class: Mollicutes Order: Mycoplasmatales
Family I Mycoplasmataceae

i) Sterol required for growth
ii) Genome size about 0.5×10^9 Da
iii) Reduced nicotinamide-adenine dinucleotide (NADH) oxidase localized in cytoplasm

Genus I *Mycoplasma* (about 50 species)
- i) Do not hydrolyze urea

Genus II *Ureaplasma*
- i) Hydrolyzes urea (one species with many serotypes)

Family II Acholeplasmataceae
- i) Sterol not required for growth
- ii) Genome size about 1.0×10^9 Da
- iii) NADH oxidase localized in membrane

Genus *Acholeplasma* (6 species)

Family III Spiroplasmataceae
- i) Helical cells formed during some phase of growth
- ii) Sterol required for growth
- iii) Genome size about 1.0×10^9 Da
- iv) NADH oxidase localized in cytoplasm

Genus *Spiroplasma* (one species)

Genera of uncertain taxonomic position:
- I *Thermoplasma* (one species)
- II *Anaeroplasma* (two species)

3.1.4 Symptoms Induced by Viruses

Plant viruses cause a variety of symptoms, depending on the host plant species, and different unrelated viruses may induce similar symptoms in the same host plant species. Dependence on the symptoms alone for the identification of viruses may lead to erroneous conclusions. However, information on host range and the reactions of diagnostic or differential hosts has been used for the identification of some viruses which have not been purified and adequately characterized.

The viruses induce primary symptoms on inoculated leaves, which exhibit chlorotic or necrotic local lesions or vein clearing. Later, when the virus becomes systemic, secondary symptoms develop on other plant parts. The secondary symptoms may be grouped as color changes, teratological symptoms, death or necrosis, and abnormal growth forms. Color changes may vary from mosaic on leaves to color breaking in flowers. Various kinds of changes in size and shape of plant part may be seen as leaf roll, leaf curl, enations, leaf crinkle, galls, and tumors (Figs. 3.8–3.10). Necrosis may be localized, as in chlorotic or necrotic lesions or ringspots on leaves or streaks on stems. Extensive necrosis may occur in phloem tissue, resulting in various growth abnormalities and gum formation. The plant species that react with local lesions have been extensively used for detection, identification, and assay of plant viruses, especially those for which nonbiological assay methods are either not available or difficult to perform (Table 4.2). The virus infection may result in conspicuous changes in the general growth and appearance of infected plants. General stunt-

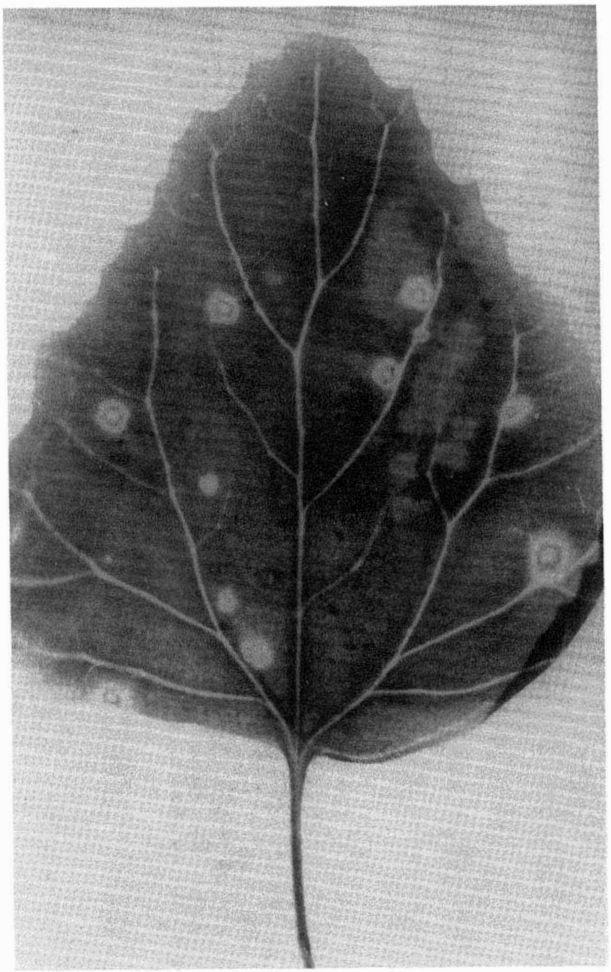
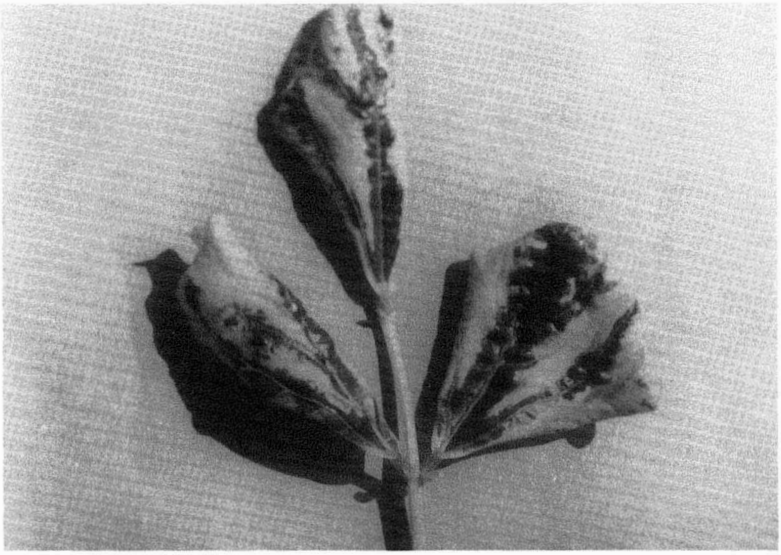


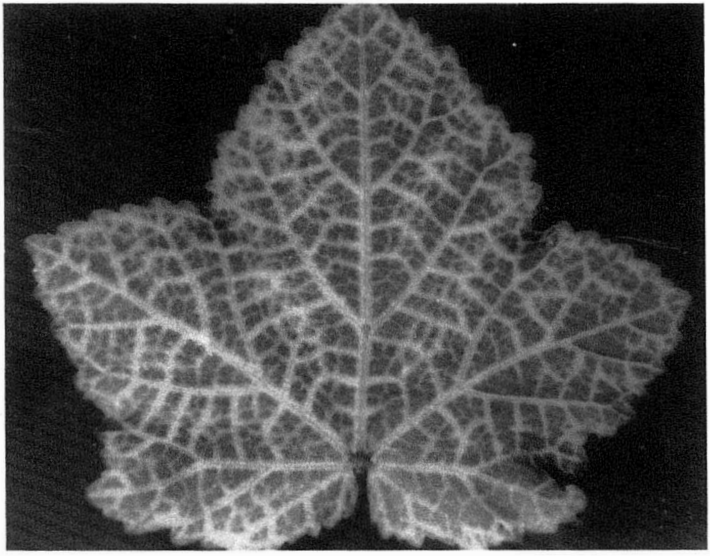
Figure 3.8 Local lesions induced by cowpea aphid-borne mosaic virus on *Chenopodium amaranticolor*.

ing of branches for entire plants, rosette nature of leaves, and bunching or crowding of leaves at the apex leading to bunchy top are some of the symptoms associated with viruses. For more details Bos (1970a) and Narayanasamy and Sabitha Doraiswamy (1996) may be consulted.

Plant viruses may be isolated from plants either infected naturally or inoculated artificially by different methods when the symptoms are expressed. Different methods of purification are followed, and they may be grouped on



(A)



(B)

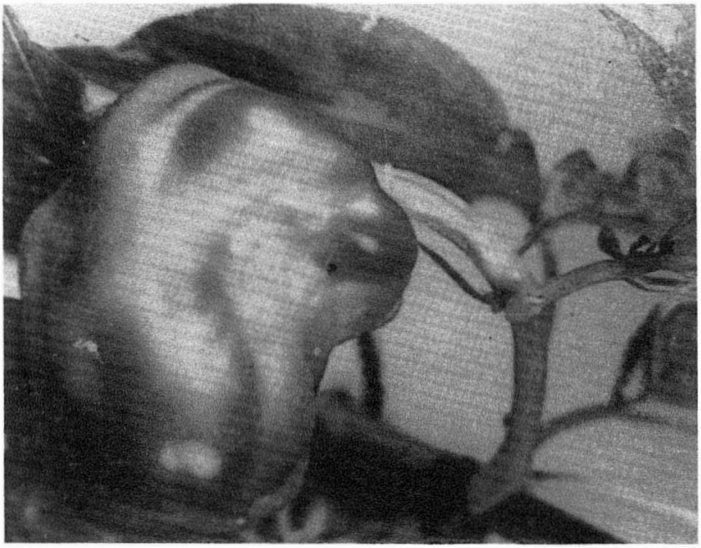
Figure 3.9 A, Vein banding caused by cowpea aphid-borne mosaic virus on cowpea CV 152; B, vein clearing caused by yellow vein mosaic virus of okra.

the basis of structural, physical, chemical, and serological properties. The particle morphological characteristics, presence or absence of envelope, nature of viral genome, and strandedness of viral nucleic acid are some of the important characters used for grouping them. Plant viruses are classified into different groups, each having a type member, definitive members, and possible members that are not fully characterized (Fig. 3.11).

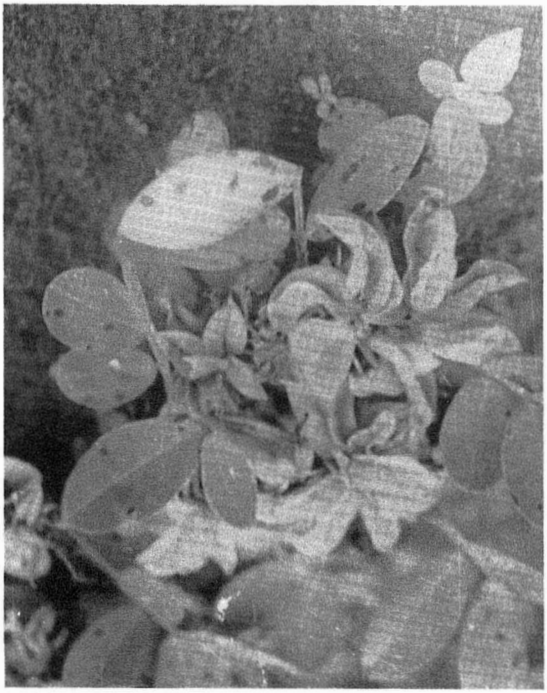
I. Nonenveloped viruses
   A. Single-stranded (ss) RNA, monopartite genome
      Isometric particles
      1. *Tymovirus* group—turnip yellow mosaic virus
      2. *Luteovirus* group—barley yellow dwarf virus
      3. Tombus virus group—tomato bushy stunt virus
      4. *Sobemovirus* group—southern bean mosaic virus

(A)

Figure 3.10 A, Yellow leaf curl virus-infected tomato (AVRDC); B, tomato spotted wilt virus-infected pepper fruit (AVRDC); C, rosette virus complex disease of peanut (ICRISAT).



(B)



(C)

Figure 3.10 Continued

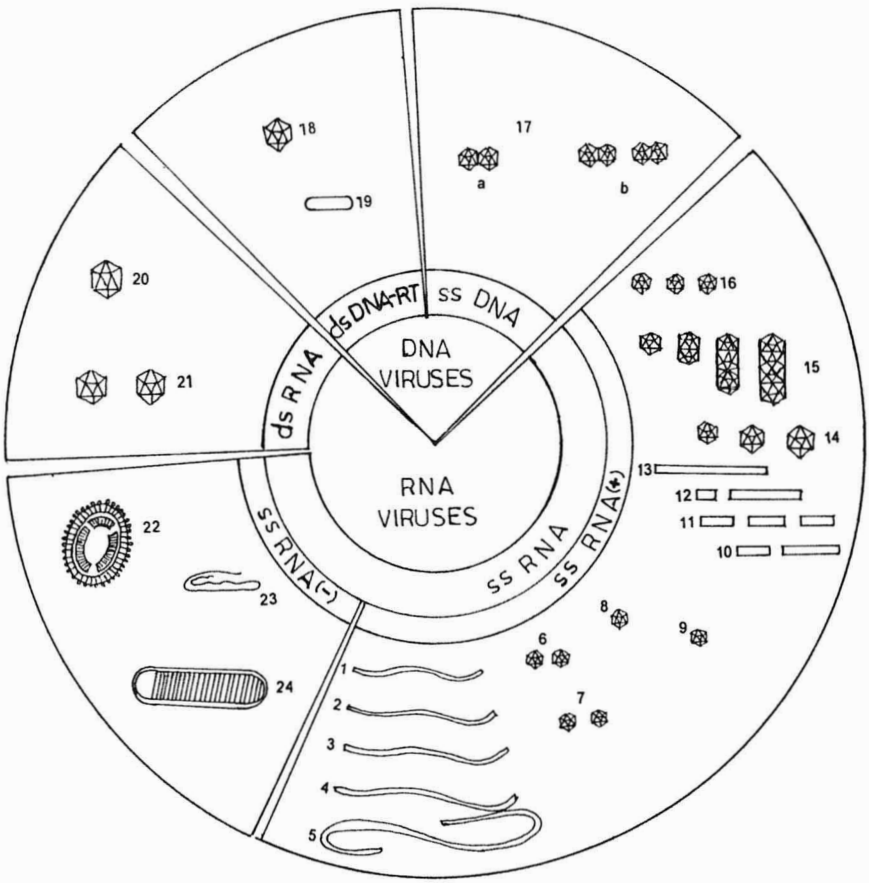


Figure 3.11 Plant virus groups/families: 1, *Potexvirus*; 2, *Capillovirus*; 3, *Carlavirus*; 4, *Potyvirus*; 5, *Closterovirus*; 6, *Enamovirus*; 7, Comoviridae; 8, Tombusviridae; 9, *Dinathovirus*, *Luteovirus*, *Machlovirus*, *Marafivirus*, *Necrovirus*, *Sobemovirus*, and *Tymovirus*; 10, *Furovirus*; 11, *Hordeivirus*; 12, *Tobravirus*; 13, *Tobamovirus*; 14, *Ilarvirus*; 15, *Alfamovirus*; 16, Bromoviridae—*Cucumovirus* and *Bromovirus*; 17, *Geminivirus*—a, subgroups I and II; b, subgroup III; 18, *Caulimovirus*; 19, *Badnavirus*; 20, *Reoviridae*; 21, *Alphacryptovirus*, *Betacryptovirus*; 22, Bunyaviridae—*Tospovirus*; 23, *Tenuivirus*; 24, Rhabdoviridae.

5. *Necrovirus* group—tobacco necrosis virus
6. *Machlovirus* group—maize chlorotic dwarf virus
7. *Carmovirus* group—carnation mottle virus
8. Cocksfoot mild mosaic virus group—cocksfoot mild mosaic virus
9. *Marafivirus* group—maize rayado fino virus

10. Parsnip yellow fleck virus group—parsnip yellow fleck virus
11. Satellite virus group—tobacco necrosis satellite virus

Rod-shaped virus particles

12. *Closterovirus* group
    Subgroup 1—beet yellows virus
    Subgroup 2—apple chlorotic leafspot virus
13. *Carlavirus* group—carnation latent virus
14. *Potyvirus* group
    Subgroup 1 (aphid-transmitted)—potato virus Y
    Subgroup 3 (mite-transmitted)—wheat streak mosaic virus
    Subgroup 4 (whitefly-transmitted)—sweet potato mild mottle virus
    Subgroup 2 (fungus-transmitted)—barley yellow mosaic virus
15. *Potexvirus* group—potato virus x
16. *Tobamovirus* group—tobacco mosaic virus
17. *Capillovirus* group—potato virus T

B. ss-RNA, bipartite genome

Isometric particles

18. Nepovirus group—tobacco ringspot virus
19. Pea enation mosaic virus group—pea enation mosaic virus
20. *Comovirus* group—cowpea mosaic virus
21. *Dianthovirus* group—carnation ringspot virus
22. Fabavirus group—broadbean wilt virus

Rod-shaped particles

23. Tobravirus group—tobacco rattle virus
24. Barley yellow mosaic virus group—barley yellow mosaic virus
25. *Furovirus* group—soil-borne wheat mosaic virus

C. ss-RNA, tripartite/multipartite genome

Isometric particles

26. *Cucumovirus* group—cucumber mosaic virus
27. *Bromovirus* group—brome mosaic virus
28. *Ilarvirus* group—tobacco streak virus

Isometric and bacilliform particles

29. Alfalfa mosaic virus group—alfalfa mosaic virus

Rod-shaped particles

30. *Hordeivirus* group—barley stripe mosaic virus
31. *Tenuivirus* group—rice stripe virus

D. Double-stranded (ds) RNA

Isometric particles

32. Reoviride (Family)
    Subgroup 1 *Phytoreovirus* (genus) wound tumor virus (type member)
    Subgroup 2 *Fijivirus* (genus) Fiji disease virus (type member)
    Subgroup 3 rice ragged stunt virus (type member)
33. Cryptovirus group
    Subgroup A white clover cryptic virus 1
    Subgroup B white clover cryptic virus 2

E. ds-DNA
Isometric particles
34. *Caulimovirus* group—cauliflower mosaic virus
F. ss-DNA
Isometric particles
35. *Geminivirus* group—maize streak virus
II. Enveloped viruses
A. ss-RNA
Bacilliform particles
36. Rhabdoviridae (Family)
Subgroup 1 (cytoplasma-associated) lettuce necrotic yellow virus
Subgroup 2 (nucleus-associated) potato yellow dwarf virus
Spherical particles
37. Bunyaviridae (family)
Tospovirus group—tomato spotted wilt virus

Detailed descriptions of the characteristics of different virus groups are provided in CMI AAB Descriptions of Plant Viruses.

3.1.5 Symptoms of Viroid Diseases

Plant viroid diseases do not exhibit any specific symptoms that can be used to differentiate them from diseases caused by viruses. This may be a possible reason why this group of disease-causing agents could not be recognized prior to the report of Diener (1971a) on potato spindle tuber disease. However, individual viroid diseases can be identified by the symptoms on given plant species. Infected potato plants may show different degrees of stunting with foliage turning slate gray with dull leaf surface. But the characteristic symptom of the disease is seen on the tubers, which are abnormally elongated, assuming spindle or cylindrical shape with prominent eyes (Diener, 1979).

Tomatoes infected by bunchy top viroid are markedly stunted. Smalling and distortion of leaflets, necrosis of leaves and stems, and crowding of leaves near the apices of branches are the other symptoms induced by this viroid (McClean, 1931).

Citrus exocortis disease is characterized by scaling of the bark below the graft union and stunting of the trees. Trifoliate orange (*Poncirus trifoliata*), and *Citrus* spp. and varieties may be used to detect latent infection (Benton et al., 1950; Olson, 1968). Chrysanthemum plants infected by stunt viroid show severe stunting, reduction in leaf size, and paling of foliage as the chief symptoms (Dimock, 1947). Chrysanthemum chlorotic mottle viroid, on the other hand, induces mild mottling or variegation of young leaves. The cv. 'Deep Ridge' can be used as the diagnostic host for this viroid (Horst, 1975).

The diagnostic symptoms of cucumber pale fruit disease are observed on fruits, which become pale, shorter, and slightly pear-shaped. The flowers are stunted and crumpled. The leaves are malformed and become chlorotic later (van Dorst and Peters, 1974).

Coconuts affected by cadang-cadang disease have small irregularly shaped lamina with bright yellow or orange spots. As the discolored spots coalesce, older leaves may show mottling or turn yellow and become brittle. The nuts become progressively smaller in size year after year, elongated, or distorted. Nut-bearing capacity is progressively reduced as the crown size is reduced with cessation of flower production. Ultimately the growing bud dies and falls off, leaving bare trunk (Price, 1971).

Hop stunt disease is recognized by shortened internodes of the main and lateral branches and curling of upper leaves (Sasaki and Shikata, 1977a).

3.2 CYTOPATHOLOGICAL CHARACTERISTICS

3.2.1 Fungal Diseases

Pathogenic fungi gain entry into susceptible host plants through natural openings or by direct penetration of the epidermal cells. Most of the obligate parasites that cause powdery mildews, downy mildews and rust absorb nutrients from the cells into which haustoria alone are formed, keeping the host cells alive for longer periods. Facultative parasites, on the other hand, produce many kinds of biologically active substances, such as enzymes, toxins, growth regulators, polysaccharides, and antibiotics. The substances may affect the cells directly or cause structural changes.

The fungi produce characteristic spore-bearing structures which arise from the internal mycelium and emerge either through stomata or by piercing of the epidermis. Most of the pathogens that cause downy mildews, powdery mildews, rusts, leafspots, and blight can be identified by examining the sporangia or conidia which are formed either on free conidiophores or in specialized structures such as sori, acervuli, or pycnidia. The pathogens that cause damping off and root rots produce macerating enzymes that produce extensive structural breakdown in affected tissues. The mycelium and spore-bearing structures are formed in these tissues. The wilt pathogens are found in the conducting vessels. They produce toxic metabolites and enzymes, which may break down the cell walls, leading to death of cells and plugging of vessels. Fungal structures, such as mycelium, microconidia, and macroconidia, may be present in the affected tissues.

Anatomical research will be useful in studying the characteristics of fungi present in the tissues of affected plants. However, there is hardly any specific

change induced in infected tissue by fungal pathogen that may have diagnostic value for the identification of the pathogen that is causing the disease in question.

3.2.2 Bacterial Diseases

After the entry of bacteria through wounds or natural openings in susceptible plants, bacteria that cause diseases such as soft rots (*Erwinia* spp.), fire blight of apple (*E. amylovora*), and wild fire of tobacco (*Pseudomonas tabaci*) live and reproduce in the intercellular spaces for some time. Then they secrete enzymes capable of breaking down the middle lamella and macerating the tissues, leading to loss of turgor, cell collapse, and ultimate death of cells. The toxins produced by these pathogens hasten the loss of water and electrolytes from the cells. As the cells are killed in large numbers, bacterial ooze may appear on the leaf surface. Lysigenous cavities may be formed as a result of breakdown of cell walls, and the cavities are filled with cellular contents and masses of bacterial cells. Cankers may arise from the cavities formed in the cortical tissues.

Some pathogenic bacteria that cause wilts invade the conducting vascular tissues, in which they are able to reproduce rapidly and spread to other organs or tissues. They secrete enzymes, toxins, or slimy extracellular polysaccharides (EPSs). These metabolites may act on the cells of vascular tissue, leading to the breakdown of cells and accumulation of cellular materials. This may cause clogging of vessels, resulting in reduction in or complete blockage of translocation of water and nutrients. The infected plants show progressive stunting, wilting, and death.

Excessive cell division (hyperplasia) and cell enlargement (hypertrophy) are the characteristic features of crown gall disease caused by *Agrobacterium tumefaciens*. Infection by this pathogen results in galls or tumors on roots, stem, and other organs. In the infected plants, diversion of nutrients to the tumor or gall tissues occurs, and normal tissue development and other essential processes are hampered. As a result, the growth and consequently the yield of affected plants are appreciably reduced.

3.2.3 Virus Diseases

Plant viruses induce characteristic macroscopic or external symptoms (see Chapter 3.1.4) that may be of diagnostic value. So also some plant viruses induce distinct histological changes in infected plants, and such changes may help to differentiate the virus in question from other pathogens that may infect the same host plant species.

3.2.3.1 Anatomical changes

Viruses such as potato leaf foll virus and sugar beet curly top virus cause phloem degeneration. Necrosis is confined to primary phloem in potatoes infected by leaf roll virus and no abnormal growth of phloem tissue is seen. There is excessive deposition of callose in the phloem of stem and tubers. Presence of callose in high concentration may be observed by staining the cells with resorcin blue. Sugar beet curly top virus causes growth abnormalities in the initial stages of phloem degeneration and necrosis of primary and secondary phloem, leading to cell collapse and lesion formation.

Grapevine leaf roll virus induces a characteristic structural abnormality in vascular tissues. Formation of tuberculae, cellulose bars and rods that traverse the lumens of interfascicular and phloem parenchyma cells, is a diagnostic symptom of this disease (Bos, 1970a). Crimson clover wound tumor disease is characterized by the abnormal development of phloem cambial cells. Phloem parenchyma forms meristematic tumor cells in the phloem of leaf, stem, and root (Lee and Black, 1955).

Pitting of the wood in apple stem pitting disease is due to the failure of some cambial initials to differentiate into normal cells. A wedge of phloem tissue is formed and becomes embedded in the newly formed xylem tissue. Later the phloem tissue becomes necrotic (Hilborn et al., 1965).

3.2.3.2 Cytological changes

Virus infections result in cytological effects such as starch accumulation, inhibition of plastid development, and chloroplast destruction. But a virus-specific effect leading to the formation of intracellular inclusions is observed in certain host plants after infection by viruses. These inclusions have characteristic features useful for the identification of viruses. The inclusions may be formed in either the cytoplasm or the nucleus of infected cells and may be either amorphous or crystalline in nature. Among the 49 criteria listed for the classification of plant viruses, characteristics of inclusion bodies and their intracellular location are included (Harrison et al., 1971). Members of 20 virus groups are known to induce inclusions which form one of the main characteristics of the respective virus group. These inclusions are useful for diagnostic and taxonomic purposes and for identification and characterization of virus-specific, noncapsid proteins and possible sites of viral synthesis (Hiebert et al., 1984).

Viruses which produce characteristic inclusions may be identified by following simple techniques. The inclusions are present in large numbers in the epidermal cells of leaves showing distinct symptoms of virus infection. Epidermal strips without fixing are stained with trypan blue, mounted in water, and examined under the microscope. Trypan blue is dissolved in hot 0.9% aque-

ous NaCl to yield a 0.5% solution, and this stock solution may be diluted in 0.9% NaCl to produce 1/2000–1/5000 dilution at the time of examination. Amorphous inclusions are deeply stained, whereas nuclei have less intense color (McWhorter, 1941b). Phloxine (1%) is recommended by Rubio-Huertos (1950). Inclusion bodies appear as bright red structures, and nuclei stain pink. A combination of pyronin (0.2%) and methyl green (0.5%) in 0.1 *M* acetate buffer, pH 5.3, can be used to stain the inclusions, differentially. Nuclei turn blue and inclusions are red, because of their chemical constitution. The blue color is due to the presence of DNA and the red to the presence of RNA (Rubio-Huertos, 1972). With different combinations of calcomine orange, Luxol brilliant green, Congo rubin-methyl green, phloxine, and methylene blue, Christie (1967) and Christie and Edwardson (1977) reported that inclusion bodies produced by tobacco mosaic virus, and tobacco etch virus could be differentiated. Christie et al. (1988) showed that by using two differential stains—Azure A and Luxol brilliant green–calcomine orange—characteristic inclusions induced by several viruses could be detected, and the causal virus may be identified by this simple technique. The maize dwarf mosaic virus, maize stripe virus, maize mosaic virus, and maize rayado fino virus could be detected and identified by examining the intracellular inclusions associated with infection by these viruses (Overman et al., 1992).

Intracellular inclusions have been examined in plant materials fixed with different fixatives. By using Dalton's fixative containing mercuric chloride and a small quantity of acetic acid, followed by washing with iodine in alcohol, excellent results have been obtained. Details regarding the composition of different fixatives and protocols are described in the review by Rubio-Huertos (1972).

By using Azure A and orange–green (calcomine orange 2 RS and Luxol brilliant green BL) combinations, the inclusions associated with viruses belonging to 17 virus groups, viz. bromovirus, carlavirus, caulimovirus, closterovirus, comovirus, cucumovirus, geminivirus, luteovirus, nepovirus, phytoreovirus, plant rhabdovirus, potexvirus, potyvirus, tobacco necrosis, tobamovirus, tombus virus, and tymovirus, can be detected in leaves infected by these viruses (Hiebert et al., 1984).

Cylindrical inclusions of many potyviruses, including tobacco etch virus (Hiebert and McDonald, 1973), bean yellow mosaic virus (Nagel et al., 1983), turnip mosaic virus (Hiebert and McDonald, 1973), and tobacco vein mottling virus (Hellman et al., 1983), have been purified. Hiebert et al. (1984) have developed a procedure for the purification of nuclear inclusions induced by tobacco etch virus. Viroplasms formed by cauliflower mosaic virus can be purified by the procedure developed by Al Ani et al. (1980).

Polyclonal antisera using rabbits have been raised against inclusion bodies induced by potyviruses and caulimoviruses. These antisera prepared against

nonstructural virus-associated proteins are useful in detecting their presence in different host plants. The immunodiffusion test (Purcifull et al., 1973), immunoflourescence test, ferritin-antibody electron microscopy (Breese and Hsu, 1971), liquid precipitin test (Hiebert et al., 1974), Western blotting, and radioimmunoassay (Towbin et al., 1979) and enzyme-linked immunosorbent assay (ELISA) (Falk and Tsai et al., 1987) have been employed for the detection of inclusion body proteins in various host plant species infected by different viruses.

As the cylindrical inclusion proteins, the amorphous inclusion proteins, and the TEV nuclear inclusion proteins account for about 70% of the total protein-coding capacity of the potyviral genome, the antisera raised against these inclusions may be used for more precise viral diagnosis and study of relationships between potyviruses. Antisera raised against virus capsid proteins represent only about 10% of potyviral protein-coding capacity, and such antisera have only limited value in establishing the relationship between potyviruses. Another advantage derived from the study of inclusion bodies, which are, in most cases, an aggregation of the virus particles inducing their formation, is the potential for purifying the viruses in a novel way as in the case of citrus tristeza virus (Lee et al., 1982).

Cytological changes induced by viruses may be studied in detail by examining ultrathin sections under an electron microscope (Chapter 7). In many host–virus combinations, specific or broad changes in ultrastructure of the cellular constituents, which represent the signature of the virus (McWhorter, 1965), may be discernible. Such cytopathological features of infected cells may be used as the basis for identification of virus groups or even individual viruses.

a. Tombus viruses. Two cytological changes occur together commonly in plants infected by tombus viruses: a) appearance of cytoplasmic multivesicular bodies and b) formation of virus-containing bleblike evaginations of the tonoplast into the vacuole (Martelli, 1981). Additionally the intranuclear presence of virus and membraneous inclusions may be observed in certain host–virus combinations.

b. Comovirususes and nepoviruses The members of the comovirus and nepovirus groups do not produce any distinct cytopathological change with which they can be distinguished. However, most of them produce vesiculate–vacuolate cytoplasmic inclusions, tubules containing rows of virus particles, and cell wall outgrowths. These changes are quite characteristic of these two groups and useful as intracellular markers of diagnostic value (Martelli and Russo, 1984). Another distinct feature of infection by como- and nepoviruses is the production of tubules enclosing virus particles. They are usually single-walled, but in some cases, for example, strawberry latent ringspot virus, double-walled tubules may be formed (Roberts and Harrison, 1970).

c. Tymoviruses. The tymoviruses form a homogeneous group and so also they induce group-specific, as well as virus-specific, cytological changes that may help in their identification. Clumping of altered chloroplasts and intranuclear accumulations of empty viral capsids are the two diagnostic markers of this group. Clumping of chloroplasts, first reported in chinese cabbage infected with turnip yellow mosaic virus (Rubio-Huertos, 1950), is observed in all hosts infected by tymoviruses. Periplastial flask-shaped vesicles bounded by a double membrane and containing finely stranded material appear as a very early change induced by these viruses (Gerola et al., 1966; Matthews, 1977). Empty virus protein shells, when produced excessively, move into the nucleus, forming large crystalline aggregates (Lesemann, 1977).

d. Luteoviruses. The luteoviruses do not appear commonly to cause any cytological changes that may be considered characteristic of this group. However, the presence of virus particles and membranous vesicles in the nuclear area and of virions in plasmodesmata has been noted consistently in infections by viruses that have been studied. Esau and Hoefert (1972) observed the accumulation of viruses around the nucleolus sometimes in crystalline array in the early stages of infection with beet western yellows virus (BWYV). The vesicles found in plants infected by BWYV and potato leaf roll virus (PLRV) are bound by a single or double membrane and have a network of fibrils resembling nuclei acid (Esau and Hoefert, 1972; Shepardson et al., 1980). On the basis of the ultrastructural differences induced, barley yellow dwarf virus (BYDV) strains can be divided into two distinct subgroups (Gill and Chong, 1979a).

e. Bromoviruses. The bromoviruses induce the production of inclusion bodies which can be recognized by light microscopy. The inclusion bodies produced by broadbean mottle virus (BBMV) consist of amorphous material, vesicles, and virions, and their locations may be the sites of viral RNA replication. Viral antigen is also present in the inclusions. The inclusion bodies formed by cowpea chlorotic mottle virus (CCMV) have a fine granular zone, proliferating endoplasmic reticulum, fibril-containing vesicles, and thin flexuous filaments. The CCMV particles are randomly scattered in the cytoplasm and do not aggregate into crystalline structures (Bancroft et al., 1969).

f. Cucumoviruses. Cytological alterations induced by cucumber mosaic virus have been studied in detail. The virus particles, usually found scattered in the cytoplasm, have a tendency to aggregate into crystals in the vacuoles (Fig. 3.12). The presence of viruses in the nuclei of many cells has been frequently observed (Honda and Matsui, 1974). The membrane-bound vesicles associated with the tonoplast may be the site of viral RNA replication (Hatta and Francki, 1981).

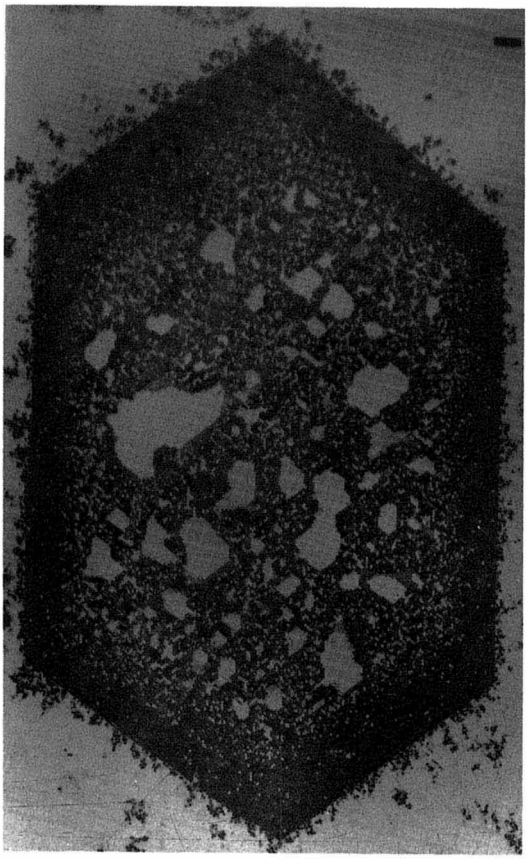


Figure 3.12 An intravacuolar virus crystal consisting of cucumber mosaic virus particles; bar represents 200 nm (Academic Press Inc., USA). (Courtesy of Martelli and Russo, 1984).

g. Sobemoviruses. No group-specific cytological change is induced by sobemoviruses. The virus particles may occasionally be found to aggregate, forming crystals. Two strains of the type member, southern bean mosaic virus, may be differentiated by their intracellular behavior: Large crystals are produced by cowpea strain in the cytoplasm and nuclei, mainly in infected cells near vascular tissues; on the other hand, the bean strain forms crystals very rarely, and only in phloem cells (Weintraub and Ragetli, 1970). Certain virus-specific changes, such as formation of flexuous tubules in rice yellow mosaic virus–infected plants (Bakker, 1975) and the network of densely stained material in sowbane mosaic–infected plants (Milne, 1967), have also been reported.

h. Dianthoviruses. Carnation ringspot virus (CRSV) induces characteristic cytological changes in infected cells. Large irregular crystalline aggregates are seen in the cytoplasm, nuclei, and nucleolus. The nuclei may also contain tubules, generally without any virus particles, but sometimes tubules may enclose a single row of virions.

i. Tobacco necrosis and satellite viruses. The intracellular behavior of the tobacco necrosis virus (TNV) and that of its satellite show distinct difference. The satellite virus forms large stable crystals in situ, whereas TNV particles are found scattered even when they are in high concentration. Probably the TNV crystals are quite unstable (Martelli and Russo, 1984).

j. Pea enation mosaic virus. Pea enation mosaic virus (PEMV), the only member of the PEMV group, induces specific cytopathological structures consisting of accumulations of single-membraned vesicles in the perinuclear space of infected cells. As the vesicles are released into the cytoplasm, an additional membrane derived from the outer lamella of the nuclear envelope is also formed around the vesicles, which have their origin on the virus nuclear membrane (DeZoeten et al., 1972). Though the vesicular aggregates may resemble those of some luteoviruses, PEMV aggregates are larger than luteovirus aggregates, which may sometimes occur in groups. The PEMV may also occasionally form paracrystalline inclusions in the endoplasmic reticulum (Rassel, 1972).

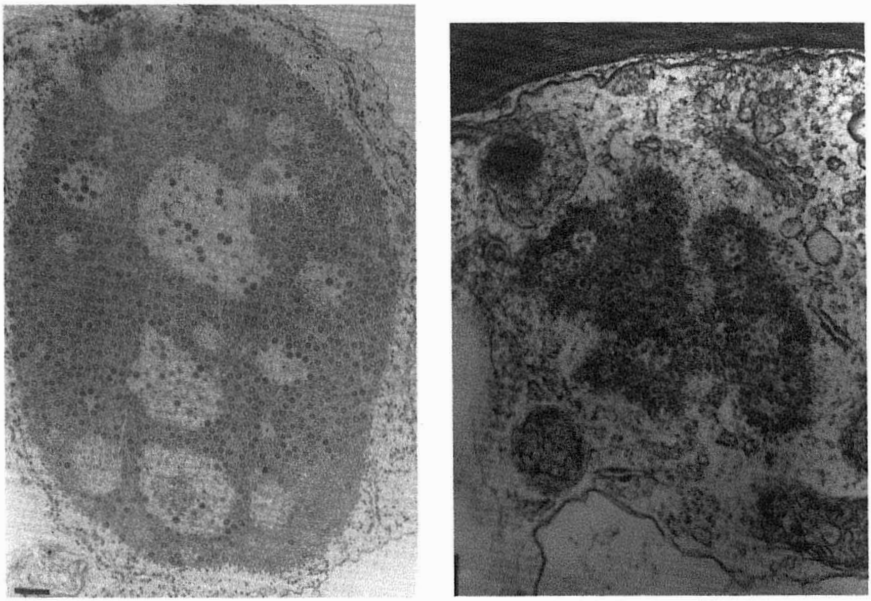
k. Alfalfa mosaic virus. Alfalfa mosaic virus (AMV) is the sole member of the alfalfa mosaic virus group, and its presence in the ultrathin sections of infected leaves can be recognized by the polymorphic particles, the shape of which varies from spherical to bacilliform. All strains of AMV do not aggregate, but the strains that do may be differentiated on the basis of the manner in which the virus particles aggregate intracellularly. Four types of virus particle aggregates have been recognized: a) short rafts of particles arranged in a hexagonal array; b) long bands of particles aligned side by side, sometimes in a stacked-layer configuration; c) aggregates consisting of whorllike structures; and d) aggregates with four parallel rows of particles packed in either an apparently rhomboid lattice or a hexagonal one (Martelli and Russo, 1984).

l. Reoviruses. Plant reoviruses, consisting of two subgroups with generic status, *Phytoreovirus* and *Fijivirus*, produce three recognizable cytopathic effects: a) proteinaceous material (viroplasm) containing dark spherical bodies (about 50 nm diameter) representing immature inner cores of virus particles accumulates; b) near the viroplasm mature virus particles aggregate into crystals; and c) tubules containing rows of virus particles are also formed. The two subgroups can be distinguished on the basis of the shape and size of viroplasms. *Phytoreovirus* induces small spherical viroplasms, whereas *Fijivirus* produces large elongated viroplasms (Shikata, 1981).

m. Caulimoviruses. The caulimoviruses are found freely scattered in the parenchymatous cells but very often aggregate to form rounded or elongated inclusion bodies in which virus particles are embedded (Fig. 3.13 A and B). The final size of the inclusions is determined by the viral genome; hence the individual virus isolates of cauliflower mosaic virus may be differented on the basis of the size of the inclusions (Shalla et al., 1980).

The characteristics of inclusions can be used for diagnosis of caulimoviruses by using a light microscope. Selection of healthy dahlia plants was successsfully accomplished by determining the presence or absence of the inclusions in the tissues (Robb, 1963).

n. Hordeiviruses. The cytological effects of barley stripe mosaic virus (BSMV), the type member of the hordeivirus group, have been studied in detail (McMullen et al., 1978). In the infected cells, small, rounded plastidial vesicles or flask-shaped invaginations of the outer lamella of the boundary



(A) (B)

Figure 3.13 A, Characteristic cytoplasmic inclusion produced by cauliflower mosaic virus; bar represents 200 nm (Academic Press, Inc., USA). (Courtesy of Martelli and Russo, 1984); B, inclusion bodies in peanut leaf cell infected by peanut chlorotic streak virus; bar represents 200 nm. (American Phytopathological Society, Minnesota, USA). (Courtesy of Reddy et al., 1993.)

membrane containing fine fibrils are formed (McMullen et al., 1978). The virus particles accumulate in either the cytoplasm or the nucleus, forming irregularly shaped or paracrystalline aggregates (Carroll, 1970). Another effect is seen on cell walls, which show irregular thickenings at the level of plasmodesmata, and at later stages extracytoplasmic sacs containing small granules are formed (McMullen et al., 1977).

o. Tobraviruses. Tobacco rattle virus (TRV), type member of the tobravirus group, induces bulky cytoplasmic inclusions consisting of mitochondria, ribosomes, and electron-dense, possibly proteinaceous material (Harrison et al., 1970). In addition, paracrystalline aggregates of long particles arranged in tiers are formed (Chang et al., 1976).

p. Tobamoviruses. Two tobamovirus subgroups, tobacco mosaic virus (TMV), representing subgroup A, and beet necrotic yellow vein virus (BNYVV), representing subgroup B, have been recognized. Tobacco mosaic virus and other viruses of subgroup A induce the production of crystalline inclusions which can be seen under a light microscope as hyaline plates with a hexagonal or rounded shape. The characteristics of the crystalline inclusions can be used for virus identification. The inclusions are composed of many stacked layers of virus particles aligned in a parallel array, as seen with an electron microscope (Warmke and Edwardson, 1966). Viruses included in subgroup B do not induce the production of any crystalline inclusions; however, BNYVV is able to produce paracrystalline aggregates (Tamada, 1975).

Among the viruses in subgroup A, only TMV is known to produce amorphous inclusions (X-bodies), which are aggregates of ribosomes, endoplasmic reticulum, and small vacuoles or vesicles; viral protein in granular or tubular form.; and small pockets of virus particles (Esau, 1968). On the other hand, many of the viruses in subgroup B induce amorphous inclusions. In the fully developed inclusions induced by BNYVV, a large number of virions are found scattered throughout the inclusion (Russo et al., 1981). Hibino et al. (1974) reported that nine isolates of soil-borne wheat mosaic virus could be divided into three groups based on the composition and relative abundance of strands of endoplasmic reticulum, tubules, vesicles, membranes, and virions.

Cucumber green mottle virus (CGMV) in subgroup A is distinct from other tobamoviruses in its ability to induce an enlargement and membraneous proliferation of mitochondria which is heavily vesiculated (Sugimura and Ushiyama, 1975).

q. Potexviruses. The characteristic cytopathological effect of potexvirus infection is the formation of irregular, fibrous, or banded aggregates of virus particles. Virions are aligned side by side and aggregated end-to-end, giving spindle shape to the fibrous inclusions, whereas virus particles are aligned in

horizontal tiers in banded inclusions which are composed almost entirely of virus particles. This cytoplasmic strands separate successive layers, giving the banded appearance to the inclusions.

Potato virus X (PVX) also induces laminate inclusions consisting of thin sheets of proteinaceous material sometimes studded with beads which are antigenically different from PVX protein (Shalla and Shepherd, 1972). Clover yellow mosaic virus (ClYMV) also produces additional amorphous inclusions in the cytoplasm and vacuoles of cells. These inclusions are composed of a protein antigenically related to the ClYMV protein (Schlegel and De Lisle, 1971).

r. Carlaviruses. The carlaviruses generally do not induce any specific cellular modification that can be considered characteristic of the group. However, many of them produce flexuous bundles of virus particles or bounded aggregates similar to those induced by potexviruses. Red clover vein mosaic virus (RCVMV) appears to be unique and differs from other members in inducing crystalline inclusions composed of polyhedral particles 10 nm in diameter than contain protein and RNA. Though the origin and significance of the constituents of polyhedral particles are not known, they are useful in diagnosing RCVMV infections (Khan et al., 1977).

s. Potyviruses. The potyvirus group is the largest, enclosing 48 definitive members and 67 possible members. Though they differ considerably in particle length and nature of vector, one single unifying ultrastructural feature, the presence of a cylindrical or pinwheel inclusions in the respective host plants infected by different viruses, has been recognized. The pinwheel inclusion has a central core from which rectangular or triangular curved plates radiate. Laminated aggregates may be formed when the plates of adjacent pinwheels may fuse to form a series of stacked laminar structure or scrolls (or tubes) may be formed when the plates roll inward. On the basis of the characteristics of configuration formed as a result of the association between pinwheels and plates, Edwardson (1981) suggested that potyviruses may be grouped into four subdivisions: a) pinwheels and scrolls; b) pinwheels and long straight laminated aggregates; c) pinwheels, scrolls, and long straight laminated aggregates; and d) pinwheels, scrolls, and short curved laminated aggregates (Fig. 3.14).

Many potyviruses, in addition to proteinaceous pinwheel inclusions, produce complex amorphous cytoplasmic inclusion bodies and secondary vacuolation of the cytoplasm. Monolayers of virus particles in parallel array delimited by two membranes may be seen in the secondary vacuoles.

In the case of some of the potyviruses, the cytological abnormalities are so specific that they may be used for the diagnosis of infection by the respective viruses. Intensely electron-opaque angular or rounded bodies surrounded by spherical ribosome-like particles are the characteristic features of bean yel-

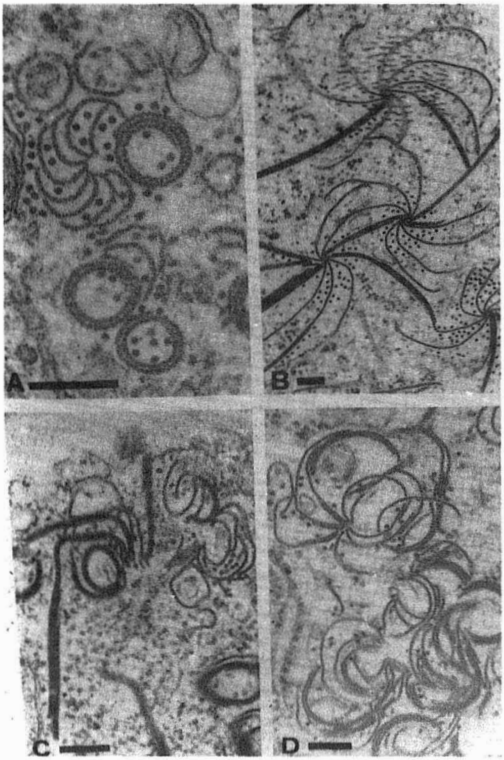


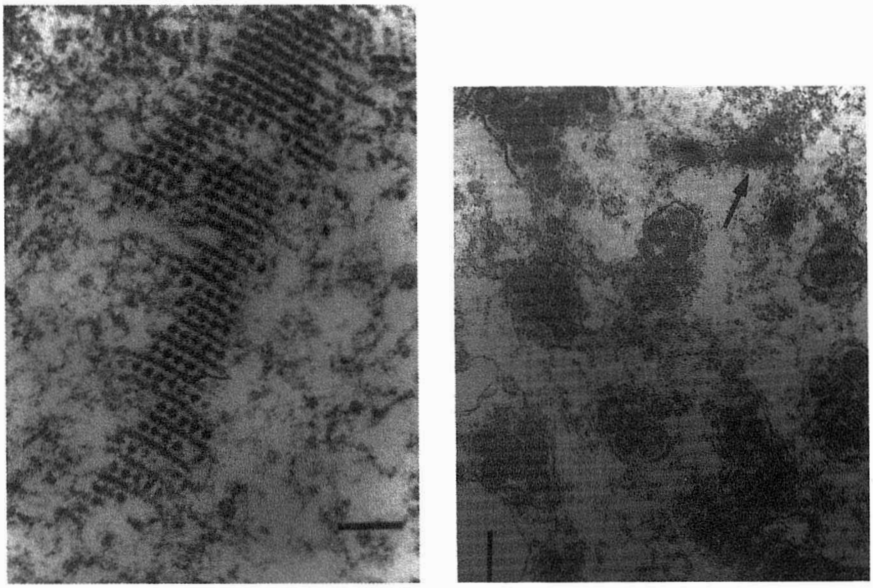
Figure 3.14 Cylindrical inclusions of potyvirus; A, pinwheels and scrolls (subdivision I); B, pinwheels and aggregates (subdivision II); C, pinwheels, scrolls, and long straight laminated aggregates (subdivision III); D, pinwheels, scrolls, and short curved laminated aggregates (subdivision IV); bar represents 100 nm (Academic Press Inc., USA). (Courtesy of Martelli and Russo, 1984.)

low mosaic virus infection. Beet mosaic virus induces characteristic satellite bodies consisting of nucleolus-related amorphous accumulation of proteinaceous material (Martelli and Russo, 1969). Accumulation of electron-dense granular material, sometimes consisting of thin rodlike structures resembling virus particles, forms a diagnostic feature of watermelon mosaic virus 1 infection (Martelli and Russo, 1976). The presence of cytoplasmic or intranuclear inclusions consisting of fimbriate bodies is a reliable indicator of infection by zucchini yellow fleck virus (Martelli and Russo, 1984). Tobacco etch virus is known to induce large crystalline inclusions in both the nuclei and the cytoplasm (Kassanis, 1939).

t. Closteroviruses. Among three closterovirus subgroups (A, B, and C) recognized, subgroup A does not induce any specific cytopathological effects, but

the viruses accumulate in high concentrations in companion cells and sieve tubes, filling almost the entire lumen of cells (Bem and Murrant, 1980). On the other hand, plants infected by viruses of subgroups B and C show the presence of virions aggregating into cross-banded structures. The virus particles are closely packed in several tiers (Esau and Hoefert, 1971), in loose wavy paracrystalline aggregates (Esau and Hoefert, 1981), or in irregular fascicles intermingled with membraneous vesicles containing a network of fine fibrils (Martelli and Russo, 1984). The inclusions induced by viruses of the B and C subgroups have diagnostic value as they are produced almost exclusively by these viruses.

u. Tomato spotted wilt virus. Tomato spotted wil virus (TSWV) is a monotypic virus group with only tomato spotted wilt virus, which has a very wide host range. The presence of large spherical particles (70–80 nm diameter) with membrane bound in groups is itself a feature useful for the identification of TSWV infection (Fig. 3.15 A and B). However, viroplasms composed of large

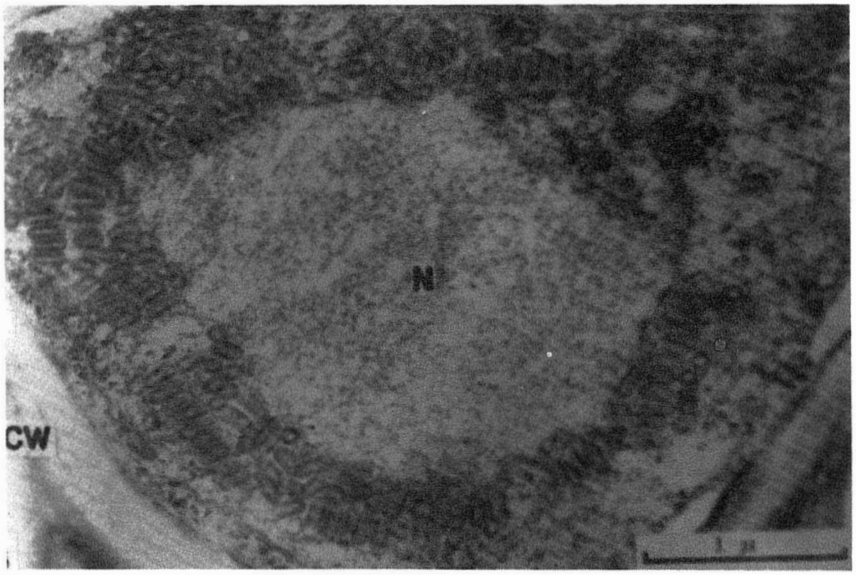


(A) (B)

Figure 3.15 A, Ordered arrays of filaments (type II) in INSV-infected *Nicotiana benthamiana* leaf cell; bar represents 100 nm, B, *Nicotiana benthamiana* leaf cells infected by tospovirus with mature virus particles within membranous cisternae; bar represents 200 nm (Blackwell Science Ltd. and British Society for Plant Pathology, UK). (Courtesy of Vaira et al., 1993.)

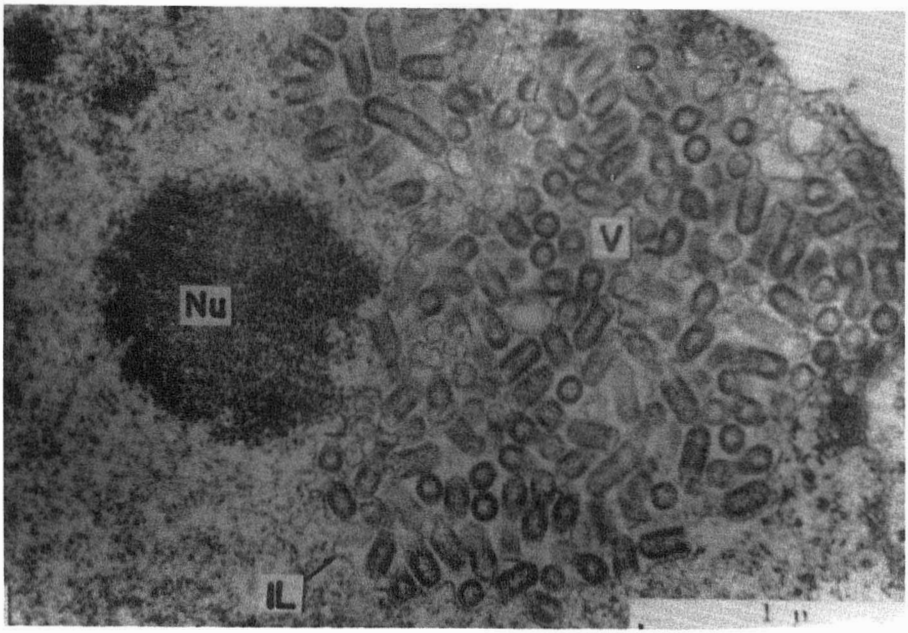
cytoplasmic aggregates of dark-staining, probably proteinaceous material (Milne, 1970) or clusters of hollow tubules intermingled with endoplasmic reticulum strands (Francki and Hatta, 1981; Vaira et al., 1993) may also be useful as markers of TSWV infection.

v. Rhabdoviruses. The characteristic particle morphological properties of the rhabdoviruses are useful diagnostic markers. The virus particles appear as spherical, elongated, or bacilliform structures when they are cut transversely or longitudinally (Chen and Shikata, 1971) (Fig. 3.16 A, B, and C). Rhabdoviruses may be divided into three subgroups based on the cytological effects induced by them: In subgroup A, the viruses acquire the membranes from endoplasmic reticulum and accumulate in the cytoplasm as small aggregates within dilated membranous cisternae. Viruses in subgroup B are initiated as buds in the inner membrane of the nuclear envelope and accumulate at the periphery of nuclei as paracrystalline aggregates composed of a high concentration of virus particles. Viruses of subgroup C are associated with the nucleus

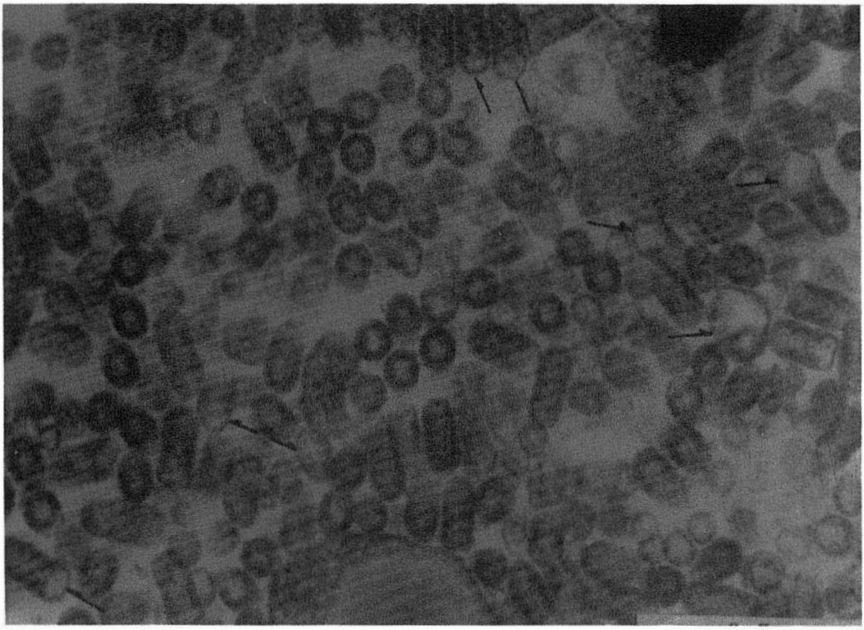


(A)

Figure 3.16 A, Rice transitory yellowing virus particles in large numbers around nuclear membrane of the leaf cell nucleus: N—nucleus, CW—cell wall; B, rice transitory yellowing virus particles in the nucleolus of infected rice leaf cell: Nu—nucleus, IL, inner lamella of nuclear membrane; C, bacilliform particles of rice transitory yellowing virus in a cell of infected rice leaf (Academic Press Inc., USA). (Courtesy of Chen and Shikata, 1971.)



(B)



(C)

Figure 3.16 Continued

and are not enveloped. They form characteristic structures known as "spokewheels" at the periphery of nuclei (Martelli and Russo, 1977; Francki et al., 1981).

SUMMARY

The external symptoms of diagnostic value induced by fungal, bacterial, viral, and mycoplasmal pathogens are described. Histological changes caused by certain viruses have diagnostic value. Cytopathological changes, including formation of intracellular and intranuclear inclusions, are characteristic of virus infections. Some of the virus groups and individual viruses can be reliably diagnosed by using differential stains or by observing ultrathin sections under an electron microscope. The viruses may be readily isolated from the inclusion bodies by new methods that have been developed.

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4
Dissemination of Plant Pathogens

Plant pathogens may be disseminated/transmitted in different ways. Fungal and bacterial pathogens may be disseminated largely by wind or water and in some cases with the help of insects. Viruses and mycoplasma-like organisms (MLOs), on the other hand, predominantly depend on vectors such as insects, mites, nematodes, and fungi for their natural spread under in vivo conditions. The infected seeds or vegetatively propagated seed materials form the most important primary sources of infection, irrespective of the nature of the pathogen. The infected seeds/seed materials may exhibit some symptoms or show the presence of the fungal or bacterial pathogens when incubated. But infection of seeds by viruses or viroids or MLOs may not be discernible, unless special methods of detection are employed. Transmission of some of the viruses and viroids through pollen, leading to seed infection, is also recognized.

4.1 TRANSMISSION OF PLANT VIRUSES

The plant viruses can be transmitted in several ways under natural and experimental conditions (Matthews, 1991; Narayanasamy and Doraiswamy, 1996). All viruses, viroids, and MLOs may be transmitted to appropriate host plant species by different grafting/budding methods. Some of the viruses and MLOs have been experimentally transmitted by using *Cuscuta* spp. This method may be useful when grafting is difficult or not possible, as in monocots.

Many viruses and all viroids may be transmitted by mechanical transmission, whereas attempts to transmit the MLOs by mechanical inoculation from plant to plant have been unsuccessful. However, the MLOs may be transmitted by mechanical inoculation from leafhopper to leafhopper (Maramorosch, 1952). All vectors of MLOs belong to the suborder Auchenorrhyncha and no

other types of vector are known. Some plant viruses, such as wound tumor virus (Black and Brakke, 1952), rice dwarf virus (Fukushi and Kimura, 1959), and northern cereal mosaic virus (Yamada and Shikata, 1969), have also been serially transmitted from insect to insect by injecting the juice of viruliferous insects.

The methods of transmission or spread of pathogens per se many not form a sound basis for distinguishing the pathogens. However, the specificity of relationship of the pathogens with their vectors, largely in the case of viruses and MLOs and of some fungi and bacteria, may indicate the identity of the pathogen. The transmission characteristics of the virus may be helpful in the identification of the virus, when considered along with other properties.

4.1.1 Mechanical Transmission

The ability of a virus to be transmitted by mechanical inoculation is considered one of the intrinsic properties of the virus concerned. The properties of the viruses in expressed sap, such as stability in vitro, thermal inactivation point (TIP), and dilution end point (DEP), have been determined for the sap-transmissible viruses. Though the usefulness of these properties for the identification of viruses is somewhat limited, they may be useful in differentiating viruses with widely different physical properties (Francki, 1980) (Table 4.1).

By selecting an appropriate method of transmission, the viruses may be transmitted to one or a range of plant species which exhibit characteristic symptoms due to local or systemic infection. The symptoms produced on certain host plant species are quite characteristic of the causal virus and are useful in differentiating such viruses. Vaira et al. (1993) reported that the two tospovirus species, tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV), can be differentiated by the type of symptoms produced on diagnostic plants (Table 4.2). The local lesion hosts are used for both detection and assay of plant viruses. The plant species which can be used as assay or diagnostic hosts for different viruses are presented in Table 4.3.

4.1.2 Transmission of Viruses by Vectors

Various groups of vectors are involved in the transmission of plant viruses and the viruses transmitted by a particular group of vectors exhibit differences in a number of other properties. The nature of vector involved in transmission and the relationship between the virus and the vector have been studied for the majority of plant viruses and MLOs (Carter, 1973; Harris, 1979; Harris and Maramorosch, 1977).

The species of vector and transmission characteristics are useful in differentiating some viruses and their strains. The vector specificity, in certain

Table 4.1 Physical Properties of Plant Viruses

Name of virus	Dilution end point (DEP)	Thermal inactivation point (TIP) 0°C	Longevity in vitro (LIV)	Reference
Agropyron mosaic virus	1:10,000–1:20,000	50	12 days	Bremer, 1964
Alfalfa mosaic virus	1:1,000–1:5,000	50–60	4 hr to 4 days	Smith, 1972
Apple chlorotic leafspot virus	1:1,000–1:5,000	55	7 hr at 24°C	Saksena and Mink, 1969
Apple stem grooving virus	1:1,000	67	2 days at 20°C	Smith, 1972
Arabis mosaic virus	1:1,000–1:100,000	55–60	15–21 days at 18–21°C	Smith, 1972
Barley mosaic virus	1:100–1:500	53–55	6 hr at 19°–20°C .	Dhanraj and Raychaudhuri 1969
Barley stripe mosaic virus	1:10,000	63	18 days	Ohmann-Kreutzberg, 1962
Bean (common) mosaic virus	1:1,000–1:10,000	56–58	24–32 hr	Smith, 1972
Bean pod mottle virus	1:10,000	70–75	62 days	Bancroft, 1962
Bean southern mosaic virus	1:500,000	90–95	32 weeks at 18°C	Smith, 1972
Bean yellow mosaic virus	1:800–1:1,000	56–60	24–32 hr	Weintraub and Ragetli, 1966
Beet mosaic virus	1:4,000	55–60	24–48 hr at 21°C	Smith, 1972
Broad bean mottle virus	1:1,000	95	3 weeks	Smith, 1972
Broadbean stain virus	1:1,000–1:10,000	60–65	10–17 days at 18°C	Gibbs et al., 1968
Brome mosaic virus	1:100,000–1:300,000	78–79	14 months at 17°C	Smith, 1972
Cacao mottle leaf virus	1:100	55–60	96 hr	Kenten and Legg, 1967
Cacao yellow mosaic virus	1:10,000	60–65	16–32 days	Brunt et al., 1965
Carnation latent virus	1:1,000	60	2 days	Hollings and Stone, 1965b
Carnation mottle virus	1:200,000	85–90	81 days at 18°C	Hollings and Stone, 1964
Carnation ringspot virus	1:100,000	85–90	16 days at 20°C	Kassanis, 1955
Carnation vein mottle virus	1:1,000	50	10 days	Hollings and Stone, 1967

(*continued*)

Table 4.1 Continued

Name of virus	Dilution end point (DEP)	Thermal inactivation point (TIP) 0°C	Longevity in vitro (LIV)	Reference
Carrot mottle virus	1:1,000	70	9–24 hr	Murrant et al., 1969
Cauliflower mosaic virus	1:2,000	70–75	14 days	Smith, 1972
Celery mosaic virus	1:100–1:1,000	55–60	6 days	Smith, 1972
Celery (western) mosaic virus	1:4,000	55–60	4–6 days	Purcifull and Shepard, 1967
Cherry chlorotic–necrotic ringspot virus	1:40–1:100	48–50	1–2 days	Kralikova and Kegler, 1967
Cherry leaf roll virus	1:100	52–55	5–10 days at 20°C	Cropley, 1961
Clover (red) mottle virus	1:1,000,000	70–75	2 weeks at 20°C	Valenta and Marcinka, 1971
Clover (red) vein mosaic virus	—	58–60	2–3 days	Osborn, 1937
Clover (white) mosaic virus	1:100,000–1:1,000,000	60	10–99 days	Bercks, 1971
Clover yellow mosaic virus	1:10,000	58–60	6–12 months	Pratt, 1961
Clover yellow vein virus	1:10,000	55	8 days at 18°C	Hollings and Nariani, 1965
Cocksfoot mottle virus	—	65	2 weeks at 20°C	Serjeant, 1967
Cowpea aphid-borne virus	1:4,000	60–62	5 days at 21°C	Lovisolo and Conti, 1966
Cowpea chlorotic mottle virus	1:10,000	65–70	1–2 days	Kuhn, 1964
	1:100,000			Bancroft, 1971
Cowpea mosaic virus	1:10,000	65–70	1–20 days	Dale, 1949
	1:100,000			Vankammen, 1971
Cucumber green mottle mosaic virus	—	80–90	1 year	Bawden and Pirie, 1937
Cucumber mosaic virus	1:10,000	60–70	3–4 days	Smith, 1972
Cymbidium mosaic virus		65–70	7 days	Smith, 1972
Dahlia mosaic virus	1:3,000	85–90	10–14 days	Brierly and Smith, 1950
Eggplant (brinjal) mosaic virus	—	78	Few weeks	Smith, 1972
Elm mottle virus	1:1,000	58–62	7 days	Schmelzer, 1969
Grapevine fan leaf virus	1:5,000–1:10,000	60–62	14–21 days	Cadman et al., 1960
Groundnut (peanut) mottle virus	1:1,000	60	24 hr at 25°C	Kuhn, 1965

Henbane mosaic virus	1:10,000 1:100,000	50–60	About 4 days	Bawden, 1951
Iris mosaic virus	1:100 1:1,000	65–70	3–4 days at 20°C	Brunt, 1968
Lettuce mosaic virus	1:100	55–60	48 hr	Couch and Gold, 1954
Lettuce necrotic yellows virus	1:100	52–54	1–8 hr	Stubbs and Grogan, 1963
Lynchnis ringspot virus	1:2,000	64–68	2–7 days	Bennett, 1959
Muskmelon vein necrosis virus	1:1,000–1:10,000	50–55	2–7 days	Freitag and Milne, 1970
Narcissus mosaic virus	1:100,000	70	12 weeks at 18°C	Brunt, 1966a
Narcissus yellow stripe virus	1:100–1:1,000	70–75	72 hr	Brunt, 1971
Onion yellow dwarf virus	1:10,000	75–80	100 hr at 29°C	Dhingra and Nariani, 1963
Papaya mosaic virus	1:10,000	73–76	6 months	Purcifull and Hiebert, 1971
Parsnip yellow fleck virus	1:1,000–1:10,000	57–60	4–7 days	Murant and Goold, 1967
Pea early browning virus	1:10,000–1:100,000	65–70	147 days	Bos and Vander Want, 1962
Pea enation mosaic virus	1:3,000	56–58	3 days	Pierce, 1935
Pea mosaic virus	1:5,000	60–64	3–4 days	Murphy and Pierce, 1937
Pea streak virus	1:1,000,000	58–60	16–32 days	Hagedorn and Walker, 1949
Pelargonium leaf curl virus	1:100	85–90	21 days at 18°C	Hollings, 1962
Plum line pattern virus	1:6,400	65	—	Paulsen and Fulton, 1968
Plum pox virus	1:10–10,000	51–54	1–2 days at 20°C	Smith, 1972
Potato aucuba mosaic virus	1:200–1:500	65	3–4 days at 15°C	Kasssanis, 1961
Potato moptop virus	1:10,000	80	14 weeks at 20°C	Jones and Harrison, 1969
Potato virus A	1:50–1:100	44–52	12–24 hr	MacLachlan et al., 1953
Potato virus S	1:100–1:1,000	50–60	3–4 days	Wetter, 1971
Potato virus X	1:100,000–1:1,000,000	70	Up to a year	Smith, 1933
Potato virus Y	1:100–1:1,000	52–55	24–48 hr	Ross, 1948
Potato yellow dwarf virus	1:1,000–1:10,000	50	2–12 hr	Smith, 1972
Prunus necrotic ringspot virus	1:50–1:100	50–55	—	Smith, 1972
Radish mosaic virus	1:14,000	65–68	14 days at 22°C	Tompkins, 1939

(*continued*)

Table 4.1 Continued

Name of virus	Dilution end point (DEP)	Thermal inactivation point (TIP) 0°C	Longevity in vitro (LIV)	Reference
Raspberry bushy dwarf virus	1:10,000	65	4 days at 22°C	Barnett and Murrant, 1970
Raspberry ringspot virus	1:100	60–65	Rapidly inactivated	Roland, 1962
Sowbane mosaic virus	1:100,000–1:100,000,000	84–86	2 months	Bennett and Costa, 1961
Soybean mosaic virus	—	64–66	4–5 days	Gardner and Kendrick, 1921
Squash mosaic virus	1:1,000,000	75	6 weeks	Freitag, 1956
Sugarcane mosaic virus	1:1,000	53–55	2–24 hr	Rafay, 1935 Adsuar, 1950
Tobacco etch virus	1:1,000–1:5,000	54–58	5–8 days	Smith, 1972
Tobacco mosaic virus	1:1,000,000	93	1 year	Smith, 1972
Tobacco necrosis virus	1:10,000–1:1,000,000	70–90	2–3 months	Kassanis, 1970
Tobacco rattle virus	1:100,000	80–85	6 weeks	Cadman and Harrison, 1959
Tobacco ringspot virus	1:1,000–1:10,000	60–65	6–10 days	Henderson, 1931
Tobacco streak virus	1:20–1:100	53	2–3 days at 22°C	Diachun and Valleau, 1950
Tobacco wilt virus	1:1,000	60–65	4 days	Badami and Kassanis, 1959
Tomato aspermy virus	1:100–1:1,000	50–55	24–28 hr	Hollings, 1955
Tomato black ring virus	1:100 1:1,000	58–62	7 days	Gibbs and Harrison, 1964
Tomato bushy stunt virus	—	80	25 days	Smith, 1935
Tomato ringspot virus	1:10–1:1,000	56–58	21–27 hr	Price, 1936
Tomato spotted wilt virus	1:10,000–1:100,000	42	About 5 hours	Best, 1968
Tulip breaking virus	1:100,000	65–70	—	Cayley, 1932
Turnip crinkle virus	1:100,000	80–85	6–7 weeks	Smith, 1972
Turnip mosaic virus	1:1,000	55–60	48–72 hr	Tompkins, 1938
Turnip yellow mosaic virus	1:100,000–1:1,000,000	70–75		Markham and Smith, 1949
Watermelon mosaic virus	1:10,000–1:30,000	55–60	9–10 days	Anderson, 1954
Wheat (soil-borne) mosaic virus	1:100–1:1,000	60–65		Smith, 1972

Table 4.2 Differentiation of Two Tospovirus Species by Reaction of Test Plant Species

	TSWV		INSV	
Test plant species	Local infection	Systemic infection	Local infection	Systemic infection
Nicotiana benthamiana	a*	b	a	a
N. clevelandii	a	c	a	b
N. tabacum cv. White Burley	a	c	a	NI
Lycopersicon esculentum cv. Marmande	a	b	(a)	NI
Datura stramonium	a	b	(d)	NI

*a, Necrotic local lesion; b, mosaic; c, necrosis; d, chlorotic local lesion; ( ), inconsistent appearance; NI, no infection.
Source: Vaira et al. (1993).

viruses, may be helpful to establish the identity of viruses with reasonable certainty. The viruses transmitted by nematodes or fungi may be identified through the vector specificity to some extent (Taylor, 1980; Teakle, 1980). A knowledge of the variations in these properties may be useful in differentiating viruses.

4.1.2.1 Viruses transmitted by aphids

The viruses transmitted by aphids show differences in the following properties: a) persistence in aphids, b) presence of latent period, c) passage through molt, d) multiplication of virus in aphid, e) mechanical inoculation, f) virus particle morphological characteristics, and g) nature of viral genome. On the basis of these properties the aphid-borne viruses may be grouped as follows:

a. Nonpersistent viruses. The vectors of nonpersistent viruses remain viruliferous for short periods, have no demonstrable latent period, and lose infectivity after molting. Fasting prior to acquisition feeding remarkably increases the period of virus retention and efficiency of transmission by the aphids. They can be mechanically transmitted from plant to plant. All viruses have single-stranded ribonucleic acid (ss-RNA) genome, except caulimoviruses, which have double-stranded deoxyribonucleic acid (ds-DNA) as genome. These nonpersistent viruses are included in the alfalfa mosaic virus, carlavirus, caulimovirus, cucumovirus, fabavirus, and potyvirus groups; they may have mono-, bi-, or tripartite genomes. These viruses fall into four groups based on particle morphological characteristics (Watson, 1972):

Table 4.3 Assay Diagnostic Hosts of Plant Viruses

Name of virus	Diagnostic hosts	Infection[a] type (local/ systemic)	Reference
Agropyron mosaic virus	*Chenopodium quinoa*	L	Bremer, 1964
Alfalfa mosaic virus	*Chenopodium amaranticolor*	L	Smith, 1972
	C. quinoa		
	Gomphrena globosa	L	Hollings, 1959
	Phaseolus vulgaris	L	
	Ocimum basilicum	L	Lovisolo, 1960
Apple chlorotic virus	*C. amaranticolor*	S	Cropley, 1964
	C. quinoa	S	Lister et al., 1964
Apple mosaic	Apple varieties Lord Lambourne, Jonathan, and Golden Delicious	S	Smith, 1972
Arabis mosaic	*Cannabis sativa*	S	Smith, 1972
	C. amaranticolor	S	
	C. murale	S	
	C. quinoa	S	
Barley stripe mosaic virus	*C. amaranticolor*	L	Hollings, 1959
	G. globosa	L	
	Spinacia sp.	L	
Bean common mosaic virus	*Phaseolus vulgaris*	S	Pierce, 1934
Bean southern mosaic virus	*Phaseolus vulgaris*	L	Shepherd, 1971
	P. lunatus	L	
	P. aureus	L	
	Cowpea	L	
Bean yellow mosaic yellow	*Tetragonia expansa*	L	Hollings, 1966
Beet curly top virus	*Datura stramonium*	S	Severin, 1929
	Nicotiana tabacum var. White Burley	S	

Beet mosaic virus	*Gomphrena globosa*	L	Hollings, 1959
	Chenopodium amaranticolor	L	
	Beta vulgaris	S	Russell, 1971
	Spinacea oleracea	L, S	
Broadbean mottle virus	*Coronilla varia*		
	Lourea vespertilionis		Smith, 1972
Brome mosaic	*Zea mays*	L, S	Smith, 1972
Cardamom (greater) mosaic streak virus	*Achorus calamus*	S	Smith, 1972
	Curcuma longa		
Carnation latent virus	*C. amaranticolor*	L, S	Wetter, 1971
	C qunioa	L, S	
Carnation mottle virus	*C. amaranticolor*	L	Hollings, 1956
Carnation ringspot virus	*G. globosa*	L	Paludan, 1965
Carnation vein mottle virus	*C. amaranticolor*	L	Smith, 1972
	C. quinoa	L	
Carrot mosaic virus	*Vigna sinensis*	L	Smith, 1972
Carrot mottle virus	*C. amaranticolor*	L	Smith, 1972
	C. quinoa	L	
Cauliflower mosaic virus	*Verbesina encelioides*	L, S	Sheperd, 1970
	Dahlia pinnata	S	
	Ageratum conyzoides	L, S	
	Zinnia elegans	S	
	Amaranthus caudatus	L, S	
	Chenopodium capitatum	S	
Celery mosaic virus	*Apium graveolens* var. *dulce* (celery)	S	Shepard and Grogan, 1971
	Conicum maculatatum	S	
	Pastinaca sativa	S	
	Dacus carota var. *sativa*	S	

(*continued*)

Table 4.3 Continued

Name of virus	Diagnostic hosts	Infection[a] type (local/ systemic)	Reference
Celery (western) mosaic virus	*C. amaranticolor*	L	Wolf, 1969, Smith, 1972
	C. quinoa	L	
	C. amaranticolor	L, S	
Cherry leaf roll virus	*C. murale*	S	Smith, 1972
	N. tabacum cv. White Burley	L, S	Smith, 1972
	Vicia faba	S	
	Prunus persica	S	Kegler et al., 1962
Chili (pepper) mosaic virus	*Beta vulgaris*	L	Nariani and Sastri, 1958
	Nicotiana glutinosa	S	
Citrus infectious variegation virus	*Cucumis sativus*	L	Desjardinis and Wallace, 1962
	C. quinoa	S	
	Crotalaria spectabilis	L	
	Petunia	S	Garnsey, 1968
Citrus tristeza	West Indian (Mexican) lime	S	Wallace, 1951
Citrus vein enation virus	Rough lemon or sour lemon	S	Wallace and Drake, 1961
Clover (white) mosaic virus	*Vigna sinensis*—cowpea var. Blackeye	L, S	Smith, 1972
	Phaseolus vulgaris		
	Pisum sativum		
Clover (red) mottle virus	*Phaseolus vulgaris*	L	Sinha, 1960
	G. globosa	S	
	C. amaranticolor	L	Smith, 1972
	C. quinoa	L	
Clover yellow mosaic virus	*Antirrhinum majus* var. Majestic	L, S	Pratt, 1961
Clover yellow vein virus	*Nicotiana clevelandii*	S	Hollings and Narrani, 1965
Cotton leaf curl virus	*Hibiscus cannabinus*	S	Tarr, 1951

Cowpea aphid-borne mosaic virus	*C. amaranticolor*	L	Lovisolo and Conti, 1966
	O. basilicum	L	
Cowpea chlorotic mottle virus	*Soja max*—soybean	L	Kuhn, 1964
Cowpea mosaic virus	*Canavalia ensiformis*	L	Dale, 1949
Cucumber mosaic virus	*C. amaranticolor*	L	Hollings, 1959
	G. globosa	L	
	Datura stramonium	S	
Dahlia mosaic virus	*Verbesina encelioides*	S	Brierley, 1951
Eggplant (brinjal) mosaic virus	*C. amaranticolor*	L	Smith, 1972
	N. clevelandii		
Grapevine fan leaf virus	*C. amaranticolor*	L, S	Harrison and Nixon, 1960
	G. globosa	L, S	Dias, 1963
Groundnut bud necrosis virus	Cowpea cv. 152	L, S	Ghanekar, et al., 1979
	Dolichos lablab	L	
Groundnut (peanut) mottle virus	Bean (*P. vulgaris*) var. Topcrop	L	Kuhn, 1964
	Cassia occidentalis	S	
Groundnut rosette virus	*C. amaranticolor*	L	Hull and Adams, 1968
	C. hybridum	L	
	C. quinoa	L	
Hop mosaic virus	*N. clevelandii*	L	Bock, 1967
Lettuce mosaic virus	*G. globosa*	L	Rohloff, 1968
	C. quinoa	S	
	Spinacia oleracea	L	Hollings, 1959
Lettuce necrotic yellows virus	*Nicotiana glutinosa*	L, S	Stubbs and Grogan, 1963
	Petunia	S	

(*continued*)

Table 4.3 Continued

Name of virus	Diagnostic hosts	Infection[a] type (local/ systemic)	Reference
Lychnis ringspot virus	Sugar beet	L, S	Bennett, 1959
	Callistephus chinensis	L	
	C. amaranticolor	L	
	C. album	L	
	C. capitatum	L	
Maize rough dwarf virus	*Hordeum vulgare*	S	Vidano et al., 1966
Maize streak disease virus	*Digitaria horizontalis*	S	Storey, 1932, 1933
Narcissus mosaic virus	*G. globosa*	L	Brunt, 1966a
	Trifolium incarnatum	S	
Narcissus yellow stripe virus	*Tetragonia expansa*	L	Brunt, 1971
	Narcissus jonquilla	S	
Nasturtium ringspot virus	*N. glutinosa*	L, S	Smith, 1950
	N. tabacum var. White Burley	L, S	
	Blackstonia perfoliata	S	Juretic et al., 1970
Onion yellow dwarf virus	*Narcissus jonquilla*	S	Hendreson, 1935
Papaya mosaic virus	*G. globosa*	L	Purcifull and Hiebert, 1971
	C. amaranticolor	L	
	Cassia occidentalis	L	
Parsnip yellow fleck virus	*Chenopodium quinoa*	L	Murant and Gold, 1967
Pea early browning virus	*G. globosa*	L, S	Bos and Van der Want, 1962
	Cowpea var. Monarch's Black Eye	L, S	
Pea enation mosaic virus	*Chenopodium amaranticolor*	L	Pierce, 1935

Pelargonium leaf curl virus	*C. amaranticolor*	L	Hollings, 1962
	Phaseolus vulgaris	L	
	N. clevelandii	L, S	
	Antirrhinum majus	L	
Plum line pattern virus	*Nicotiana megalosiphon*	S	Paulsen and Fulton, 1968
	Vigna cylindrica	S	
Plum pox virus	*Prunus persica*	S	Smith, 1972
	Chenopodium foetidum	L	
Potato aucuba mosaic virus	Potato var. Irish Chieftain	S	Clinch et al., 1936
	Tomato var. Kondure Red	S	
	Capsicum annuum	S	Maris and Rozendaal, 1956
Potato leaf roll virus	*Datura tatula*	S	Maramorosch, 1955
	Physalis angulata		
	Potato var. Earlaine	S	
Potato moptop virus	*C. amaranticolor*	L	Harrison and Jones, 1970
Potato spindle tuber viroid	*Solanum rostratum*	S	Singh and Bagnall, 1968
Potato virus A	*N. glutinosa*	S	Sommereyns, 1959
	Solanum demissum	L	Webb and Buck, 1955
	N. tabacum cv. Samsun	S	Smith, 1972
	N. tabacum cv. White Burley		
Potato virus S	*C. album*	L	Wetter, 1971
	C. amaranticolor		
	C. quinoa		
	Solanum rostratum		
	Cyamopsis psoraloides		

(*continued*)

Table 4.3 Continued

Name of virus	Diagnostic hosts	Infection[a] type (local/ systemic)	Reference
Celery (western) mosaic virus	*C. amaranticolor*	L	Wolf, 1969, Smith, 1972
	C. quinoa	L	
	C. amaranticolor	L, S	
Cherry leaf roll virus	*C. murale*	S	Smith, 1972
	N. tabacum cv. White Burley	L, S	Smith, 1972
	Vicia faba	S	
	Prunus persica	S	Kegler et al., 1966
Chili (pepper) mosaic virus	*Beta vulgaris*	L	Nariani and Sastri, 1958
	Nicotiana glutinosa	S	
Citrus infectious variegation virus	*Cucumis sativus*	L	Desjardinis and Wallace, 1962
	C. quinoa	S	
	Crotalaria spectabilis	L	
	Petunia	S	Garnsey, 1968
Citrus tristeza	West Indian (Mexican) lime	S	Wallace, 1951
Citrus vein enation virus	Rough lemon or sour lemon	S	Wallace and Drake, 1961
Clover (white) mosaic virus	*Vigna sinensis*—cowpea var. Blackeye	L, S	Smith, 1972
	Phaseolus vulgaris		
	Pisum sativum		
Clover (red) mottle virus	*Phaseolus vulgaris*	L	Sinha, 1960
	G. globosa	S	
	C. amaranticolor	L	Smith, 1972
	C. quinoa	L	
Clover yellow mosaic virus	*Antirrhinum majus* var. Majestic	L, S	Pratt, 1961
Clover yellow vein virus	*Nicotiana clevelandii*	S	Hollings and Narrani, 1965
Cotton leaf curl virus	*Hibiscus cannabinus*	S	Tarr, 1951

Rose mosaic virus	*Cyamopsis psoraloides*	L	Fulton, 1952
Sowbane mosaic virus	*C. murale*	L, S	Bennett and Costa, 1961
	C. quinoa	L, S	
Squash mosaic virus	*Cucurbita pepo, Citrullus lanatus*, *Cucumis sativum*	S	Smith, 1972
Strawberry crinkle virus	*Fragaria vesca*	S	Frazier, 1968
Strawberry mild yellow-edge virus	*Fragaria vesca*	S	Prentice, 1948
Strawberry mottle virus	*Fragaria vesca*	S	Prentice and Harris, 1946
Sugarcane mosaic virus	*Holcus sorghum*	S	Costa and Penteado, 1951
Tobacco broad ringspot virus	*Helianthus annuus*	L, S	Johnson and Fulton, 1942
Tobacco etch virus	*C. amaranticolor*	L	Holmes, 1946
	Physalis peruviana	L	
	N. tabacum	L, S	Smith, 1972
	C. annuum	S	
	D. stramonium		
	L. esculentum		
Tobacco mosaic virus	*Nicotiana glutinosa*	L	Holmes, 1931
	N. tabacum var. Xanthi	L	
	Phaseolus vulgaris var. Pinto bean	L	Piacitelli and Santilli, 1961
Tobacco necrosis virus A	*Phaseolus vulgaris*	L	Lovisolo, 1966
	Ocimum basilicum	L	Hollings and Stone, 1965c
	Torenia fournieri	L	
Tobacco rattle virus	*C. amaranticolor*	L	Cadman and Harrison, 1959
	Phaseolus vulgaris	L	
Tobacco ringspot virus	*C. amaranticolor*	L	Smith, 1972
	C. quinoa	L	
	Cowpea	L, S	
	Cucumber	L, S	

(*continued*)

Table 4.3 Continued

Name of virus	Diagnostic hosts	Infection[a] type (local/ systemic)	Reference
Tobacco streak virus	*Cyamopsis psoraloides*	L	Fulton, 1948, Smith, 1972
	Nicotiana tabacum	L	
	Vigna cylindrica		
Tomato aspermy virus	*Tetragonia expansa*— New Zealand spinach	L	Smith, 1972
	C. amaranticolor	L	Hollings and Stone, 1971
	N. glutinosa	L, S	
Tomato black ring virus	*Cucumis sativus*	S	Roland, 1969
	Chenopodium foliosum	S	Roland, 1969
	G. globosa	S	Roland, 1969
	Vicia faba	S	Roland, 1969
	Phlox drummondii	S	Schmelzer, 1970
Tomato bunchy top virus	*N. glutinosa*	S	McClean, 1935a
	Petunia sp.	S	
Tomato bushy stunt virus	Cowpea	L	Schmelzer, 1958
	Datura stramonium	L, S	

Tomato ringspot virus	*C. amaranticolor*	L	Samson and Iamle, 1942
	C. quinoa	L	
	Cowpea		
Tomato spotted wilt virus	*Petunia* sp.	L	Best, 1968
	N. glutinosa	L	Best, 1968
Turnip crinkle virus	*C. amaranticolor*	L	Hollings and Stone, 1963
Turnip mosaic virus	*C. amaranticolor*	L	Smith, 1972
	N. tabacum	L	
	N. glutinosa	S	
Turnip yellow mosaic virus	*Brassica pekinesis*	L, S	Diener and Jenifer, 1964
	Cleome spinosa	L	
Watermelon mosaic virus (western)	*Lavatera trimestris*	L	Schmelzer, 1965
	C. album	L	Demski, 1968
	C. strictum	L	Demski, 1968
Wheat (soil-borne) mosaic virus	*Triticum spelta*—Red Winter	S	McKinney, 1953
	C. amaranticolor	L	Smith, 1972
	C. quinoa	L	

[a]L, local; S, systemic.

i) Rigid rods: carnation latent, potato viruses S and M, and red clover vein mosaic viruses
ii) Long flexible rods: beet mosaic, henbane mosaic, potato virus Y, and turnip mosaic viruses
iii) Isometric: cucumber mosaic and cauliflower mosaic viruses
iv) Isometric–bacilliform: alfalfa mosaic virus

b. Semipersistent viruses. The semipersistent viruses differ from nonpersistent viruses in having longer periods of retention by aphids, longer periods of acquisition feeding, and the absence of any influence of fasting prior to acquisition feeding. They are not readily transmitted by mechanical inoculation as the nonpersistent viruses are. Beet yellows and citrus tristeza viruses have long flexible rod-shaped particles showing differences in the length of virus particles. Cauliflower mosaic virus is transmitted both nonpersistently and semipersistently by the same aphid species (*Brevicoryne brassicae*); such transmission is known as bimodal transmission (Chalfant and Chapman, 1962). Pea seed-borne mosaic virus also is transmitted bimodally, by *Macrosiphum euphorbiae* (Lim and Hagedorn, 1977).

c. Circulative (nonpropagative) viruses. The circulative viruses have a definite latent period in the aphid vector and pass through molting. Some are transmitted by mechanical inoculation with difficulty. They do not multiply in the vector. Pea enation mosaic virus (monotypic virus group) and luteovirus are transmitted in a nonpropagative circulative manner (Sylvester and Richardson, 1966; Miyamoto and Miyamoto, 1966; Watson and Okusanya, 1967). The barley yellow dwarf, carrot mottle, groundnut rosette, parsnip mottle, pea enation mosaic, and potato leafroll viruses are spherical.

d. Propagative viruses. Many viruses in Rhabdoviridae have been reported to have longer latent periods and to multiply in the aphids. They are not transmitted mechanically from plant to plant, but some viruses may be successfully inoculated into aphids. Lettuce necrotic yellows virus (LNYV) and sowthistle yellow vein virus (SYVV), transmitted by *Hyperomyzus lactucae* (O'Laughlin and Chambers, 1967; Sylvester, 1973), have bacilliform particles. Eggs of the vector carry SYVV (Sylvester, 1969), which is able to multiply in the primary cultures of aphid cells (Peters and Black, 1970). Successful serial passage of strawberry crinkle virus by needle inoculation to the aphid *Chaetosiphon jacobi* was reported by Sylvester et al. (1974), indicating the possible multiplication of the virus in the vector.

4.1.2.2 Viruses transmitted by leafhoppers

According to the transmission characteristics, the leafhopper-borne viruses may be divided into three groups. The properties of the viruses may be useful to

differentiate those with similar transmission characteristics. None of the leafhopper-borne viruses is transmitted in a nonpersistent manner. Some of the propagative viruses have been successfully inoculated into the leafhoppers, and a few are known to pass through eggs to successive generations (transovarial or congenital transmission).

a. Semipersistent viruses. Rice tungro-associated viruses are transmitted by *Nephotettix virescens* semipersistently (Ling, 1972). Studies by Hibino and Cabunagan (1986) showed that rice tungro spherical virus (RTSV), one component of the rice tungro complex, is independently transmitted by the leafhopper, whereas rice tungro bacilliform virus (RTBV), another component of the complex, can be transmitted only if the leafhopper has already acquired RTSV. These two viruses differ in other properties, such as the virus particle morphological features and the nature of the virus genome. The RTSV has spherical particles with ss-RNA as genome, whereas RTBV particles are bacilliform in shape, containing ss-DNA as genome (Hibino et al., 1991). Maize chlorotic dwarf virus (MCDV) is transmitted in a semipersistent manner by *Graminella nigrifrons* (Choudhury and Rosenkranz, 1983). The virus particles are isometric with ss-RNA as genome (Nault and Ammar, 1989).

b. Circulative (nonpropagative) viruses. The circulative viruses are ingested into the body of the leafhoppers, which transmit them after varying periods of latency. There is no convincing evidence for the multiplication of the virus in the insect body. Beet curly top, *Chloris* striate mosaic, and maize streak viruses belong to the geminivirus group and have paired, small isometric particles with a diameter of 18–22 nm (Mumford, 1974; Goodman, 1981). They are transmitted by different leafhopper species (Bennett, 1963; Goodman, 1981; Harrison, 1985; Nault, 1991).

c. Propagative viruses. Viruses belonging to the Reoviridae, and Rhabdoviridae families, and Marafivirus, and Tenuivirus groups have a definite latent period and are able to multiply in the body of their vectors. Some of them have been reported to be transovarially transmitted to several susccessive generations.

1. *Reoviridae.* The Reoviridae are spherical in shape but larger than other isometric viruses and have ds-RNA as the genome. The clover wound tumor virus (WTV) particles have ds-RNA enclosed in a capsid with a diameter of 60 nm composed of 32 capsomeres (Bils and Hall, 1962; Black, 1965). The virus induces characteristic cytopathic changes in the nerve cells (Hirumi et al., 1967). By injecting the leafhopper extracts serially into virus-free *Agallia constricta*, Black and Brakke (1952) demonstrated that WTV multiplied in the body of the vector insects. Multiplication of WTV in plant and leafhopper, by causing the formation of viroplasmic loci, has been reported by Shikata and Maramorosch (1967).

Transovarial transmission of rice dwarf virus (RDV) through eggs up to several successive generations was reported by Fukushi (1933, 1940). The RDV particles are spherical with a diameter of 70 nm and have ds-RNA as the genome (Shikata, 1962; Whitcomb, 1972). The presence of virus particles in mycetome and the fat body of leafhoppers was observed by Nasu (1963). Maize rough dwarf vius (MRDV) particles have similar size to RDV. The presence of MRDV in different tissues and its multiplication in the delphacid planthopper were demonstrated by Vidano (1970). Sugarcane Fiji disease virus (FDV) transmitted by *Perkinsiella saccharicada* and rice ragged stunt virus transmitted by *Nilaparvata lugens* are also able to multiply in the vector insects (Conti, 1984).

2. *Rhabdoviridae.* The Rhabdoviridae have bacilliform nucleoprotein cores enclosed in three layered membranes on which short projections are seen. Potato yellow dwarf virus (PYDV) transmitted by different species of agallian leafhoppers has bacilliform particles with dimensions of 380 × 75 nm (MacLeold et al., 1966). Two strains of the virus present in eastern North America are transmitted by *Agallia constricta* and *Aceratagallia sanguinolenta* (Black, 1941). Transovarial transmission of PYDV is known (Black, 1953). Multiplication of the virus in cells of *A. constricta* was reported by Chiu et al. (1970). By sap inoculation PYDV can be transmitted to *Nicotiana rustica*, which reacts with local lesions initially and with systemic symptoms later (Black, 1953).

Maize mosaic virus, transmitted by delphacids, has bacilliform particles measuring 242 ± (10 × 48) ± 10 nm, and the presence of the virus particles in both plant and vector tissues has been observed (Herold and Munz, 1965). Wheat striate mosaic virus transmitted by deltocephaline leafhoppers also has bacilliform particles measuring 260 × 80 nm (Lee, 1968).

3. *Marafiviruses.* Maize rayado fino virus is transmitted by the leafhopper *Dalbulus maidis*. The presence of the virus in the body of 80% of the vector, after acquisition feed, has been detected by ELISA tests, but only about 10%–34% of the insects are able to transmit the virus. No cytopathological effects due to the virus are known. The virus has isometric particles with 30 nm diameter and ss-RNA as genome (Gamez and Leon, 1988).

4. *Tenuiviruses.* Maize stripe virus is transmitted by *Peregrinus maidis*; its multiplication in the vector has been indicated by ELISA. The presence of the virus has been detected in different organs and salivary glands of the vector (Nault and Gordon, 1988). Transovarial transmission of rice stripe virus in the vector leafhopper *Laodelphax striatellus* is known (Gingery, 1988). The virus has thin coiled filamentous particles measuring 3 × 950 – 1350 nm and ss-RNA as genome (Toriyama, 1983a, 1983b).

4.1.2.3 Viruses transmitted by whiteflies

Whiteflies transmit different groups of plant viruses which cause many economically important diseases of crops in tropical and subtropical countries. None of

the viruses transmitted by whiteflies has a nonpersistent relationship with its vetor; most seem to be of circulative (nonpropagative) type, and no evidence suggesting the multiplication of the virus in the vector has been obtained so far. Only three species of whiteflies have been reported as vectors of plant viruses; *Bemisia tabaci* is the most frequently reported vector species. Many of the geminiviruses and some viruses belonging to the carlavirus, closterovirus, luteovirus, nepovirus, and potyvirus groups and a DNA-containing rod-shaped virus are transmitted by whiteflies (Duffus, 1985). The virus particle morphological features and nature of the viral genome will be useful to differentiate the viruses transmitted by whiteflies.

4.1.2.4 Viruses transmitted by beetles

Beetles have biting mouthparts and differ from other groups of vector insects which suck the sap while feeding on plants. As the beetles do not have salivary glands, the virus along with plant materials is returned to the mouth by regurgitation. During this process the mouthparts are contaminated with virus particles released from plant tissues. The viruses transmitted by beetles do not appear to have a definite latent period or to be able to multiply in the vector. There is a marked degree of specificity between viruses and beetles (Fulton et al., 1987); they can be easily transmitted and remain stable under in vitro conditions. The beetles transmit viruses belonging to the bromovirus, comovirus, sobemovirus, and tymovirus groups. These viruses have small isometric particles with 25–30 nm diameter and ss-RNA as genome.

4.1.2.5 Viruses transmitted by thrips

The relationship between the viruses and vector thrips is somewhat unique in the sense that only the larvae can acquire the virus from infected plants, not the adults. Both the thrips species and the tomato spotted wilt virus (TSWV) have wide host range. *Thrips tabaci* and three species of *Frankliniella* transmit TSWV more efficiently (Sakimura, 1962). There is no evidence for the multiplication of TSWV in the vector, but the virus is retained by thrips till their death. The virus is not passed through eggs; TSWV can be transmitted by sap inoculation by taking suitable precautions to prevent inactivation, as the virus has only a short period of longevity in vitro. Transmission by thrips may be lost if the virus is repeatedly transmitted by sap inoculation. Peanut bud necrosis virus, earlier considered as TSWV, has been shown to be a distinct virus belonging to tospovirus group (Reddy et al., 1992).

Other viruses transmitted by thrips include tobacco ringspot virus (nepovirus group) and tobacco streak virus (ilarvirus group) (Bergeson et al., 1964; Kaiser et al., 1982). Tobacco ringspot virus is transmitted by a nematode vector also. The presence of envelope for the tospoviruses is a distinguishing feature.

Tobacco ringspot virus and tobacco streak virus have isometric particles without an envelope and have bipartite and tripartite genomes, respectively.

4.1.2.6 Viruses transmitted by eriophyid mites

The relationships between the viruses and their mite vectors have been studied only to a limited extent because of the small size of the mites. Nine viruses have been reported to be transmitted by seven different species of mites (Slykhuis, 1972). Of these, transmission of wheat streak mosaic potyvirus (WSMV) by *Aceria tulipae* has been studied well. *Aceria tulipae* also transmits uncharacterized wheat spot mosaic virus. The WSMV has flexuous rod-shaped particles and is transmitted even after molting by the mite. Only the nymphs can acquire the virus, and there is no convincing evidence indicating the replication of the virus in the mites. The WSMV can be detected in the midgut, body cavity, and salivary glands (Paliwal, 1980). Wheat spot mosaic virus also has a similar relationship with the vector mites. Wheat streak mosaic virus, agropyron mosaic virus, and ryegrass mosaic virus (the latter two viruses are transmitted by *Abacarus hystrix*) may also be transmitted by mechanical inoculation (Slykhuis, 1972).

4.1.2.7 Viruses transmitted by nematodes

Nematodes that transmit plant viruses are all free-living ectoparasites and belong to three genera, viz. *Xiphinema*, *Longidorus*, and *Trichodorus*. The viruses can be acquired and transmitted by all stages of the nematodes. A longer acquisition and inoculation access period results in a greater percentage of transmission. The viruses are retained by the nematode vector for several weeks or months, if the nematodes are kept in fallow soil (Taylor, 1972). Nematode-transmitted viruses are divided into two groups based on particle morphological characteristics. Nepoviruses, with isometric particles, are transmitted by different species of *Xiphinema* and *Longidorus*, whereas tobraviruses, with rod-shaped particles, are transmitted by species of *Trichodorus*. Serologically related viruses, such as grape fan leaf virus and arabis mosaic virus, require *X. index* and *X. coxi*, respectively, indicating specificity of transmission by nematodes (Dias and Harrison, 1963). It is likely that the coat protein of the virus has a role in determining the specificity of transmission. The virus particle morphological features and transmission characteristics may help to differentiate nematode-transmitted viruses.

Many viruses are readily transmitted by mechanical inoculation, and some are seed- and pollen-borne in many host plant species, especially weed plants, which have epidemiological importance.

4.1.2.8 Viruses transmitted by fungi

Studying the characteristics of transmission of plant viruses by fungal vectors has been found to be difficult because of the limitations associated with virus–fungus interactions. In some cases adequate experimental evidence of involvement of the fungus in virus transmission is not available. The fungi reported as vectors of viruses belong to three genera, *Olpidium*, *Polymyxa*, and *Spongospora*. The viruses transmitted by them form a heterogenous group. *Olpidium* spp. transmit isometric viruses, including tobacco necrosis, satellite, cucumber necrosis, and tobacco stunt viruses, whereas rod-shaped viruses, such as wheat mosaic, potato moptop, peanut clump, and potato virus X, are transmitted by the plasmodiophorous genera *Polymyxa* and *Spongospora* (Brunt and Shikata, 1986); some of the viruses may be transmitted by other means also. Pea false leaf roll is also transmitted by aphids and through seeds (Thottapilly and Schumutterer, 1968), whereas potato virus X mainly spreads through leaf contact under field conditions (Teakle, 1972).

4.2 TRANSMISSION OF VIROIDS

Viroids may be transmitted by the following methods under natural or experimental conditions.

4.2.1 Grafting

Potato spindle tuber viroid (PSTVd) can be transmitted by side grafting from infected *Datura stramonium* to tomato plants (O'Brien and Raymer, 1964). However, it is readily transmitted by mechanical inoculation from potato to other susceptible plant species. Other viroids that are transmitted by grafting are the citrus exocortis and chrysanthemum chlorotic mottle viroids. Mechanical transmission is the preferred method of experimental transmission in the case of most viroids.

4.2.2 Dodder Transmission

Some of the viroids have been transmitted by using parasitic dodders (Table 4.4).

4.2.3 Arthropod Transmission

Though early reports suggested the involvement of arthropods in the spread of viroid disease, none of the viroids has been conclusively shown to have any arthropod vector.

Table 4.4 Transmission of Viroids by Dodder

Viroid	Dodder	Reference
Citrus exocortis	*Cuscuta subinclusa*	Weathers, 1965
Chrysanthemum stunt	*C. gronovii*	Keller, 1953
Cucumber pale fruit	*C. subinclusa*	Peters Runia, 1974

4.2.4 Seed Transmission

Potato spindle tuber viroid and tomato bunchy stunt viroid have been found to be transmitted through seeds of infected host plants (Table 4.5).

In the case of other viroids, evidence for seed transmission is not convincing.

4.2.5 Mechanical Inoculation

Most of the plant viroids are readily transmitted by sap inoculation, although some require special methods or appropriate host plant species for successful transmission. Citrus exocortis viroid is inoculated by a contaminated knife or razor blade (Garnsey and Whidden, 1973). Coconut cadang-cadang viroid requires a high-pressure injector for transmission to seedlings (Randles et al., 1977).

4.2.6 Host Range of Viroids

The viroids may have narrow or wide host range indicating the variation in the infecting potential and extent of distribution. The PSTVd has a wide host range

Table 4.5 Seed and Pollen Transmission of Viroids

		Transmission through		
Viroid	Host plant	seed	pollen	Reference
Tomato bunchy top viroid	*Solanum incanum*	+	-	McClean, 1931
	Physalis peruviana	+	-	
Potato spindle tuber viroid	Potato	+	+	Benson and Singh, 1964
	Tomato	+	-	Fernow et al., 1970

distributed in 12 families and 157 species, whereas the burdock stunt (BSVd), avocado sunblotch (ASBVd), coconut cadang-cadang (CCCvd), and chrysanthemum chlorotic mottle (CCMVd) viroids have a narrow host range limited to members of only one family (Table 4.6). Among the 19 families tested, only 3, the Compositae, Cucurbitaceae, and Solanaceae, are susceptible to more than one viroid, and the members of 16 families are susceptible to only one viroid. This specificity of host range may be useful to differentiate the viroids, when considered with other properties. The Solanaceae include members susceptible to a maximum number of viroids (i.e., 8 of 12 viroids).

For the identification of viroids some methods followed for plant viruses cannot be used, because of the absence of the protein coat in viroids. Electron microscopy and serological techniques employed for detection, identification, and assay of plant viruses are not useful for the viroid diseases. Among the methods available diagnostic hosts are commonly used for viroid identification, as in the case of viruses.

Table 4.6 Host Range of Plant Viroids

Families susceptible	Viroids											
	ASB	BS	CCC	CCM	CE	CPF	CS	CV	HS	PST	TBT	TPM
Amaranthaceae	—	—	—	—	—	—	—	—	—	1	—	—
Boraginaceae	—	—	—	—	—	—	—	—	—	1	—	—
Campanulaceae	—	—	—	—	—	—	—	—	—	1	—	—
Caryophyllaceae	—	—	—	—	—	—	—	—	—	1	—	—
Compositae	—	2	—	1	2	2	43	—	—	1	1	1
Convolvulaceae	—	—	—	—	—	—	—	—	—	1	—	—
Cucurbitaceae	—	—	—	—	4	30	—	—	10	2	—	—
Dipsaceae	—	—	—	—	—	—	—	—	—	1	—	—
Gesneriaceae	—	—	—	—	—	—	—	1	—	—	—	—
Lauraceae	2	—	—	—	—	—	—	—	—	—	—	—
Leguminosae	—	—	—	—	2	—	—	—	—	—	—	—
Moraceae	—	—	—	—	—	—	—	—	2	—	—	—
Palmae	—	—	4	—	—	—	—	—	—	—	—	—
Rutaceae	—	—	—	—	14	—	—	—	—	—	—	—
Sapindaceae	—	—	—	—	—	—	—	—	—	1	—	—
Scrophulariaceae	—	—	—	—	—	—	—	—	—	6	—	—
Solanaceae	—	—	—	—	21	11	9	2	1	140	24	3
Umbelliferaceae	—	—	—	—	2	—	—	—	—	—	—	—
Valerianaceae	—	—	—	—	—	—	—	—	—	1	—	—

Source: Peters and Runia (1985).

4.2.6.1 Diagnostic hosts

Most of the viroids cause, in their natural hosts, symptoms which are likely to be similar to those induced by other pathogens, and some cultivars do not exhibit any recognizable symptoms. In such cases use of indicator plants becomes necessary for early detection of infection and identification of the viroid involved, especially for building up disease-free seed stocks, as in the case of potatoes free of spindle tuber viroid and citrus free of exocortis viroid. Diagnostic hosts for different viroids are listed in Table 4.7.

Hop stunt disease may be more reliably detected by cucumber bioassay than by symptom diagnosis or α-acid analysis. For the cucumber bioassay technique, a temperature-controlled greenhouse or plastic house at 30°C is required and the plants are observed for a period of 1 month. The HSVd can be detected by cucumber bioassy in a sample containing a mixture of one HSVd-infected leaf disc and 200 HSVd-free leaf discs (Sasaki and Shikata, 1980). This technique has been used for HSVd detection in samples from fields (Sasaki et al., 1981).

4.3 TRANSMISSION OF BACTERIAL PATHOGENS

Bacterial plant pathogens may be transmitted through infected seeds or propagative materials, rain/irrigation water, infected plant debris, and in some cases insects. Transmission by insects may be either generalized or specialized. Such association with insects may be useful for differentiating some of the bacterial pathogens. The natural avenues of entry for the bacteria into plant hosts are stomata, water pores, lenticels, and flower nectaries; the feeding and oviposition wounds also form important entry points. The insects may be mechanical carriers of bacteria on their body, and in some cases there may be a mutualistic relationship between the insect and the bacteria. The bacteria may remain in the hibernating insects in the absence of susceptible hosts or during adverse environmental conditions (Carter, 1973). The bacterial pathogens which have some kind of association with insects for dissemination are listed in Table 4.8.

4.4 TRANSMISSION OF FUNGAL PATHOGENS

The role played by insects in the transmission of fungal plant pathogens is relatively less important except in a few cases. Most are disseminated by wind, water, humans, and animals. Of course the infected seeds and seed materials transported by humans to different locations form the most important sources of infection. The fungi which are adapted to dissemination by insects produce

Table 4.7 Diagnostic Hosts for Viroid Detection and Identification

Name of viroid	Diagnostic host	Method of testing	Reference
Potato spindle tuber viroid (PSTVd)	*Lycopersicon esculentum* cv. Rutgers/Allerfruheste	Sap inoculation	Raymer and O'Brien, 1962 Singh, 1970
	Freil and *Scopolia sinensis*	Sap inoculation	Singh, 1971
Tomato bunchy top viroid (TBTVd)	*L. esculentum* cv. Rutgers	Sap inoculation	Bensen, et al., 1965
Citrus exocortis viroid (CEVd)	*Citrus medica* cv. Etrog	Grafting	Calavan et al., 1964
	Gynura aurantica	Sap inoculation	Weathers and Greer, 1972
Chrysanthemum stunt viroid (CSVd)	*Chrysanthemum morifolium* cv. Mistlefoe	Sap inoculation	Kaller, 1953
Chrysanthemum chlorotic mottle viroid (ChCMVd)	*C. morifolium* cv. Deep Ridge	Sap inoculation	Dimock et al., 1971
Cucumber pale fruit viroid (CPFVd)	*Cucumis sativus* cv. Sporu	Sap inoculation	VanDorst and Peters, 1974
Hop stunt viroid (HSVd)	*C. sativus*	Sap inoculation	Sasaki and Shikata, 1977a

Source: Diener (1979).

Table 4.8 Insect Transmission of Plant Bacterial Pathogens

Disease	Bacterial pathogen	Insects associated	Nature of association	Reference
Apple fireblight	*Erwinia amylovora*	*Drosophila melanogaster* *Musca domestica* *Lucilia sericata*	General	Ark and Thomas, 1936
Walnut blight	*Xanthomonas juglandis*	*Eriophyes tristriatus* var. *erinea*	General	Rudolph, 1943
Beans haloblight	*Pseudomonas medicaginis* var. *phaseolicola*	*Heliothrips femoralis*	General	Buchanan, 1942
Potato	*Corynebacterium sepedonicum*	*Melanoplus differentialis* *Epicauta pennsylvanica* *Leptinotarsa decemlineata*	General	List and Kreutzer, 1942
Apple bacterial rot	*Pseudomonas melophthora*	*Rhagoletis pomonella*	General	Allen and Riker, 1932
Citrus canker	*Xanthomonas citri*	*Phyllocnistic citrella*	General	Sohi and Sandhu, 1968
Cucurbit bacterial wilt	*Erwinia tracheiphila*	*Diabrotica vittata* *D. doudecimpunctata*	Specialized: required for inoculation, dissemination, and and overwintering	Rand and Cash, 1920; Gould, 1994
Corn bacterial wilt and Stewart's leaf blight	*Xanthomonas stewartii*	*Chaetocneme pulicara* *C. denticulata*	Specialized: required for dissemination and overwintering	Elliott and Poos, 1934
Potato blackleg	*Erwinia carotovora*	*Hylemyia cilicrura*	Specialized; mutualistic symbiosis	Leach, 1933
Oliverknot	*Pseudomonas savastonoi*	*Dacus oleae*	Specialized; mutualistic symbiosis	Petri, 1910

Table 4.9 Insect Transmission of Fungal Pathogens

Disease	Fungal Pathogen	Insects associated	Nature of association	Reference
1. *Incident to pollination*				
Fig endosepsis	*Fusarium moniliforme* var. *fici*	*Blastophaga psenes*	Incidental to pollination	Caldis, 1927
2. *Traumatic injury*				
Azalea flowerspot	*Ovulinia azaleae*	*Heterothrips azaleae*	Through wounds produced by insects	Smith and Weiss, 1942
3. *Feeding on fungal masses*				
Cereal ergot	*Claviceps purpurea*	*Sciara thomae*	Through communication	Mercier, 1911
4. *Feeding and oviposition wounds*				
Cucumber anthracnose	*Colletotrichum lagenarium*	*Diabrotica undecimpunctata howardii*	Through feeding wounds	Liby and Ellis, 1954
Cotton boll rot	*Fusarium moniliforme*	*Anthonomus grandis*	Through feeding and oviposition wounds	Bagga and Laster, 1968
		Heliothiszeae		
	Alternaria tenuis	*Lygus lineolaris*		
Sugarcane red rot	*Colletotrichum falcatum*	*Diatraea saccharalis*	Through feeding wounds	Abbott, 1955
		Anacentrinus subnudus		
Apple brown rot	*Sclerotium fructigena*	*Forficula auricularia*	Through feeding injuries	Croxall et al., 1951
Cocoa dieback	*Calonectria rigidiscula*	*Sahlbergella singularis*	Through feeding wounds	Crowdy, 1947
		Distantiella theobroma		Owen, 1956

(*continued*)

Table 4.9 Continued

Disease	Fungal Pathogen	Insects associated	Nature of association	Reference
5. *Feeding punctures*				
Corn and sorghum downy mildew	*Sclerospora sorghi*	*Rhopalosiphon maidis* *Schizaphis graminum*	Through feeding punctures	Naqvi and Futrell, 1970
Pine burn blight	*Diplodia pinea*	*Aphrophora parallela*	Through feeding punctures	Haddow and Newman, 1942
Cotton internal boll disease	*Nematospora gossypii* *Nematospora* (spp.)	*Dysdercus* (spp.)	Through feeding punctures	Frazer, 1944 Mendes, 1956
Coffee bean rot	*Nematospora* spp.	*Antestia lineaticollis*	Through feeding punctures	LePelley, 1942
Soybean yeast spot	*Nematospora corylii*	*Acrosternum hilare*	Through feeding punctures	Clarke and Wilde, 1971
Rice grain discoloration	*Nematospora corylii*	*Oebalus pugnax*	Through feeding punctures	Daugherty, and Foster, 1966
Cotton lint rot	*Nigrospora oryzae*	*Stieroptes reniformis*	Through feeding punctures	Laemonlen, 1969
6. *Symbiotic association*				
Apple fruit rot and canker	*Gloeosporium perennans*	*Eriosoma lanigerum*	Symbiotic	Zeller and Childs, 1925
Oak wilt	*Ceratocystis fagacearum*	Many species belonging to Nitidulidae, Scolytidae, and Drosophiladae	Required for spermatization and transmission	Dorsey and Leach, 1956
Dutch elm	*Ceratostomella ulmi*	*Hylurgopinus rufipes* *Scolytus multistriatus*	Required for transmission	Hoffman and Moses, 1940

spores in sticky masses which attract the insects. The spores are often deposited in the wounds formed during feeding or oviposition. The fungal pathogens may also initiate infection a) incident to pollination, b) through traumatic injury, c) as a result of internal and external contamination of the insect caused by feeding on fungal masses, d) through feeding punctures, and e) as the result of a symbiotic association between the fungus and insect (Carter, 1973). The fungi–insects associations leading to transmission of and infection by the fungal pathogens are presented in Table 4.9.

SUMMARY

The primary method of dissemination/transmission may vary, depending on the nature of the pathogens. However, all of them may be transmitted through infected seeds and seed materials. Fungal and bacterial pathogens are largely disseminated by wind, water, and soil, whereas viruses depend on arthropod vectors and soil-borne nematodes or fungi for their spread, in addition to their transmission through mechanical inoculation, grafting/budding, and parasitic dodders. The methods of transmission or dissemination of pathogens, as such, may not be useful for differentiating them. However, the specificity of the relationship of the pathogens with their vectors may indicate the identity of pathogens in certain cases. Various modes of transmission of plant viruses, physical properties in expressed plant sap, diagnostic hosts, and transmission characteristics associated with different virus–vector interactions as the basis of virus identification are described. The mechanical/biological relationships of pathogens with their vectors are discussed.

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5
Cross-Protection

When a plant already infected with one strain of a virus is inoculated with another strain of the same virus, symptoms due to the challenging strain usually fail to appear. Tobaccos inoculated with a strain of tobacco mosaic virus (McKinney, 1929) or potato virus X (Salaman, 1933) did not develop any additional symptoms when inoculated later with another strain of the respective virus. This phenomenon, known as cross-protection, is one of the biological properties of the viruses used to establish relationships among viruses and their strains.

The degree of cross-protection, as a measure of relatedness of strains, may be determined quantitatively by inoculating a systemically invading strain and local lesion-forming strain on an appropriate host plant species. Thus, relationships between tobacco mosaic virus strains can be assessed by using *Nicotiana sylvestris*, which is systemically infected by type strain, and local lesions are induced by aucuba mosaic strain. The half-leaf method of testing may be followed (Kunkel, 1934). The type strain is inoculated in one-half of the leaf and 2–3 days later the whole leaf is challenge-inoculated with the local lesion-forming strain. The half-leaf inoculated earlier with the systemic strain will develop very few lesions as compared to the unprotected opposite half leaf.

When the systemic spread of the virus is slow, an indicator local-lesion-forming strain may be employed, as in the case of cucumber mosaic virus (CMV) for the identification of its strains. Very young plants of *Zinnia elegans* are inoculated with systemic strains of CMV and 12–14 days later the leaves are challenged with local-lesion-forming strains. Relationships between two systemic strains may be established, if the macroscopic symptoms caused by them are quite distinct. Cross-protection between strains of potato virus X (PVX) may be demonstrated by inoculating the mild systemic strain on *Datura*

stramonium plants followed by severe strains causing necrotic mottle mosaic symptoms (Loebenstein, 1972).

Cross-protection may be observed in persistent viruses transmitted by vectors, but not by mechanical inoculation. Potato plants inoculated with a mild strain of potato leaf roll virus (PLRV) are protected against severe strains of PLRV introduced by *Myzus persicae* (Harrison, 1958).

When the external symptoms induced by strains of a virus are not markedly different, differences in cytopathic effects caused by strains of the virus may form the basis to distinguish them. Bean yellow mosaic virus forms intranuclear inclusions in the epidermal cells of leaves of *Vicia faba*, and the inclusions can be readily seen by staining with trypan blue. The occurrence and size of the inclusions differ, depending on the strain of bean yellow mosaic virus. If plants are already infected by one strain of the virus, formation of inclusion by another strain is inhibited, indicating the cross-protection offered by the challenged strain against the challenging strain. On the other hand, such protection is not afforded to other unrelated viruses affecting bean (Mueller and Koenig, 1965).

Although cross-protection against strains of many plant viruses has been reported, some viruses do not exhibit such protection against their strains. Strains of sugar beet curly top virus do not afford protection in neither sugar beet nor water pimpernel (*Samolus parviflorus*) (Bennett, 1955). Tobacco (potato) veinal necrosis virus infection does not offer protection to tobacco or potato plants against serologically related strains (Klinowski and Schmelzer, 1960). Only some strains of PVY, not all strains, can protect plants from other severe strains of the virus (Loebenstein, 1972). None of the strains of tobacco streak virus is able to protect tobacco against other strains (Fulton, 1978).

Interference between strains of a viroid and also between unrelated viroids has been observed (Niblett et al., 1978). Tomato plants inoculated with a very mild strain of potato spindle tuber viroid (PSTVd) were protected against its severe strain when challenged 2 weeks after inoculation with mild strain of PSTVd (Fernow, 1967). When both mild and severe strains were inoculated simultaneously, the severe strain suppressed the development of mild strain, though the inoculum contained very high concentrations (100-fold) of the mild strain (Branch et al., 1988). Interference between a mild exocortis agent (CVd-IIa) and the cachexia agent (Cvd-IIb) has been observed (Semancik et al., 1992). The mild isolate CVd-IIa could interfere with the replication and/or accumulation of the severe cachexia agent CVd-IIb in citron (*Citrus medica*), indicating the possibility of employing CVd-IIa for the control of cachexia in commercial plantings.

The results of cross-protection tests have to be interpreted cautiously, because of interference between unrelated viruses (Bos, 1970b) and absence of

cross-protection between related strains of some viruses. It is desirable to use reciprocal inoculation procedures by introducing the viruses or strains sequentially into one series of plants and reversing the order of inoculation in the second series of plants. High or complete protection can be considered good evidence for a close relation between the strains or viruses tested. Absence or a low degree of protection may not give a conclusive picture, and the relationship or lack of relationship between the viruses has to be established by other tests.

SUMMARY

Interference occurs generally between related viruses or strains, resulting in protection of plants against the challenging virus or strains. This phenomenon of cross-protection is used as a basis to establish relationships between viruses or strains. The extent of reduction in disease intensity or failure of formation of intracellular inclusions induced by challenging strains will indicate the degree of cross-protection, which may be used as a measure of relatedness between strains. The possibility of using cross-protection for the management of virus disease is indicated.

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6
Chemodiagnostic Methods

6.1 DETECTION OF PLANT VIRUSES

Different kinds of chemicals have been used to distinguish plants infected by viruses and MLOs. Lindner (1961) has reviewed the various chemical tests that have been used for the diagnosis of virus infection in plants. These tests depend on the reaction between the chemicals used for detection and the compounds present in infected plants, which results in the development of a recognizable color either in the extracts or in tissues. These tests can be grouped according to the nature of the compounds involved.

6.1.1 Protein Test

Virus-infected plants have protein profiles different from those of healthy plants. Accumulation of free amino acids and amides in infected plants has been reported by many workers (Diener, 1963; John, 1963; Narayanasamy and Ramakrishnan, 1966). Potato tubers infected by leaf roll virus had two- to threefold higher concentrations of glutamine than comparable healthy tubers (Allison, 1953). On the basis of the higher glutamine content of diseased tubers, Cornuet (1953) developed a color test using Nessler's reagent to distinguish diseased tubers. Chiu et al. (1958) reported that apple trees infected by rough skin disease could be identified by a test based on higher arginine content. Such a test is, however, not used because of the inconsistent results obtained.

6.1.2 Carbohydrate Test

The starch iodine lesion test was developed by Holmes (1931) to detect the diffused lesions formed by potato virus X and tomato spotted wilt virus. This

test is based on the reduced photosynthetic activity and poor translocation of starch from infected tissues. The infected leaves are decolorized by hot alcohol and then dipped into iodine in potassium iodide solution. This test was modified and used for the detection of rice tungro virus infection. The rice leaves are excised before sunrise, and the cut ends of the leaves are dipped into iodine in potassium iodide solution. Dark blue streaks appear in the infected leaves (IRRI, 1983). The iodine test, though simple, cannot be reliably applied for the detection of rice tungro virus infection under varied conditions (Narayanasamy, 1989).

The accumulation of sugars in different tissues of virus-infected plants has been the basis of detection of virus infection in certain host plants. Starch accumulates after citrus tristeza virus infection in the parenchyma above the bud union of sweet orange grafted on sour orange root stock. Applying 3% alcohol solution of iodine after scraping the bark slightly at the bud union helps to identify infected plants (Bitancourt, 1944). Mature potato leaves infected by leaf roll virus contain higher concentrations of glucose, which reacts with aniline phthalate, producing a distinctive blackish brown color (Martin, 1954).

The presence of pentoses in cells of tissues of virus-infected plants is revealed by the color formed by reaction with phloroglucinol and concentrated HCl. Free hand sections of stem or petiole are immersed in a 1% alcoholic solution of phloroglucinol for 1 min followed by treatment with the concentrated HCl until the xylem stains red. In healthy tissue xylem cells alone are stained, whereas the phloem cells, in addition to xylem, turn pinkish in infected plants. These tests have been found to be useful in detecting potato leaf roll infection in potato, exocortis infection in *Poncirus trifoliata*, and bud necrosis virus infection in groundnut (Childs et al., 1958; Lindner, 1961; Narayanasamy and Natarajan, 1974). The accumulation of the intermediary compound 4-methyl-*d*-glucuronic acid in leaves of grapevine infected by leaf roll virus could be detected by paper chromatography in 625 infected plants but not in 700 healthy plants or in plants infected by other diseases, indicating the specific nature of the test (Ochs, 1960). The presence of two unidentified chemical compounds in citrus leaves infected by decline disease could be detected by paper chromatography. The abnormal compounds were detected prior to the appearance of virus symptoms, making this technique useful for disease diagnosis (Newhall, 1975).

6.1.3 Enzyme Test

Stimulation of oxidases in virus-infected plants is frequently observed (Diener, 1963). A solution of 2,6-dichlorophenol indophenol is decolorized with sodium bisulfite and the extracts from diseased and healthy leaves are added. The blue color reappears much earlier in tubes containing the extracts of diseased plants.

Symptomless infection of dahlia mosaic virus infection in dahlia could be detected with a high degree of accuracy by this technique. Tests with potato shoots infected by potato virus Y had 95% agreement with serological tests on 1000 tubers (Lindner, 1961).

Rapid reduction of the dye 2,3,5-triphenyl tetrazolium chloride by the virus-infected tissues forms the basis for identification of plants infected by southern bean mosaic virus, common bean mosaic virus, bean pod mottle virus, sugar beet yellows and curly top viruses, and tobacco mosaic virus (Beal et al., 1955) and banana bunchy top and infectious chlorosis viruses (Summanwar and Mazama, 1982). Association of greater specific activity of acid phosphatase with clover yellow mosaic virus infection was reported by Tu (1976). The infected leaves and root nodules exhibited a 100% more acid phosphatase activity than healthy controls.

6.1.4 Polyphenol Test

Virus infection is known to increase the polyphenol contents in infected plants. The test developed by Lindner et al. (1950) consists of heating the leaf discs in acidulated alcohol to give a red color. Whole leaves are tested by heating in alcohol containing formaldehyde and then in sodium hydroxide. The polyphenols remain fixed in leaves, giving a blue color. The color that develops may not, however, be specific for any particular substance.

6.1.5 Nucleic Acid Test

The ultraviolet (UV) absorption spectra of leaf extracts form the basis of differentiating virus-infected plants. Maximum absorption occurs at 260 nm with leaf extracts of plants infected by mosaic viruses, whereas extracts of tissues infected by yellows type disease have the peak at 280 nm (Lindner, 1961). After removal of chlorophylls by heating in alcohol, leaf discs are extracted with acidulated alcohol. The absorption curves are prepared for the extracts. This technique was employed for rapid assay of tobacco mosaic virus (TMV) in tomato and quantitative assay of stone fruit ringspot virus in cucumber and for detection of TMV and potato virus X in tobacco. Kimura and Black (1972) reported that UV absorbance of extracted RNA was correlated with the counts of purified clover wound tumor virus particles determined by electron microscopy.

6.1.6 Fluorescence Test

Fluorescence associated with the extracts of certain plants infected by viruses seems to have diagnostic value, and this property has been used to detect car-

nation mosaic virus infection. A portion of the vegetative shoot in distilled water is autoclaved for 45 min at 1.06 kg/cm^2 pressure. The extract is then mixed with *n*-butanol and a drop of ammonium hydroxide is added to the mixture. A light pink color appears between the water and *n*-butanol phases when viewed under UV light. Extracts from healthy shoots do not show any fluorescence. When the results of the fluorescence and infectivity tests were compared, it was observed that the fluorescence test detected infection in 35,670 plants, whereas 35,678 plants were found infected out of a total of 35,763 plants tested over a period of 3 years (Thomas et al., 1951).

The possibility of using a remote sensing infrared thermometer for previsual diagnosis of citrus young tree decline was tested by Edwards and Ducharme (1974). It was observed that the temperature of trees in early decline was virtually identical to that of healthy trees. They concluded that tree remote temperature measured within the spectral range of 6.5–20 μm was not a reliable indicator for making these diagnoses.

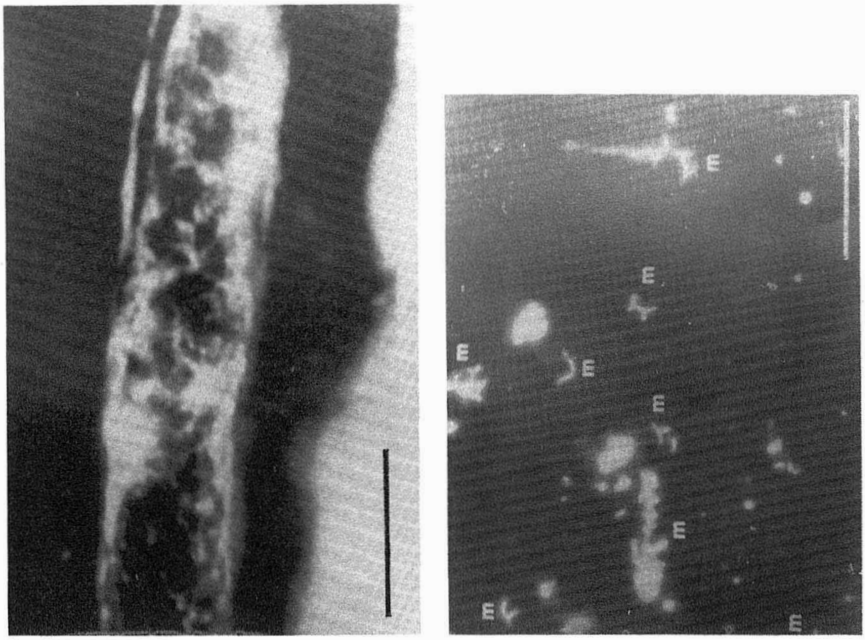
6.2 DETECTION OF MYCOPLASMA-LIKE ORGANISMS

6.2.1 Histochemical Methods

Use of Dienes's stain for the detection of MLO infection was first reported by Deeley et al. (1979). The presence of MLOs in different plant species was detected by using Dienes's stain: rice yellow dwarf (Srinivasan, 1982; Reddy, 1986; Rao, 1988), brinjal little leaf and sandal spike (Srinivasan, 1982), and coconut root (wilt) (Solomon and Govindan Kutty, 1991). Dark blue color is retained in the phloem cells of MLO-infected plants, indicating the positive reaction.

The presence of *Spiroplasma citri*, MLOs causing aster yellows (AY), pear decline (PD), and tomato big bud (TB); or clover club leaf (CCL) agent could be observed in the sieve elements of infected plants by treating the midribs with macerating enzymes (cellulase and macerozyme) and then separating the sieve elements. The helical motile spiroplasma, pleomorphic MLOs, and slender rod-shaped CCL agent could be visualized under dark field microscope (Lee and Davis, 1983) (Figs. 6.1A and B and 6.2). This simple technique is useful to study morphological and other properties of viable cells of these pathogens present in the sieve elements.

Hiruki and da Rocha (1986) developed a histochemical method for diagnosing MLO infections in *Catharanthus roseus* by using a fluorescent DNA binding stain, 4,6-diamidino-2-phenyl indole-2 HCl (DAPI). The rapid compression technique developed by Dale (1988) can be employed for the rapid detection of MLOs. Excised leaf midrib sections are firmly compressed and crushed by a small spatula. The vascular system is then removed with fine forceps and



(A) (B)

Figure 6.1 Typical cells of *Spiroplasma citri* in infected sieve element of *Catharanthus roseus*; bar represents 10 μm; B, single and clumped MLO released from osmotically shocked sieve tube elements of pear decline infected plant; bar represents 10 μm (American Phytopathological Society, Minnesota, USA). (Courtesy of Lee and Davis, 1983.)

fixed in Karnovsky's fixative for 20 min. The vascular tissue, after rinsing in phosphate buffer for 5 min is placed into DAPI solution for 3–5 min. After staining, the tissue is mounted in a drop of DAPI solution on a glass slide and examined under a fluorescence micrscope. By this technique AY-MLO in aster, blue berry stunt MLO in periwinkle, and *Spiroplasma citri* in periwinkle can be detected.

Using DAPI, infection of MLOs in several plant species has been detected. Sinclair et al. (1989) found the DAPI fluorescence test superior to Dienes's stain for histological detection of aster yellows MLO in lilac witches'-broom. In different plant species MLOs also could be diagnosed by using the DAPI fluorescence test (Hibben et al., 1991). Sinclair et al. (1992) reported that the DAPI fluorescence test detected ash yellows MLO in white ash trees as consistently as the DNA probes in the dot hybridization technique. Griffiths et al. (1994) used the DAPI fluorescence test to detect MLOs in six species of

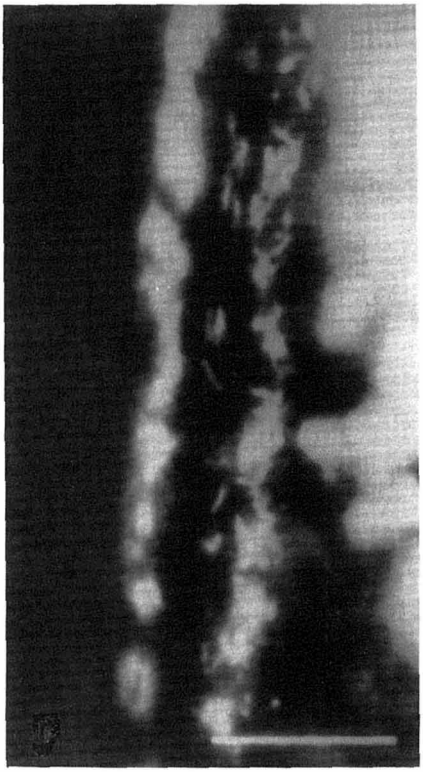


Figure 6.2 Sieve elements from clover club leaf-infected *Catharanthus roseus* plants containing rod-shaped bacterial cells. bar represents 10 μm (American Phytopathological Society, Minnesota, USA). (Courtesy of Lee and Davis, 1983.)

ash and lilac in 13 locations in the United States. The histochemical methods are rapid and relatively sensitive but are nonspecific in certain cases. They can be, however, used for preliminary diagnosis of MLO infection, when good-quality antiserum is not available.

6.3 DETECTION OF FUNGAL PATHOGENS

Fungal pathogens present in plant tissues are examined directly under microscopes either by making sections or after isolating them in appropriate media. In most cases, conventional staining procedures or selective media for isolation may be sufficient and no special histochemical methods are necessary. Moreover, it is possible for sufficiently trained plant pathologists to make a rapid

presumptive identification of the pathogens from characteristic disease symptoms. However, some fungal pathogens may not induce distinctive symptoms, and it may be difficult to isolate and identify them in culture. Some fungi may grow very slowly in culture, taking weeks or even months to produce spore-bearing structures required for identification. Under such conditions alternative reliable methods are required for detection and identification of fungal pathogens.

6.3.1 Use of Selective Media

The growth and development of a fungal pathogen may be specifically encouraged by providing a selective medium. Cultural differences and enhanced micromorphological features were observed when Czapek solution agar containing 20% saccharose was used to grow *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans*. *Gibberella fujikuroi* could be readily distinguished from the other two species on the basis of the differences in colony color and texture. Lowering of the pH of the medium from 7.7 to 4.4 intensified these differences without affecting micromorphological characteristics adversely (Clear and Patrick, 1992). Duffy and Weller (1994) developed a semiselective medium (R-PDA) consisting of dilute potato-dextrose agar amended with 100 μg/ml of rifampicin and 10 μg/ml of tolclofosmethyl, useful for isolation and diagnosis of the presence of *Gaeumannomyces graminis* var. *tritici*, which was able to alter the color of rifampicin in R-PDA from orange to purple in about 24 hr. It was found that R-PDA was more effective in isolating *G. graminis* var. *tritici* than the semiselective medium for *Gaeumannomyces graminis tritici* (SM-GGT3), another selective medium used earlier.

6.3.2 Biochemical Test

The presence and viability of spores of fungal pathogens on seeds can be determined by fluorescein diacetate assay (FDA). Lipase activity is detected consistently in the extracts of viable teliospores of *Tilletia controversa*, but not in extracts of autoclaved spores. Detection of lipase activity is consistent when 4-methyl-umbelliferyl-palmitate is used as a substrate. Fluorescein diacetate assay can be used to get the results rapidly, and the time required to assess the viability of a teliospore population may be reduced to 1 hr from the 2 months needed for other methods of assay (Chastain and King, 1990).

Oliver et al. (1993) produced strains of *Cladosporium fulvum*, which infects tomatoes, and *Leptosphaeria maculans*, pathogen of brassica crops, that constitutively expressed β-glucronidase, the activity of which was used to detect histochemically the presence of fungal hyphae in host plant tissues. In addition, the β-glucuronidase activity of *C. fulvum* could be used to quantify

fungal biomass in the cotyledons of infected seedlings. The enzyme β-glucuronidase is stable, and its activity can be quantified by fluorimetric assays by using the substrate 4-methyl-umbelliferyl β-D-glucuronide (MUG). It can also be detected histochemically by using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as the substrate. This approach is useful, because this enzyme is essentially absent from all fungi and plants tested.

Wheat loose smut pathogen (*Ustilago nuda*) can be detected in seed embryos by extracting them in 5% NaOH and concentrating them. The embryos are stained with trypan blue to reveal the presence of fungal mycelia (Khanzada and Mathur, 1988). The presence of intercellular mycelium and haustoria in smut-infected sugarcane tissues could be visualized by staining with trypan blue. This test is useful for rapid diagnosis of smut infection under field conditions (Sinha et al., 1982; Padmanabhan et al., 1995).

6.3.3 Isozyme Analysis

The usefulness of isozyme electrophoresis for the detection and identification of fungal pathogens such as *Peronosclerospora* spp. (Bonde et al., 1984), *Phytophthora cinnamomi* (Old et al., 1984, 1988), *Fusarium oxysporum* (Bosland and Williams, 1987), and *Trichoderma* spp. (Stasz et al., 1989) has been assessed. The enzymes are separated by electrophoresis in a horizontal starch gel. Isozyme patterns are recorded according to their relative mobility, and each band is considered as an allele of a specific locus. The bands are then labeled alphabetically from the slowest to the fastest.

Bonde et al. (1989) reported that isozyme patterns could be used to distinguish between teliospores produced by *Tilletia indica* and *T. barclayana* present in stored grains, storage facilities, and transportation vehicles. The isozyme patterns were determined by horizontal starch-gel electrophoresis and staining. Oudemans and Coffey (1991a, 1991b) suggested the use of isozyme analyses for classifying 12 papillate *Phytophthora* species. Oudemans and Coffey (1991a) reported that in terms of isozyme analysis *P. cambivora*, *P. cinnamomi*, and *P. cactorum* were clearly separated and each species could be further subdivided into electrophoretic types (ETs). Three enzymes, viz. phosphoglucose isomerase, malate dehydrogenase, and lactate dehydrogenase, when fractionated by cellulose acetate electrophoresis, were found to have diagnostic potential, permitting clear differentiation of these three species. Further studies by Oudemans and Coffey (1991b) to examine intraspecific diversity and interspecific relatedness of different papilate species of *Phytophthora* indicated interspecific relationships which cannot be predicted on the basis of morphological comparisons alone. *Phytophthora medii* and *P. botryosa* clustered together, indicating a very close genetic relatedness; *P. katsurae* and *P. heveae*

also formed a single cluster; *P. capsici* and *P. citrophthora* formed another distinct cluster.

6.3.4 Cell Wall Glycoprotein Analysis

Snow rot disease of winter cereals is caused by *Pythium graminicola*, *P. iwayamai*, *P. okanoganense*, *P. paddicum*, *P. vanterpoolii*, and *P. volutum*. The cell wall glycoproteins of these six species (about 25–40 kDa) form the major component among cell wall proteins of each species. The electrophoretic patterns of the glycoproteins, detected with Coomassie brilliant blue, lectin, and antibody, exhibited sufficient interspecific polymorphism and intraspecific stability to allow identification and classification of the six *Pythium* spp. that cause snow rot disease (Takenaka and Kawasaki, 1994).

6.4 DETECTION OF BACTERIAL PATHOGENS

Bacterial pathogens induce variable symptoms, and some diseases have long incubation periods before symptoms are expressed. It is not unusual to see close similarities between symptoms of bacterial infection and other biotic and abiotic factors. Identification of bacteria requires, as a first step, the isolation of bacteria, followed by a series of physiological or biochemical tests and a pathogenicity test. To distinguish pathovars, type of symptoms induced by the bacterial isolate in a particular host plant species/cultivar and host range of the bacterial isolate have to be studied. The identification of bacterial pathogens by these studies is very time-consuming and laborious and sometimes inconclusive.

6.4.1 Use of Selective Media

It is possible to isolate different bacterial pathogens by using specific media (Schaad, 1988). Suitable selective medium has to be identified for each bacterial species. Of the 13 media tested, Nauman et al. (1988) found that nutrient agar containing 5% saccharose (NASA), NASA + 2 ppm crystal violet, Kings agar B, and Kings agar Bf were most suitable for isolating and detecting *Pseudomonas syringae* pv. *phaseolicola* in *Phaseolus vulgaris* seeds. The pathogen could be detected at a concentration of $< 10^2$ cell/ml in the water used to rinse the seeds for 6 hr at 22°C, whereas immunofluorescence and enzyme-linked immunosorbent assay (ELISA) tests could detect it only at a minimum concentration of 10^5 cells/ml.

Jansing and Rudolph (1990) developed a sensitive and quick test to determine the bean seed infection by *Pseudomonas syringae* pv. *phaseolicola*.

Seeds are soaked in physiological saline solution for 20 hr at 4°–6°C. Saline solution, concentrated by centrifugation, is streaked on a semisolid selective medium which supports the growth of all strains of the pathogen. The pathovars are distinguished by determining phaseotoxin production by *Escherichia coli* bioassay. The pathogen may be detected even when 1 out of 50,000 seeds is infected by the bacterial pathogen.

6.4.2 Biochemical Tests

In addition to the morphological features, several other biochemical/physiological properties have to be studied for the proper identification of bacteria. The following are the important characteristics useful for identification and classification.

6.4.2.1 Properties of bacteria in culture

a. Size of bacterial cells. The size of the bacteria may vary, depending on cultural conditions. However, the size measurements will be useful, if the age of culture, constituents and pH of the nutrient medium, incubation temperature, and staining method are similar, when comparisons are made between different bacterial isolates/species.

b. Location of flagella. The presence or absence of flagella and their location/distribution in the bacterial cells are characteristic of the bacterial species. The procedure of Zettonow as modified by Stapp (1966) can be employed for staining the flagella. The flagella may be located as a single polar flagellum at one end of the bacterial cell or a group of flagella either at one end (lophotrichous) or at both ends (amphitrichous) of the bacterial cells. In some cases the flagella may be distributed all over the bacterial cell (peritrichous).

c. Gram Staining. The retention of Gram's stain by the bacterial cells is one of the important properties used for the identification of the bacteria. The Gram's complex, consisting of magnesium-ribonucleoprotein, which retains the stain is present in the gram-positive bacteria, whereas magnesium is present in the ionic form in gram-negative bacteria. Among the bacterial plant pathogens bacteria belonging to the genus *Corynebacterium* are gram-positive, whereas those in other genera are gram-negative.

d. Presence of food reserve materials in bacteria. Volutin, fat, glycogen, and iogen are the reserve materials present in bacterial cells. Of these, volutin and fat alone have been observed in plant pathogenic bacteria. Volutin is colorless, viscous, and less refractive than the fat globules seen in bacteria. It is not stained by the fat stains and is different from glycogen in its reaction with Lugol. Presence of volutin in *Agrobacterium tumefaciens* can be observed. Fat

in the bacterial cells may be seen as highly refractive globules varying in size. When stained with dimethyl paraphenyl diamine, globules are stained blue.

e. Reduction of nitrates. Nitrates are reduced to nitrates, ammonia, and finally free nitrogen by bacterial activities. The ability to reduce nitrates accompanied by visible gas formation is another property used to classify the bacteria.

f. Hydrogen sulfide production. Production of hydrogen sulfide in the liquid cultures of bacteria is tested by using a strip of lead acetate paper which turns brown to black after incubation.

g. Production of indole. Production of indole in the liquid medium containing tryptophan by the activities of bacteria is determined by adding Ehrlich reagent after incubation of the culture for 1 to 7 days. Appearance of cherry red color indicates the production of indole.

h. Utilization of carbon and nitrogen compounds. The ability of different species of bacteria to utilize different carbon compounds as sources of energy varies, and such differences may be useful in the identification of pathogenic bacteria. Production of acid or gas or both or neither from a certain compound by the bacteria is determined. Quantitative determinations of gas production are made by using ferment tubes with a liquid substrate. Acid production is detected by indicators such as litmus bromothymol blue and bromocresol purple in 0.4% alcoholic solutions.

The nitrogen sources that support the multiplication of bacteria are also studied by providing different organic and inorganic sources. Different amino acids are also incorporated to find out the suitable amino acids required for the growth of the bacteria. The differences in the requirements of carbon and nitrogen sources for optimal growth of bacteria may help in the identification of bacterial species.

i. Starch hydrolysis. The extent of diastase activity is assessed by using Lugol's iodine after incubation of the bacterial culture in medium containing starch. Phytopathogenic bacteria such as *Xanthomonas begoniae* have strong diastatic activity.

j. Lipolytic activity. The bacterial culture is inoculated on agar medium in a petri dish, and olive oil, cotton seed oil, or castor oil is sprayed with an atomizer to produce fine droplets. The transparent oil droplets turn granular and opaque if the bacterial species has lipolytic activity. This property has been used for differentiating the bacterial species.

k. Action on litmus milk. The litmus solution is prepared by dissolving litmus granules (80 mg) in 40% alcohol (300 ml). A mixture of skimmed milk

with low butter content and 2% litmus solution is taken in sterilized test tubes, inoculated with the test bacterial culture, and incubated for 10 days at room temperature. Change of color to red indicates a positive reaction.

l. Gelatin liquefaction. Gelatin medium consisting of beef extract (3.0 g), peptone (5.0 g), gelatin (120.0 g), and distilled water (1000 ml) is transferred to test tubes at 10 ml/tube after sterilization. The test bacterium is stab-inoculated and incubated for 5 days at 20°C. The medium is liquefied, indicating a positive reaction.

m. Ammonia production. The test bacterium is inoculated on peptone nitrate broth, placed into test tubes, and incubated for 72 hr at room temperature. Formation of reddish brown precipitate when Nessler's reagent is added indicates the production of ammonia by the bacterial pathogen.

n. Kovocs's oxidase test (Kovocs, 1956). Place Whatman No. 1 filter paper in a sterilized petri dish and add 3–4 drops of freshly prepared 1% aqueous solution of tetramethyl paraphenylenediamine dihydrochloride at the center of the filter paper. Transfer a loopful of concentrated bacterial growth from a 24–48 hour old culture and rub as a strip of 1 cm in length across the impregnated filter paper. The appearance of purple color indicates oxidase activity of the test bacteria.

o. Thornley's arginine dehydrolase activity. Thornley's arginine medium, containing peptone (1.0 g), NaCl (5.0 g), K_2HPO_4 (0.49 g), L-arginine HCl (10.0 g), phenyl red (0.01 g), phenol red (0.019 g), agar (3.0 g), and distilled water (1000 ml), pH 7.2, is stab-inoculated with a loopful of bacterial culture, covered with sterile molten petroleum jelly (Vaseline), and incubated for 72 hr at room temperature. A positive reaction is inferred from the appearance of red color.

p. Tyrosinase activity. Dye's medium (Appendix 2(iv)B) in slants is inoculated with the test bacterium. Tyrosine in the medium is converted into brown colored melanin by the activity of tyrosinase if the bacterial pathogen has the enzyme.

The important characteristics of the genera of plant pathogenic bacteria are presented in Table 6.1.

6.4.2.2 Properties of bacterial cells

a. Direct colony thin layer chromatography (Matsuyama et al., 1993a). One loopful of bacterial culture is applied directly to the origin line on silica gel thin layer chromatography (TLC) plate and dried completely. The plate is developed at 25°C for 10 min, using chloroform methanol (2:1, v/v) till the solvent moves

up to 6 cm from the origin line and dries. After scraping out the bacterial cells the TLC is run in the same direction, using chloroform methanol water (60:25:4, v/v/v), for about 1 1/2 hours. After drying the plate, ninhydrin is sprayed and dried at 100°C for 10 min for the development of spots. The lipid profiles of various bacterial species tested show distinct differences (Fig. 6.3). *Erwinia chrysanthemi* and *E. carotovora* subsp. *carotovora* show marked differences in their lipid profiles. On the basis of lipid profile differences three major types of pseudomonads can be distinguished: The chromatograms of *P. gladioli* pv. *gladioli*, *P. glumae*, *P. plantarii*, *P. caryophylli*, and *P. cepacia* resemble each other, and they are designated Cepacia type. The *P. solanacearum* is categorized as Solanacearum type, whereas other pseudomonads are included in the Syringae type (Matsuyama and Furuya, 1993b). Striking differences are seen between the chromatograms of gram-positive (*Clavibacter* spp.) and gram-negative bacteria. The chromatograms of *Erwinia* spp. are quite distinct from those of *Xanthomonas* spp. and *Agrobacterium tumefaciens* (Matsuyama et al., 1993a). (Fig. 6.4). The usefulness of the direct colony TLC method for rapid identification of different *Pseudomonas* spp. in ribosomal RNA (rRNA) homology group II was reported by Matsuyama (1995). By employing the high-performance liquid chromatography (HPLC) technique, two phytopathogenic bacteria, *Clavibacter* and *Erwinia*, could be rapidly differentiated. Distinct differences were also observed at species level in *Erwinia* (Matsuyama, 1995).

b. Polyacrylamide gel electrophoresis. Two major phenotypic groups could be identified by using polyacrylamide gel electrophoresis (PAGE) and silver staining of sodium dedecyl sulfate of lysed cells of *Xanthomonas campestris* pv. *vesicatoria*. Broad dark gray bands with molecular weight (MW) 32–35 kDa and 25.57 kDa designated α and β are present in different strains. The α band is present in 192 or 197 tomato race 1 strains and the β-band is seen in all 55 strains of tomato race 2. Moreover race 1 strains expressing the α band cannot hydrolyze starch (Amy^-) or degrade pectate (Pec^-), whereas most race 2 strains are Amy^+ and Pec^+. Silver staining of protein profiles and testing for amylolytic activity may be useful to differentiate strains of *X. campestris* pv. *vesicatoria* (Bouzar et al., 1994).

c. Bacteriophages. Bacteriophages infect bacteria, causing lysis of the susceptible bacterial cells that leads to the formation of plaques in bacterial cultures. Bacteriophages that infect plant pathogenic bacteria have been isolated from infected leaves, irrigation water, and soil. Most of the phages that infect *Xanthomonas campestris* pv. *oryzae* are tadpole-shaped with a polyhedral head and tail. A filamentous phage Xf was reported by Kuo et al. (1967).

The OP_1 phage that infects *X. campestris* pv. *oryzae* which causes rice bacterial blight disease can be employed for detection of the presence and quan-

Table 6.1 Important Characteristics of Important Genera of Plant Pathogenic Bacteria

Characteristics	*Agrobacterium*	*Corynebacterium*
1. Morphology	Small rods with 1–4 peritrichous flagella	Straight or slightly curved rods with club-shaped swellings frequently
2. Gram staining	Negative	Positive
3. Motility	Motile	Nonmotile
4. Oxygen requirement	Aerobic	Generally aerobic; some may be microaerophilic or even anaerobic
5. Gas production	No visible gas production	
6. Acid production	Not detectable by litmus	Slight
7. CO_2 production	Produced in synthetic media detectable with bromothymol blue or bromocresol purple	Oxidizes glucose, producing CO_2 + H_2O without visible gas production
8. Liquefaction	Liquefies gelatin very slowly or not at all	May or may not liquefy gelatin
9. Nitrogen utilization	No fixation of free nitrogen; nitrates or ammonium salts are utilized	May or may not produce nitrites from nitrates
10. Optimum temperature	25°–30°C	
11. Habitat	Soil, plant roots, and stems	Mostly pathogens of humans and domestic animals; some are plant pathogens
12. Chief symptoms	Hypertrophy, galls	Rotting of tissues
13. Important species	*A. tumefaciens* *A. rhizogenes* *A. radiobacter*	*C. sepedonicum* *C. michiganense*

Characteristics	*Erwinia*	*Pseudomonas*	*Xanthomonas*
1. Morphology	Rod-shaped	Rods with monotrichous, lophotrichous flagella; presence of diffusible fluorescent pigments of greenish, bluish pink, yellowish colors or nondiffusible bright red or yellow pigments	Rods usually monotrichous with yellow water-insoluble pigments
2. Gram's staining	Negative	Negative	Negative
3. Motility	Motile	Motile	Motile
4. Oxygen requirements		Aerobic	Aerobic
5. Gas production	Visible gas may or may not be produced		Gas produced by some species
6. Acid production	Acid produced	Acid produced	Acid produced from sugars
7. Liquefaction	May or may not liquefy gelatin		Some species may liquefy pectin
8. Utilization of nitrogen	May or may not produce nitrites from nitrates	Nitrates reduced to nitrites, ammonia, or free nitrogen	
9. Habitat	Plants	Soil, water, pathogens of plants and animals	Many are plant pathogens
10. Chief symptoms	Dry necrosis galls, wilts, and soft rots	Wilts	Necroses
12. Important plant pathogens	*Erwinia amylovora* E. *cartovora carotovora*	*P. solanacearum* *P. phaseolicola* *P. pisi* *P. tabaci*	*X. campestris* pv. *oryzae* X. c. malvacearum *X. juglandis*

Source: Stapp (1966).

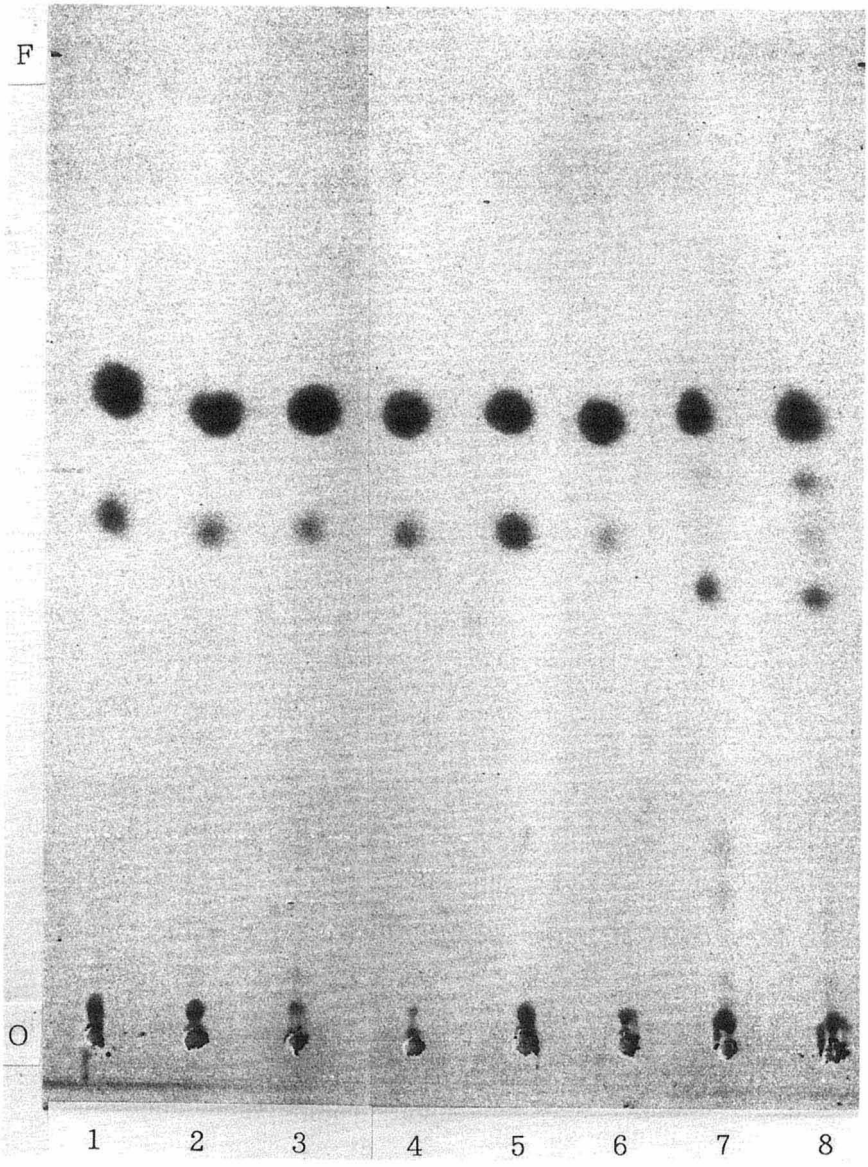


Figure 6.3 Thin layer chromatogram of lipids from phytopathogenic bacteria: 1, *Pseudomonas solanacearum* 6515; 2, *P. solanacearum* 6511; 3, *P. solanacearum* 6509; 4, *P. solanacearum* Ku 7502-1; 5, *P. solanacearum* C319SR; 6, *P. solanacearum* BY-4; 7, *P. plantarii* AZ 8201; 8, *P. glumae* 2. (Courtesy of Matsuyama et al. 1993a.)

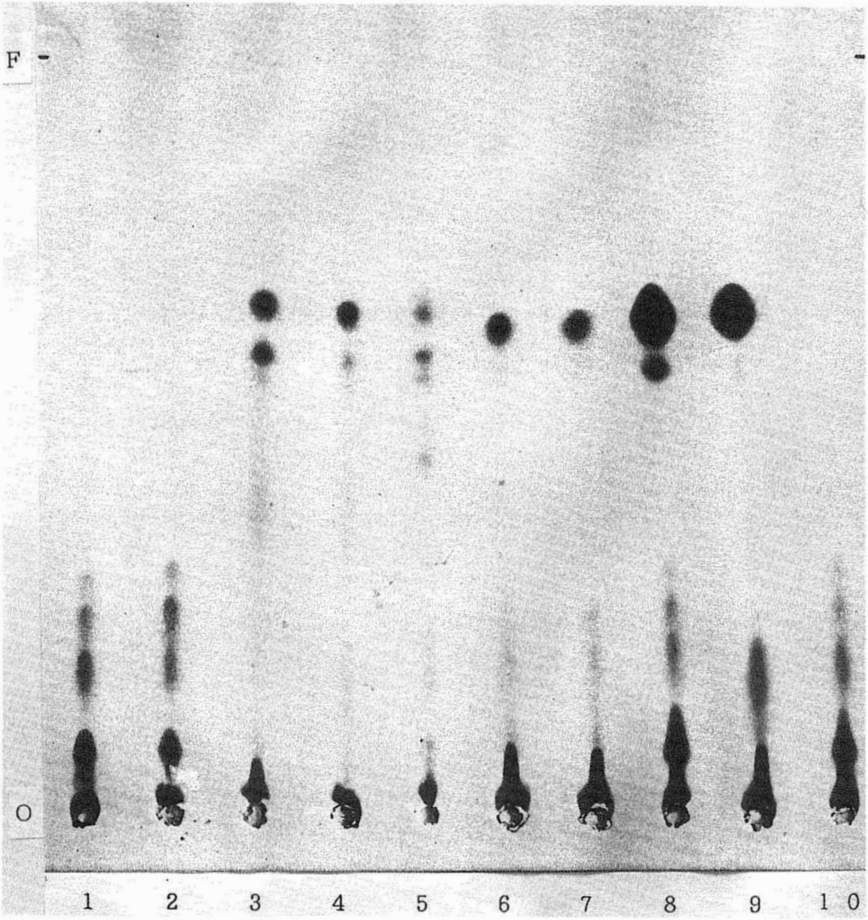


Figure 6.4 Thin layer chromatogram of lipids from phytopathogenic bacteria: 1, *Clavibacter michiganensis* subsp. *sepedonicus* 1; 2, *Clavibacter* sp. 5215; 3, *Agrobacterium tumefaciens* 1sk; 4, *A. tumefaciens* Uh; 5, *A. tumefaciens* Ku7411; 6, *Xanthomonas campestris* pv. *citri* Ku 7512-2, N 6829-1-3; 7, *X. campestris* pv. *citri* N 6829-1-3; 8, *Erwinia carotovora* subsp. *carotovora* 489-4; 9, *E. chrysanthemi* pv. *chrysanthemi* Ku8601-L1; 10, *Clavibacter michiganensis* subsp. *michigannesis* N 6206. (Courtesy of Matsuyama et al., 1993b.)

titative estimation of bacterial population (Wakimoto, 1957). On the basis of sensitivity to phages, *X. campestris* pv. *oryzae* could be differentiated into 15 lysotypes. However, the sensitivity of the bacterial strains to phages and their serological reactions are neither related to one another nor to the virulence of the strain (Ou, 1985). On the other hand, Freigoun et al. (1994) reported that

phage sensitivity of *Xanthomonas campestris* pv. *malvacearum*, causative agent of cotton bacterial blight, was related to the pathogenic potential of two races prevalent in Sudan. These races were found to be quite distinct in their phage sensitivity. Race 1 was lysed by three or rarely four of the six phages used for typing, whereas race 2 was sensitive to all six phages. The phages may be utilized for both ecological and epidemiological studies to detect and determine the population of pathogenic bacteria.

6.4.2.3 Biochemical tests with host plant tissues

a. Fluorescent markers. Indexing of plant materials for the presence of citrus greening and exocortis, using fluorescent marker substance, was reported by Schwarz (1968). Alcohol extracts of leaves or stem bark are concentrated, after filtering through a sintered glass funnel. The extracts and the fluorescent marker gentisic acid (GeA) are spotted on silica gel thin layer chromatography plates and run with water-saturated *n*-butanol. Buffered sodium borate (pH 8.7) is used to develop color, and the plates are examined under UV light at 365 nm for the presence of fluorescent violet spots corresponding to the marker. Hooker et al. (1993) reevaluated the reliability of this procedure as a method of diagnosing greening disease. The severity of foliar symptoms was correlated significantly with the amount of GeA in young and old bark tissues. This method is found to be reliable when GeA levels are more than 300 μg/g of tissue and when it is used along with diagnostic criteria under greenhouse conditions.

6.5 DETECTION OF VIROIDS

Methods that depend on the possibility of locating the viroid itself or the presence of specific products of host–viroid interaction have been suggested for detection of infection by viroids.

6.5.1 Phloroglucinol Test

The ray cells in the bark tissue of citrus infected by citrus exocortis viroid (CEVd) contain compounds that can react with aldehyde decoupling reagents such as phloroglueinol–concentrated HCl. The reaction leads to the development of characteristic color which may be visible under the microscope. Cross-sections of the bark are immersed in alcoholic phloroglucinol solution then treated with concentrated HCl for a few seconds. A positive color reaction may be seen

well before the onset of scaling of bark, which is the early visible symptom in CEVd-infected plants. A very high correlation (98.6%) between color reaction and presence of CEVd was reported by Childs et al. (1958). Such viruses as psorosis, xylopsorosis, and tristeza do not seem to affect the test results. However, use of diagnostic hosts for CEVd detection appears to be preferred by researchers (Diener, 1979).

6.5.2 Polyacrylamide Gel Electrophoresis Technique

Potato spindle tuber viroid (PSTVd) is detected in potato and tomato plants by extracting the cellular nucleic acids from both healthy and infected plants. The nucleic acids are then separated by the conventional polyacrylamide gel electrophoresis (PAGE) system, and the gel is stained to reveal the presence of different bands of nucleic acids. The PSTVd, as a nucleic acid, appears as a separate distinct band only in the samples from infected tissue; it is absent in the comparable healthy tissues. This technique can be used to detect the presence of both mild and severe strains of PSTVd. Several elite or basic seed stocks of potato have been freed of PSTVd by eliminating infected tubers by the PAGE system in certification programs (Morris and Wright, 1975). The presence of PSTVd in true seeds could be detected by return electrophoresis, which was found to be comparable with or superior to nucleic acid hybridization (Singh et al., 1988).

For the detection of coconut cadang-cadang viroid (CCCVd), leaf samples are blended with 0.1 *M* Na_2SO_3 and precipitated with polyethylene glycol (PEG) 6000 and ammonium sulfate. The PEG-insoluble fraction is then extracted in phenol-SDS-chloroform and the nucleic acids are recovered by ethanol precipitation and further fractionated with 2 *M* LiCl. The nucleic acids are then subjected to either PAGE or blot hydridaztion. In the PAGE system, toludene blue used for staining the gels detects about 0.1 μg of cadang-cadang RNA (ccRNA) per band. The differences in the electrophoretic mobility of cadang-cadang isolates can be used as the basis for distinguishing them (Randles, 1985).

The viroid molecules differ distinctly from normal host RNAs in their electrophoretic mobility in nondenaturating and partially denaturating gels. Schumacher et al. (1983) used this property for PAGE analysis. The leaf extracts are run first in nondenaturating gels in one direction and are run either in the reverse direction or at 90° to the first direction under denaturating conditions. Viroid bands can be separated easily from host nucleic acid. Using the silver staining procedure, viroids can be detected at 600 pg level, and with purified ccRNA1 as low as 0.4 to 1.6 ng may be detected.

SUMMARY

Chemodiagnostic tests depend on the reaction between chemicals and certain compound(s) present in the infected plants, resulting in the development of a visible color reaction. Detection of plant viruses, mycoplasma-like organisms, fungi, and bacteria by using different chemodiagnostic tests involving the use of dyes, different kinds of chemicals, selective media, and fluorescent markers has been reported by many workers. The chemodiagnostic tests, in general, are less specific and sensitive than tests that depend on serological properties and variations in genomic nucleic acid sequences of test pathogens. However, isozyme analysis, polyacrylamide gel electrophoresis, and direct colony thin layer chromatography have been found to yield reliable results for the detection and differentiation of certain pathogens.

APPENDIX 6(i): STAINS FOR BACTERIAL PATHOGENS

A.	Gram's stain (Hucker's modified method)	
	Solution A: Crystal violet (90% dye content)	2.0 g
	Ethyl alcohol (95%)	20.0 ml
	Solution B: Ammonium oxalate	0.8 g
	Distilled water	80.0 ml
	Mix solutions A and B.	
B.	Lugol's solution (mordant)	
	Iodine	1.0 g
	Potassium iodide	2.0 g
	Distilled water	300.0 ml
C.	Counterstain	
	Safranin (2.5% solution in 95% alcohol)	10.0 ml
	Distilled water	100.0 ml
D.	Flagella stain	
	20% Aqueous tannic acid	100.0 ml
	Ferrous sulfate	20.0 g
	10% Basic fuchsin in alcohol	10.0 g
	Distilled water	40.0 ml

Ferrous sulfate is dissolved in water by warming, followed by addition of other ingredients.

E.	Loeffler's flagella stain	
	1% Basic fuchsin in alcohol	20.0 ml
	3% Aniline water	80.0 ml

F. Spore stain

Malachite green	5.0 g
Distilled water	100.0 ml

Safranin

Safranin 0 (2.5% solution in 95% ethyl alcohol)	10.0 ml
Distilled water	100.0 ml

G. Negative stain

Nigrosin (water-soluble)	10.0 g
Distilled water	0.5 ml
Formalin	0.5 ml

APPENDIX 6(ii): STAINING METHODS FOR BACTERIAL PATHOGENS

A. Gram's staining
   i) Prepare thin smears of the bacteria on grease-free clean slides; air dry and heat the smears.
   ii) Cover the smears with crystal violet solution for 30 sec; pour off the stain and wash the slides in distilled water for a few seconds.
   iii) Cover the slide with iodine solution (mordant) for 30 sec; wash the slides with 95% ethyl alcohol by adding in drops till no more color appears in the fluid from the slide; wash the slides in distilled water and drain the water.
   iv) Cover the slides with safranin (counterstain) for 30 sec; wash with distilled water, dry the slides by blotting with filter paper, and air dry.
   v) Examine the bacteria under the oil immersion lens; gram-positive bacteria retain crystal violet stain (dark purple to blue color), and gram-negative bacteria are stained by safranin and appear pink in color.

B. Flagella staining
   i) Use new grease-free slides; place a loopful of freshly prepared bacterial suspension on one end of the slide, tilt the slide slowly, and spread the bacterial suspension as a thin film and air dry.
   ii) Cover the slide with mordant solution for 10 min and wash the slide gently with distilled water.
   iii) Flood the slide with carbolfuchsin for 5 min; wash gently with distilled water and air dry.
   iv) Examine the slide under the oil immersion lens.

C. Bacterial spore staining
   i) Prepare smears of the bacteria on grease-free glass slides; air dry the slide and fix smears by heating.

ii) Cover the slides with malachite green solution; heat the slides to steaming for 5 min, adding more stain as it is evaporated; wash the slides gently in running tap water for a few minutes.
iii) Counterstain with safranin solution for 30 sec; wash the smear with distilled water and dry the slide with filter paper by blotting.
iv) Examine the slide under an oil immersion lens; endospores may be green; vegetative cells appear red.

D. Negative staining
i) Transfer a drop of nigrosin solution to one end of a grease-free clean glass slide; place a loopful of bacterial suspension on the stain drop and mix well.
ii) Using another slide, prepare a smear of the bacteria–nigrosin mixture on the slide and allow the smear to air dry.
iii) Examine the smear under an oil immersion lens; bacterial cells appear colorless on a dark blue background.

APPENDIX 6(iii): DIRECT COLONY THIN LAYER CHROMATOGRAPHY FOR IDENTIFICATION OF PHYTOPATHOGENIC BACTERIA (MATSUYAMA ET AL., 1993)

i) Take a loopful of bacterial colony from the slant; paste the culture directly on the origin line in the silica gel thin layer plate of 20 × 20 or 10 × 20 cm and dry completely; place samples at a spacing of 1.5 cm.
ii) Incubate the plates on moistened glass vessels and immerse the sample side of the plate in the chloroform–methanol mixture (2:1 v/v) to a depth of less than 1 cm; run the chromatogram at 25°C in the incubator for 10 min until the solvent front reaches the 6 cm line from the origin spot.
iii) Scrape the bacterial cells in the origin spot completely; run the chromatogram in the same direction again; using a chloroform–methanol–water (60:25:4, v/v/v) solvent system for about 90 min at 25°C in the incubator.
iv) Dry the plates well, spray ninhydrin, and develop the color by heating in chromatographic chamber at 100°C for 10 min.
v) Determine the ratio between solvent and solute fronts (Rf) values for each spot in the chromatogram.

7
Electron Microscopy

The electron microscope (Fig. 7.1) is analogous to the light microscope (Chapter 2) in principle. The solid ground glass lenses present in the light microscope are replaced by magnetic field lenses in the electron microscope: the light source of the light microscope is replaced by an electron beam in an electron microscope (Fig. 7.2). The wavelength of visible light (white light) is 540 nm and that of ultraviolet is 260 nm, whereas the wavelength of the beam of electron is usually less than 0.1 nm, depending on the accelerating voltage. The wavelength of electrons in an electron microscope using 60 kV will be about 0.005 nm. The resolving power of the microscope is inversely proportional to the wavelength of light being used. This is the reason for the high resolving power of the electron microscope, which can magnify objects about 200,000 times, whereas even the best light microscope may magnify only up to 1500 times. Electron microscopes that are now available can be used to distinguish objects 1 nm apart with sufficient density to hold back electrons. The image of the object is focused on a fluorescent screen, photographed, and then enlarged for detailed examination. The image of the object cannot be viewed directly as in the case of light microscope. Because of the high resolving power of the electron microscope, it has been possible to study the ultrastructure of biological materials such as individual cells and molecules such as proteins and nucleic acids whose structure had been known only through biochemical analyses.

Electron microscopy has been found to be useful to study the histopathological characteristics and ultrastructural changes induced by pathogens. Virus particle morphological features and characteristics of intracellular inclusions have been studied to differentiate viruses. In certain cases, the cytopathic effects of viruses are so characteristic that the causative virus can be reasonably identified. Geminiviruses cause characteristic cytopathic effects such as formation of macrotubules in the nuclei of infected cells, as in *Euphorbia* mosaic

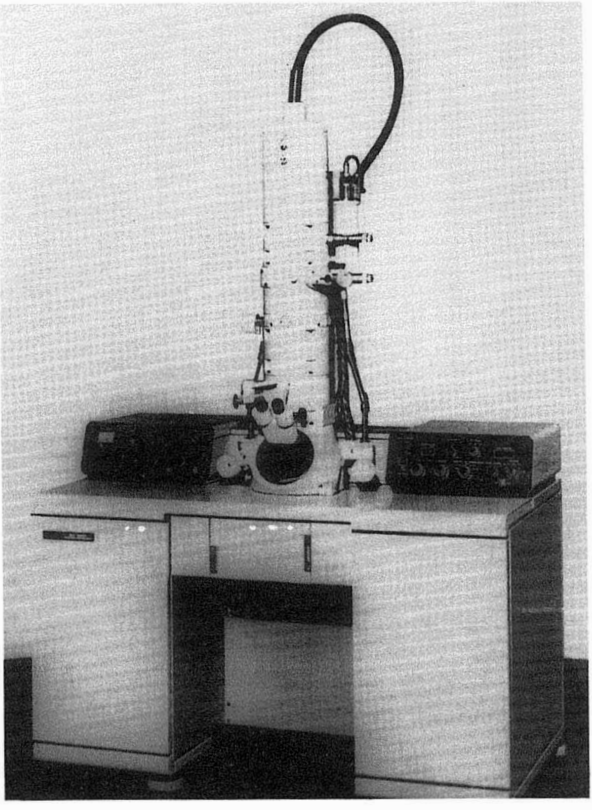


Figure 7.1 Transmission electron microscope (TEM) model Jeol—JEM 100 SK.

virus-infected *Datura stramonium* plants (Kim and Lee, 1992). Electron microscopy can be used to reveal the presence of virus particles in extracts of individual local lesions caused by the transcripts of a full-length complimentary DNA (cDNA) copy of clover yellow mosaic potex virus RNA (Holy and Abou Haider, 1993).

The essentail features of major components of the electron microscope (Fig. 7.3) are described in the following discussion.

The electron gun is the illumination source of the electron microscope. The electron source should be small and have high brightness and stability. The electrons are accelerated by the electric potential difference between the filament and anode. The electrons are concentrated at a "cross-over point," from which they are later emitted. A condenser lens is used to cause the electron beams to converge from the electron gun and to illuminate the specimen to a desired level. Generally the field of view in high magnification is limited to a

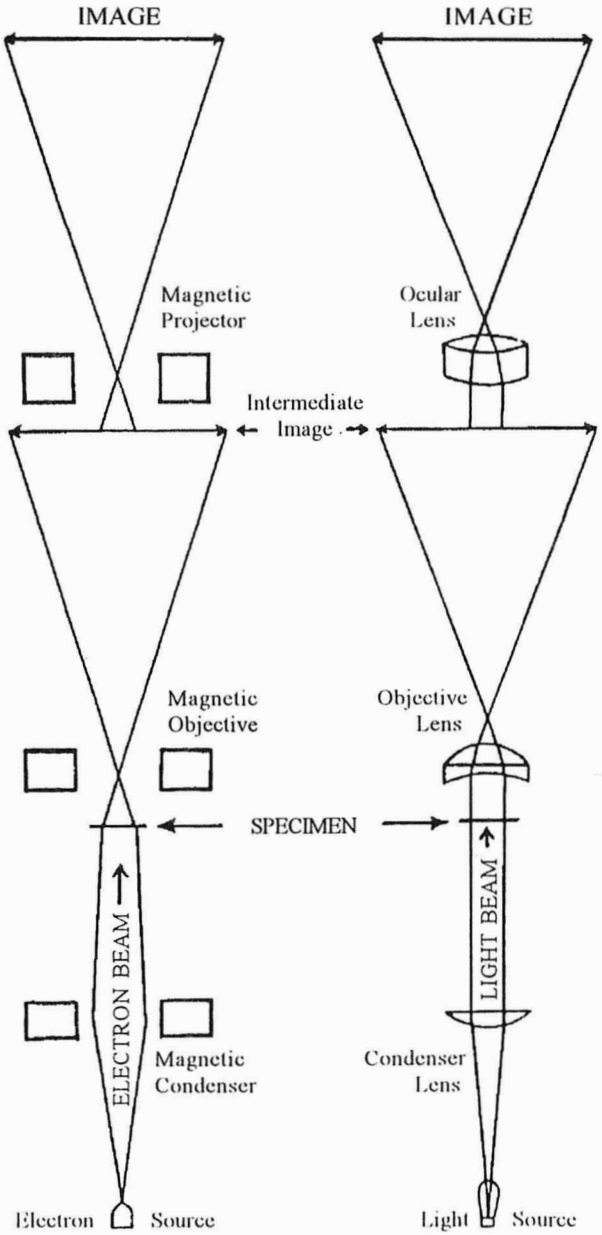


Figure 7.2 Comparison of image formation in light and electron microscopes.

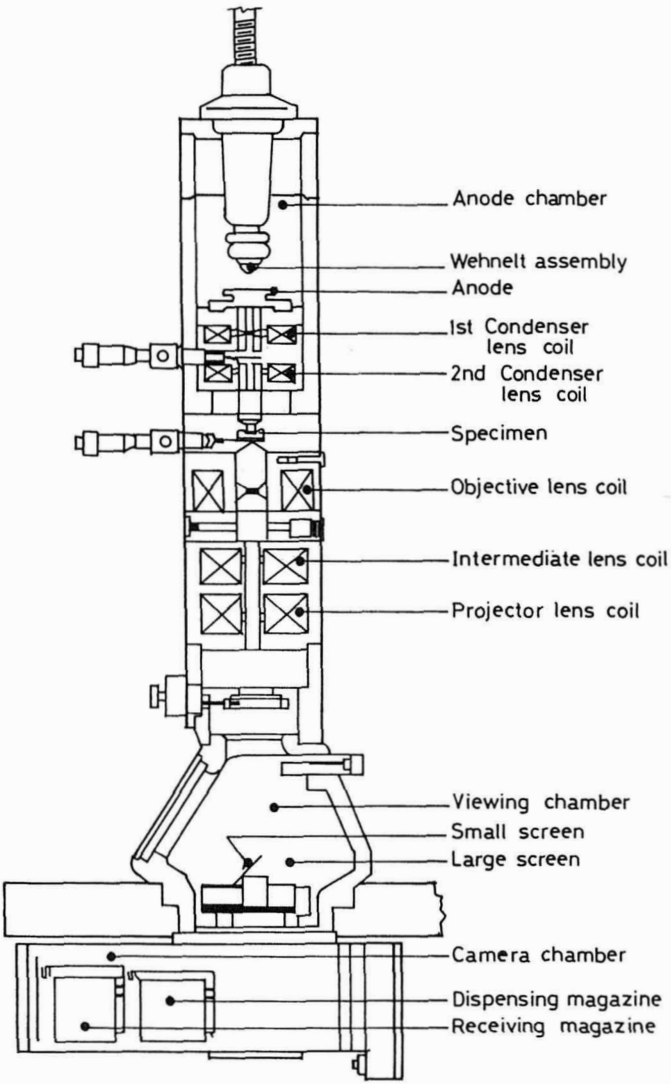


Figure 7.3 Schematic diagram of components of the transmission electron microscope.

very small area and the illumination area also has to be small. A double-condenser lens is provided to achieve this requirement.

The specimen chamber contains a stage that can be used easily for rapid exchange of specimens. This stage holds the specimen holder in a stable condition and moves smoothly for easy selection of the field of view. External

vibration, which adversely affects the resolving power of the high-performance electron microscope, should be eliminated. The specimen chamber is so constructed as to accommodate many attachments for wider applications.

The image-forming lens system consists of three lenses, namely, the objective lens, intermediate lens, and projector lens. The objective lens, located immediately under the specimen, forms a first-stage image which is further enlarged by the intermediate lens. The final image is formed on a fluorescent screen or photographic film by the high-magnification projector lens. In certain models (e.g., JEM) a four-stage image-forming lens system with a two-stage intermediate lens is provided for high performance. Focusing is done by altering the exciting current for the objective lens. Variations in magnifications are due to the intermediate lengs. But in the case of low magnification, it can be altered by deactivating one of the system lenses or by changing the projector lens. The optical axes of both lenses must align with each other, and misalignment of the axes must be rectified immediately.

The viewing chamber and camera chamber are required to observe the images and to record them in film. The images of the specimen cannot be directly observed by the naked eye. They are converted into light images and focused on a fluorescent screen for observation or on films with high resolving power. A camera equipped with an airlock mechanism and capacity for rapid film exchange should be used to prevent breaking of the vacuum column.

The electrons are easily scattered by air. Hence it is essential to maintain a high degree of vacuum, which is achieved by operating oil rotary pumps or oil diffusion pumps. A poor vacuum may cause high-tension electrical discharge, specimen contamination, contrast reduction, or damage to the specimen. The electron microscope has an electric circuit consisting of four main parts: a) a high-voltage power supply for electron beam acceleration, b) a lens power supply for eletron excitation, c) a deflecting coil power supply for electron beam deflection, and d) other circuits, including a vacuum control circuit, vacuum pump power supply, camera drive circuit, automatic exposure circuit, and electron gun filament heating circuit. For further details, see Siegel (1964), Kay (1965), and Sjostrands (1967).

7.1 PREPARATION OF SUPPORT FILMS

Electroplated copper or nickel grids (400 mesh) are covered with carbon alone or with a plastic film (e.g., Formvar, polyvinyl formaldehyde) alone or are reinforced with carbon. Parlodion, pyroxylin, and necoloidine films supported with carbon are equally good. It is better to use the support films soon after they are coated with carbon, since during storage they become hydrophobic, resulting in unsatisfactory specimen adhesion and negative staining.

7.2 CALIBRATION OF THE ELECTRON MICROSCOPE

The electron microscope has to be calibrated for determination of size measurements and magnification. For this, different standard have been used. A diffraction grating replica with 2160 lines/mm can be used as an external standard to calibrate magnifications up to 40,000×. Beef liver catalase is available as a crystal lattice which may be negatively stained and used as an external or internal standard. Tobacco mosaic virus particles whose modal length has been already determined can be used as an internal standard for calibration of the electron microscope. There are other standards (Milne, 1972), which are not commonly used.

7.3 TECHNIQUES FOR IDENTIFICATION OF PLANT VIRUSES

7.3.1 Preparation of Ultrathin Sections

The steps in the process of fixation, embedding, sectioning, and staining are time-consuming, and these procedures have limited use for rapid detection of plant viruses and phytoplasmas (mycoplasm-like organisms).

7.3.1.1 Fixation

Several fixatives have been used for fixing plant materials infected by viruses. Buffered 1%–2% solution of osmium tetroxide or 2%–5% solution of glutaraldehyde has frequently been used. Phosphate buffer (0.2 *M*) with pH 5.8–7.4 and cacodylate buffer with pH 7.4 are used to prepare the fixatives. Fixation time depends on the tissue and fixative, but short durations are preferable. Thin leaves are fixed with glutaraldehyde for 1 hr after infiltration, and 1 mm strips of leaves are further fixed in osmium tetroxide for 3 hr in the cold or at room temperature. Excess fixative is washed out of the tissue with buffer.

7.3.1.2 Dehydration

The fixed tissue has to be dehydrated by using a graded series of 50%, 70%, 90%, and 100% acetone or ethanol. The tissue is soaked in each concentration of acetone or ethanol for 10 min and finally in three changes of water-free solvent.

7.3.1.3 Embedding

Different kinds of embedding resins, such as methacrylates, epon, or araldite, have been used. Epon or araldite is used more frequently. In the case of epon,

two stock solutions, viz., A, which contains 63 ml Epon 812 (Epicote 812) plus 100 ml dodecenyl succinic anhydride (DDSA), and B, which contains 100 ml Epon 812 plus 89 ml methyl nadic anhydride (MNA), are prepared. They are mixed in different proportions to produce hard or soft blocks; solution B increases hardness. The optimal proportion is 4 parts of A and 6 parts of B. An accelerator, such as 1.5% tridimethyl aminomethyl phenol (DMP 30) or 3% benzyldimethylamine (BDMA), is added to hasten curing. The blocks are hardened at 40°C–60°C for 2–3 days in closed capsules.

Different formulations of araldite have been used, depending on the plant materials. The embedding formulation contains araldite CY 212 (10 ml), DDSA (10 ml), dibutyl phthalate (1.0 ml), and DMP 30 (2.4% by volume). The resin is cured for about 24 hr at 60°C in closed capsules.

7.3.1.4 Sectioning

Diamond or glass knives are used for cutting ultrathin sections in an ultramicrotome. The knife may be set up permanently with its water bath. The gelatin capsule is removed by soaking in water. The sections, as they are cut, float onto a water bath, which is in contact with the knife edge. Generally the water bath is made of a strip of adhesive tape or metal foil. The water bath is filled with a mixture of acetone and water or water only. The thickness of the sections is roughly determined by interference color. Gray-, silver-, and gold-colored sections may have a thickness of 50 nm, 50–80 nm, and 150 nm, respectively. Thinner sections are required for better resolution. The sections are then transferred to grids coated with Formvar or parlodion and stained.

7.3.2 Negative Staining

Though negative staining is simple in principle, several factors, including individual skill, may appreciably affect the results. Many negative stains are available.

7.3.2.1 Phosphotungstate

Sodium or potassium phosphotungstate (PTA) aqueous solution at 2% concentration adjusted to pH 7.0 with NaOH or KOH has been used to examine many plant viruses. However, it is not useful for PTA-labile viruses such as alfalfa mosaic virus, tomato spotted wilt virus, cucumoviruses, rhabdoviruses, fijiviruses, and some members of the geminivirus, ilarvirus, and closterovirus groups (Francki et al., 1984). Floating the grids with adsorbed virus particles on a drop of 0.1% glutaraldehyde for 5 minutes before staining with PTA may reduce the damage due to PTA (Milne, 1984).

7.3.2.2 Uranyl acetate

Aqueous solution of uranyl acetate (UA) usually at 2% concentration (sometimes as low as 0.1%) without adjusting the pH (which may be around 4.2) is used as a negative stain for most of the plant viruses (Fig. 7.4). But rhabdoviruses are sometimes sensitive to UA and may be stripped of their envelopes, leaving the helical nucleocapsid. The stain should be stored in a dark bottle, as UA is unstable in strong light. Uranyl acetate gives better contrast and higher resolution than PTA. Prestain rinsing of the grids with 0.01 *M* phosphate buffer, pH 7.0, or with 0.1 *M* $CaCl_2$ is recommended.

7.3.2.3 Uranyl formate

The stain uranyl formate (UF) has properties similar to those of UA and the stain solution has to be prepared afresh every day. Uranyl formate is preferred for helical viruses and alfalfa mosaic virus, since it is found to be more effective than UA.

7.3.2.4 Ammonium molybdate

Ammonium molybdate (AM) is used at 2% concentration with varying pH levels (4–9) adjusted with HCl or ammonia and is stable at room temperature.

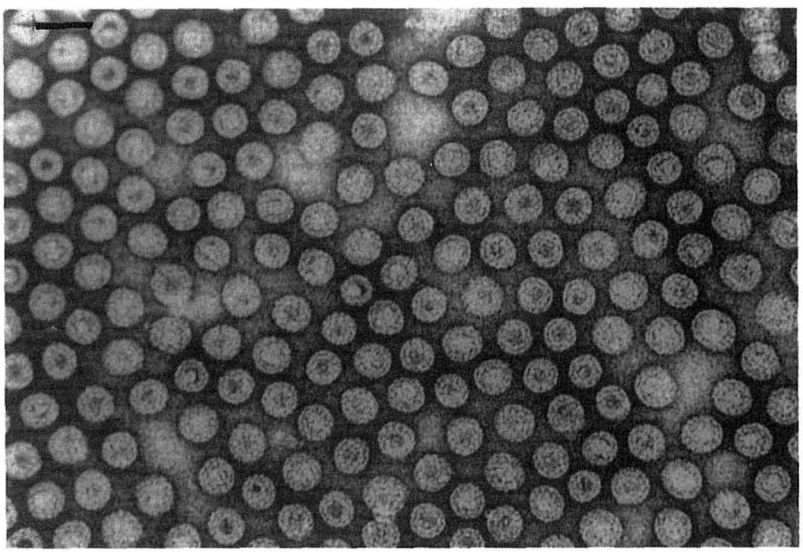


Figure 7.4 Purified peanut chlorotic streak virus particles stained with uranyl acetate; bar represents 100 nm (American Phytopathological Society, Minnesota, USA). (Courtesy of Reddy et al., 1993).

It gives relatively poor contrast generally. However, AM has been used extensively because it may be mixed directly with virus suspensions and used to stain PTA-labile viruses. In the case of barley yellow striate mosaic rhabdovirus, AM was found to be better than PTA and UA (Milne, 1984).

7.3.2.5 Methylamine tungstate

The stain methylamine tungstate (MT) is used at 2% concentration and pH 6.5 (Oliver, 1973); it can be mixed directly with a virus sample. In the case of such viruses as cucumber mosaic virus, cauliflower mosaic virus, and bean common mosaic virus, more virus particles (10–20 times) may be required when MT is used than when UA is used, after a water rinse. However, the contrast is poor and there is frequent stain precipitation.

7.3.2.6 Sodium silicotungstate

A 2% aqueous solution of sodium silicotungstate (SST) adjusted to pH 7.0 is used. It is found to be preferable for some high-resolution studies of antibodies and antigen–antibody binding (Harris and Horne, 1986). It may produce images that give contrast to upper side of virus particles.

The virus particles are negatively stained with any of the stains described as follows: The grid is held with forceps and the filmed face is rinsed with a few drops of glass-distilled water, followed by 5 drops of negative stain. The grid is then dried by holding a piece of absorbent paper (filter paper) to the edge. Excess stain may be washed with drops of distilled water as before. Alternatively the grid may be briefly floated on a series of small water drops (Milne, 1993a).

7.3.3 Metal Shadowing or Shadow Casting

The metal shadowing or shadow casting method is more laborious, severely limits resolution, and obscures inner details. The grid with the virus preparation is placed face up and horizontal in a vacuum. The specimen is coated with a thin layer of electron-dense material (heavy metals such as gold, platinum, or palladium or alloys) by evaporation of thin filaments placed above and to one side. The evaporated atoms travel in approximately straight lines parallel to each other and coat the exposed surfaces of virus particles. Because metals are opaque to the electron beam, the beam casts a shadow, giving a three-dimensional effect to the material to be photographed under the electron microscope (Fig. 7.5). With the development of negative staining methods, metal shadowing has limited use as a diagnostic or taxonomic aid for determination of shape and fine structures of virus particles. The metal shadowing technique

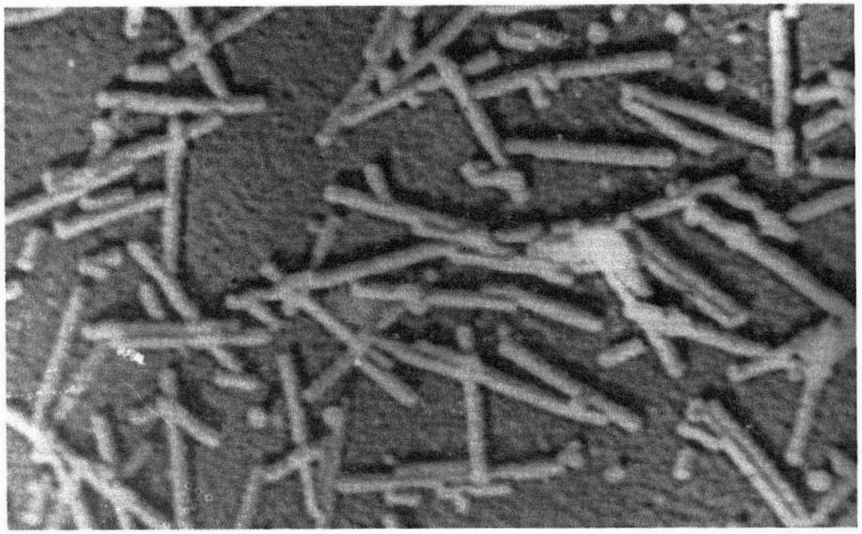


Figure 7.5 Purified tobacco mosaic virus particles shadowed with chromium (Courtesy of Eishiro Shikata).

is particularly useful for coating nucleic acid molecules, which cannot be contrasted easily with negative or positive staining. Rotary shadowing is done to coat the specimen at different angles (Milne, 1972).

7.3.4 Dip Method (Brandes, 1957; Hitchborn and Hills, 1965)

The dip method is a simple technique that can be used to check the presence of virus in plants quickly. A freshly cut leaf or an epidermal strip is passed through the surface of a drop of water placed on a glass slide. Some of the heavier cellular contents flow into the drop; particulate materials such as virus particles form a film on the surface of the water drop. A filmed grid is then touched to the surface and negatively stained. In a variant of this method, the cut leaf may be passed directly through a drop of negative stain placed on the grid. The grid is then drained and dried. Dip preparation may help to identify viruses quickly, as in watermelon infected by a strain of tomato spotted wilt virus (Honda et al., 1989).

7.3.5 Leaf-Dip Serology

Leaf-dip serology, developed by Ball and Brakke (1968), is based on the principle of the leaf-dip method of Brandes (1954). The antiserum is diluted with

0.001 *M* ammonium acetate solution to produce a 1:1000 dilution. A drop of this serum is placed on a carbon-backed collodion film on a specimen grid. Pieces of leaf tissue (1.5–2.0 × 0.5 cm) are cut from diseased leaves, and the narrowest end is drawn through the antiserum drop for 1 or 2 sec. The drops are then air-dried and a drop of a solution containing 1 part of 1% vanadatomolybdate, pH 3, plus 3 parts of 2% potassium phopshotungstate is used to stain the virus–antiserum mixture and then dried. The infection of tobacco mosaic virus (TMV) and barley stripe mosaic virus can be detected by observing the virus particles (Ball, 1971). This method can also be used for determining the dilution end point of the antiserum and the relationship between strains of elongated viruses (Langenberg, 1974). Though this method is simple, the immunosorbent electron microscopy, decoration, and gold-labeling techniques have many advantages and are being widely used.

7.3.6 Immunosorbent Electron Microscopy

Derrick (1972, 1973) developed the method of serologically specific electron microscopy (SSEM), later redesignated immunosorbent electron microscopy (ISEM) by Roberts and Harrison (1979). The grids are coated with (Formvar [polyvinyl formaldehyde] or Parlodion) strengthened with a layer of evaporated carbon. Crude antiserum raised against the virus to be detected or assayed or fractionated α-globulins may be used to sensitize the coated grids. The optimal dilution of crude antiserum may be around 1:1000 to 1:5000. The antisera and virus preparation may be diluted using 0.05 *M* Tris-HCl buffer, pH 7.2 (Brlanksy and Derrick, 1979). Other buffers, viz., 0.1 *M* phosphate, pH 7.0 (Milne and Luisoni, 1977); 0.06 *M* phosphate, pH 6.5 (Roberts and Harrison, 1979); and sodium carbonate buffer, pH 9.6 (used in enzyme-linked immunosorbent assay [ELISA] for coating) (Thomas, 1980), have also been used. The grids are then coated with the antibodies by an appropriate dilution of antiserum for 5 min at room temperature (Milne and Luisoni, 1977). Incubation of grids at 37°C results in firm attachment of virus particles to the grids and better spreading of negative stain (Roberts, 1981). Sugarcane bacilliform virus was detected in suspected sugarcane clones by trapping virus particles on sensitized grids, followed by staining with 2% uranyl acetate. The grids were carefully rinsed with either buffer or water (Viswanathan et al., 1996) (Fig 7.6).

The virus preparations are commonly diluted in 0.05 *M* Tris-HC1 buffer, pH 7.2, or 0.1 *M* phosphate buffer, pH 7.0. The pH of the buffer is an important factor influencing the number of particles trapped on the grids, and optimal pH is determined for different host–virus combinations (Cohen et al., 1982). Addition of 0.1 *M* EDTA to 0.1 *M* phosphate buffer, pH 7.0, is necessary for trapping turnip mosaic virus isolates from chinese cabbage (Lesemann and Vetten, 1984) and papaya ringspot virus (Gonsalves and Ishii, 1980). Some

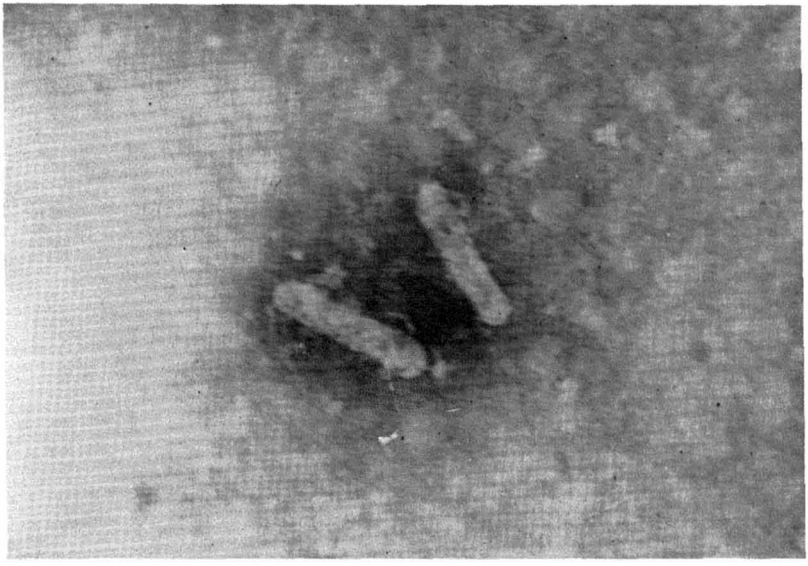


Figure 7.6 Sugarcane bacilliform virus (SCBV) particles trapped with antiserum to SCBV; bar represents 400 nm (Courtesy of Viswanathan.)

plants may contain substances that may prevent trapping of virus particles on the grids. Addition of 2.5% nicotine to the sap of plum or grapevine helped to detect plum pox virus (Noel et al., 1978) and grape fan leaf virus (Russo et al., 1982). Likewise, detection of viruses in saps of plants in Rosaceae, grapevine, and poplar required the incorporation of 2% polyvinyl pyrrolidone (Milne, 1981).

The periods for which the grids are incubated with virus preparation may vary from 15 min to several hours (or overnight); for rapid diagnosis incubation for 15 min may be sufficient (Milne and Luisoni, 1977). The sensitivity of the test, however, increases with increase in the incubation period at room temperature or preferably at 37°C (Roberts and Harrison, 1979). Incorporation of sodium azide (1:5000) is recommended to prevent bacterial growth during long incubation periods (Milne and Lesemann, 1984). The grids are rinsed as after incubation with antiserum.

Metal shadowing (Derrick, 1973) and positive staining (Derrick and Brlansky, 1976) were the methods formerly used to provide good contrast. Milne and Lesemann (1984) have recommended the use of negative stains for high resolution and good particle preservation. Uranyl acetate (2%) and sodium phosphotungstate (2%), neutral or pH 6.5, are effective in the case of many

viruses. For viruses such as alfalfa mosaic virus, tomato spotted wilt virus, ilarviruses, cucumoviruses, and some of the rhabdoviruses and geminiviruses which are labile in PTA, 2% ammonium molybdate at pH 6.0–7.0 can be used for staining the virus particles (Roberts, 1981).

The comparative efficacy of detection of arabis mosaic virus, *Prunus* necrotic ringspot virus, and strawberry latent ringspot virus by ISEM, ELISA, and infectivity assay was assessed by Thomas (1980). Thomas found that ISEM was 10 to 20 times more sensitive than ELISA in detecting the virus in purified preparations and extracts from infected plants (Table 7.1).

Two modifications of the regular ISEM procedure have been reported to increase the sensitivity and usefulness of the technique. The grids are precoated with protein A obtained from *Staphylococcus aureus*, which has the specific affinity of binding to the Fc portion of the immunoglobulin G (IgG) molecule. A significant increase in the number of virus particles trapped by protein A–coated grids is seen (Gough and Shukla, 1980). This procedure is useful when the antiserum is of low titer. In another modification, known as the decoration step, the virus particles already adsorbed on the grid are coated with IgG molecules. The grids are incubated with the same antiserum at a dilution between 1:10 and 1:100 for 15 min at room temperature. The virus particles are decorated with a halo of IgG molecules (Milne and Luisoni 1975). The virus particles may be more easily identified by this additional step introduced into the regular ISEM procedure.

Immunosorbent electron microscopy has helped to detect and differentiate viruses rapidly. Detection of cymbidium mosaic virus in crude sap of in-

Table 7.1 Comparative Efficacy of Detection of Plant Viruses by ISEM, ELISA, and Infectivity Assay

	Minimum concentration of detectable virus (ng/ml)		Maximum reacting dilution of sap (reciprocal)		
Virus	ISEM	ELISA	ISEM	ELISA	Infectivity
Arabis mosaic virus	0.5	5.0	320,000	40,000	100,000
Prunus necrotic ringspot virus	4.0	4.0	80,000	10,000	400
Strawberry latent ringspot virus	50.0	1000.0	128,000	5,500	64,000

Source: Thomas (1980).

fected orchid leaves by ISEM was about twice as sensitive as detection by ELISA (Hsu et al., 1992). Virus particles resembling those of geminiviruses were observed in extracts of plants infected by bhendi yellow vein mosaic, croton yellow vein mosaic, dolichos yellow mosaic, horsegram yellow mosaic, Indian cassava mosaic, and tomato leaf curl viruses. These viruses positively reacted with the MABs generated against African cassava mosaic geminivirus (Harrison et al., 1991; Muniyappa et al., 1991). The chickpea chlorotic dwarf virus was identified as a new leafhopper-borne geminivirus, since it did not react with the antiserum against sugar beet curly top virus (Horn et al., 1993).

Detection of virus infection and assay of viruses by ISEM have certain advantages: a) Direct visualization of virus particles is possible; b) sensitivity of detection is comparable to and better than that of ELISA, which may sometimes give false-positive results in certain cases; c) crude antiserum without fractionation or with low titer can also be used; d) very small volumes of antiserum (5–10 μl) or antigen samples (1 μl) are sufficient for the tests; e) the results may be obtained within 2 hr; f) the presence of antibodies against host material will not interfere with the results; g) using the decoration step, it is possible to detect the contaminating virus, if any, present in the plant sample. Though there are several advantages of using ISEM for detection of virus infection, it is not being used widely, because it is labor-intensive and requires costly equipment and expertise to handle the equipment. So it may not be suitable for the routine testing of large number of samples.

7.3.7 Decoration Technique

Milne and Luisoni (1975) developed the method of "decoration" of plant viruses for their rapid identification. A combination of immunosorbent electron microscopy (ISEM) and decoration resulted in greater resolution. This procedure, in principle, consists of trapping the virus particles on sensitized grids, followed by staining of the viruses with a negative stain, and incubation with the same or a different antibody to give a visible antibody halo (decoration). The decoration may be further refined with gold particles conjugated either to a secondary antibody or to protein A (PA) (Milne, 1992).

The decoration technique may be used for detection and quantitative estimation of viruses. The virus particles extracted or suspended in an appropriate buffer are adsorbed to sensitized filmed grids. If necessary, the virus particles on the grid may be fixed with 0.1% buffered glutaraldhyde (GA) for 15 min at room temperature. The grid is rinsed with 0.1 *M* phosphate buffer or 0.05 *M* borate buffer, pH 8.1, then incubated with a suitable dilution of antibody at room temperature for 15 min, followed by rinsing with water and stain-

ing with 1% aqueous uranyl acetate. The excess stain is drained at the edge with filter paper and allowed to dry.

The decoration technique was found to be useful for the detection of several potyviruses and carlaviruses. This technique may be employed for a) serological identification of viruses, b) detection of virus mixtures, c) detection of partial degradation of coat proteins, d) quantitative estimation of degrees of relationship between viruses, e) measurement of antiserum titer, f) localization of particular antigens (viral gene products on the viral surface), and g) enhancement of the virus particle's conspicuousness by increasing its size and electron density as an aid for rapid diagnosis. Xu and Li (1992) reported that ISEM combined with decoration was a suitable method for distinguishing strains of ribgrass mosaic virus and establishing the extent of the serological relationship between related viruses. Employing the combined immunosorbent and double-decoration method, TMV and PVY were detected at 0.184 ng/ml and 0.128 ng/ml, respectively, in purified preparations within 1 hr and using as low a magnification as 2000 times (Chen et al., 1990).

7.3.8 Gold Labeling

Though the decoration technique is sufficient for the detection of many plant viruses, labeling the decorating antibody with gold offers additional advantages in the case of larger viruses such as rhabdoviruses and tospoviruses. Pares and Whitecross (1982) introduced the gold label antibody decoration (GLAD) procedure for gold labeling of viruses in suspensions. This technique was further refined by Louro and Lesemann (1984) and van Lent and Verduin (1985). Gold labels with varying diameters ranging from 5 to 20 nm have been used. Gold may be conjugated directly to the primary or coating antibody, but more usually, it is attached to protein A (PA) or a secondary antibody (goat antirabbit IgG).

The virus particles are adsorbed on filmed nickel grids and rinsed with 0.1 *M* phosphate-buffered saline solution containing 0.05% Tween 20 (PBST). The grids are incubated for 15 min with 0.1%–1.0% aqueous bovine serum albumin (BSA) to block nonspecific protein adsorption sites and rinsed again with PBST. The grids are then incubated for 15 min with drops of antiserum (raised against the particular virus) diluted in 0.1 *M* phosphate buffer, pH 7 (PB), and rinsed with PBST. The grids are incubated with protein A gold (PAG) in PBS for 60 min, followed by rinsing successively in PBST and water. The preparation is then stained with uranyl acetate (1%) to enhance the contrast (Milne, 1992, 1993).

The presence of alfalfa mosaic virus antigen could be detected in the cytoplasm and vacuoles of ovule integuments, microspores, mature pollen grains, and anther tapetum cells of infected alfalfa plants. Raftlike aggregates of virus particles and large crystalline bodies were observed in the cytoplasm of the pollen grains and anther tapetum cells, whereas nonaggregated virus particles were detected in the vacuoles and cytoplasm of ovule integument cells (Pesic et al., 1988). The virus associated with pear vein yellow disease was considered to belong to the closterovirus group, because the antiserum raised against the apple stem pitting virus produced positive gold labeling of the virus aggregates (Giunchedi and Pollini, 1992). The presence of virus in infected tissues can be detected by labeling capsid structural proteins or entire coat protein. By treating with polyclonal antibodies (PABs) to three major structural proteins G, N, and M of *Festuca* leaf streak rhabdovirus, followed by protein A gold labeling, all the structural proteins could be detected in virions present at the periphery of viroplasms (Lunsgard, 1992). Gold-labeled antibodies to coat protein (Cp) of tobacco vein mottling potyvirus (TVMV) were bound to cylindrical inclusions and individual virions produced in protoplasts inoculated with TVMV-RNA. In inoculated leaves also, antibodies to TVMV-Cp were bound to cylindrical inclusions and aggregates of virions in the cytoplasm (Ammar et al., 1994).

The clues for location of the virus particles in vector tissues and evidence for multiplication of plant viruses in the vector may be observed by using immunochemical methods. Ullman et al. (1992) reported that although adults of *Frankliniella occidentalis* ingested TSWV, the virus was not retained, whereas TSWV could be detected serologically in the midgut epithelium and hemocoel of larvae fed on TSWV-infected plants, indicating that the midgut of the adult thrips may be a barrier preventing the virus from reaching the hemocoel. Using a specific antibody to nonstructural proteins (NSs) of TSWV demonstrated the presence of NSs in thrips cells, and the immunochemical evidence observed by Ullman et al. (1993) indicates that TSWV replicates in vector cells. In situ immunolabeling of salivary glands and other tissues of adult *F. occidentalis* showed the accumulation of large amounts of nucleocapsid and nonstructural proteins and the presence of several vesicles with virus particles in the salivary glands, indicating that the salivary glands may be a major site of virus replication (Wijkamp et al., 1993). The presence of potato leaf roll virus particles in the intestinal epithelium could be visualized by the immunogold labeling and immunofluorescence techniques, suggesting that intestinal cells might be the pathway for PLRV transport from gut lumen into hemocoel (Garret et al., 1993). Immunogold labeling was employed to locate barley mild mosaic virus particles in the zoospores of the fungal vector *Polymyxa graminis*. Labeled bundles of presumed virus particles were observed in about 1% of the zoospores

released from the plant roots, and in zoospores inside zoosporangia (Jianping et al., 1991).

7.3.9 Gel Double-Diffusion Precipitin Bands

The precipitin bands developed when the antigen and antibody interact in agar gel can be examined after negative staining under the electron microscope. Two procedures may be followed.

7.3.9.1 Excavation method (Watson et al., 1966)

A small amount of preciptin band formed in an agar plate (0.5 × 2 × 1 mm) is scooped out by a scalpel or razor blade and homogenized in 5 µl buffer. The homogenate is adsorbed to a filmed grid, which is gently rinsed with water and negatively stained. Clumps of virus particles linked by antibody bridges are visible under the electron microscope.

7.3.9.2 Direct printing (Milne, 1993b)

A filmed grid is gently placed over the preciptin band formed in an agar plate and left for about 15 min. The grid is then taken, rinsed gently with water, and examined.

7.4 TECHNIQUES FOR DETECTION OF MYCOPLASMA-LIKE ORGANISMS

The plant pathogenic MLOs are generally larger than viruses with varying shapes and sizes ranging from 50 nm to 1000 nm in diameter. When extracted from infected plant tissues, the MLO bodies may become flattened or fragmented, resembling degraded host debris derived from chloroplasts, mitochondria, or endoplasmic reticulum, thus posing a formidable problem for establishing the identity of the MLO reliably. Moreover, none of the plant MLOs, barring the spiroplasma, has been isolated in pure culture, making it difficult to study the distinct morphological features of different MLOs, which are morphologically indistinguishable in the characteristics determined by electron microscopy (Hiruki, 1988; Whitcomb and Tully, 1989; Clark, 1992). Hence detection and identification of MLOs require the use of immunosorbent electron microscopy and immunogold labeling, and in recent years, antisera have been raised against several MLOs, facilitating the application of these techniques.

7.4.1 Immunosorbent Electron Microscopy

Derrick and Brlansky (1976) showed that corn stunt spiroplasma could be trapped by ISEM; this finding led to the successful demonstration that MLOs could be trapped and identified by ISEM (Milne, 1992). Sinha and Benhamou (1983) could trap aster yellows (AY) MLO bodies, using the homologous antiserum, from partially purified extracts of AY-infected aster plants. The AY MLO and peach X MLO were detected by ISEM in individual vector leafhoppers, *Macrosteles fascifrons* and *Paraphelpsius irroratus*, respectively. However, some of the hoppers that had access to infected plants did not transmit the MLO and were ISEM-negative (Sinha, 1988). The presence of the MLO that causes flavescence dorée (FD) disease of grapevine was detected by ISEM in extracts of both infected grapevine and vector leafhoppers *Euscelidius variegatus* (Caudwell et al., 1983). The antiserum raised against FD-enriched material from infective leafhoppers was used to detect the MLO in plants, whereas the antiserum to material from infected *Vicia faba* plants was used to detect the MLO in leafhoppers to prevent the effects of host-directed antibodies (Lherminier et al., 1990).

7.4.2 Immunogold Labeling Technique/Gold Label Antibody Decoration

The availability of both polyclonal and monoclonal antibodies specific for several MLOs has helped to improve and refine serological techniques that can be employed for reliable detection and identification of MLOs. The protein A gold labeling procedure has been found to yield reliable and convincing results. Lherminier et al. (1990) reported that flavescence dorée MLO infecting grapevine could be detected by gold labeling of trapped MLO bodies. Vera and Milne (1994) developed the protocol for immunotrapping, gold labeling, and electon microscopy of MLOs from crude preparations of infected plants or vector insects. The European aster yellows (EAY) MLO in periwinkle plants (*Catharanthus roseus*) and in vector leafhopper. *Macrosteles quadripunctulatus* and Australian tomato big bud (TBB) in infected tomato plants were detected and differentiated. The MLOs of EAY and TBB are morphologically indistinguishable, but by using the immunogold labeling technique these two MLOs can be distinguished (Figs. 7.7 and 7.8) (Appendix 7 [i]). Milne et al. (1995) reported that by immunogold labeling of thin sections (postembedding labeling) and labeling of bulk tissues before embedding and sectioning (preembedding labeling), primula yellows (PY), tomato big bud (TBB), and bermudagrass white leaf (BGWL) MLOs could be reliably differentiated (Fig. 7.9) (Appendix 7 [ii]).

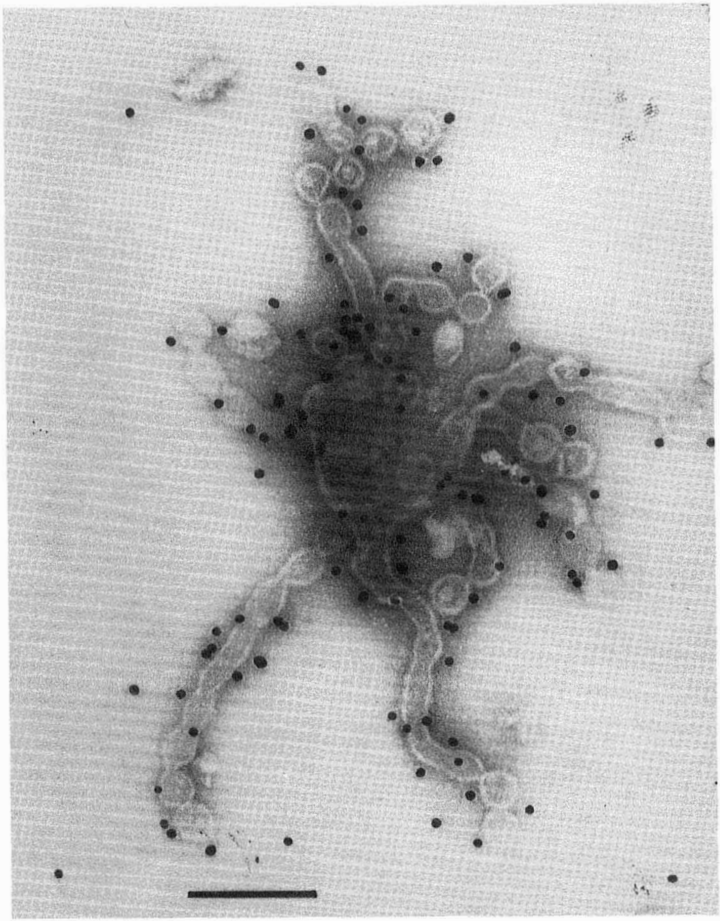


Figure 7.7 European aster yellows (EAY) labeled with gold and negatively stained with 0.5% ammonium molybydate; bar represents 200 nm (Blackwell Science Ltd. and British Society for Plant Pathology, UK). (Courtesy of Vera and Milne, 1994.)

7.5 TECHNIQUES FOR DETECTION OF FUNGAL PATHOGENS

Immunosorbent electron microscopy and protein A gold labeling have been used to detect surface antigens of fungal pathogens. Colloidal gold was linked to chitinase or lectins to detect the presence of chitin on fungal cell walls in root cells infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Chamberland et al., 1985). When two antisera, one against the surface components of *Botrytis cinerea* and the other raised against the fimbriae of the smut fungus *Ustilago*

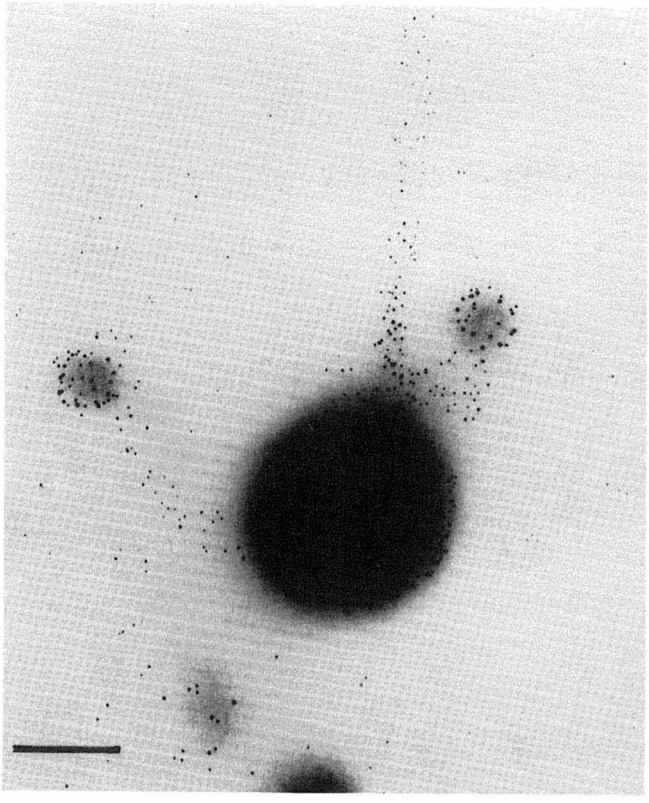


Figure 7.8 EAY MLO in sap from *Catharanthus roseus* labeled with gold without staining; bar represents 200 nm (Blackwell Science Ltd. and British Society for Plant Pathology, UK). (Courtesy of Vera and Milne, 1994.)

violacea, labeled with protein A–gold complex were used, heavy gold labeling of host cells in infected tissue, but not cells in healthy tissue, was observed (Appendix 7 [iii]). The labeled host cells, in many cases, were not penetrated by the pathogen and the nearest hypha was some distance away from labeled cells. This may be the result of movement of the surface antigen of *B. cinerea* into host tissue in advance of the pathogen (Svircev et al., 1986).

7.6 TECHNIQUE FOR DETECTION OF BACTERIAL PATHOGENS

The immunogold labeling procedure for the detection and identification of bacterial pathogen has been used only to a limited extent. Fuerst and Perry

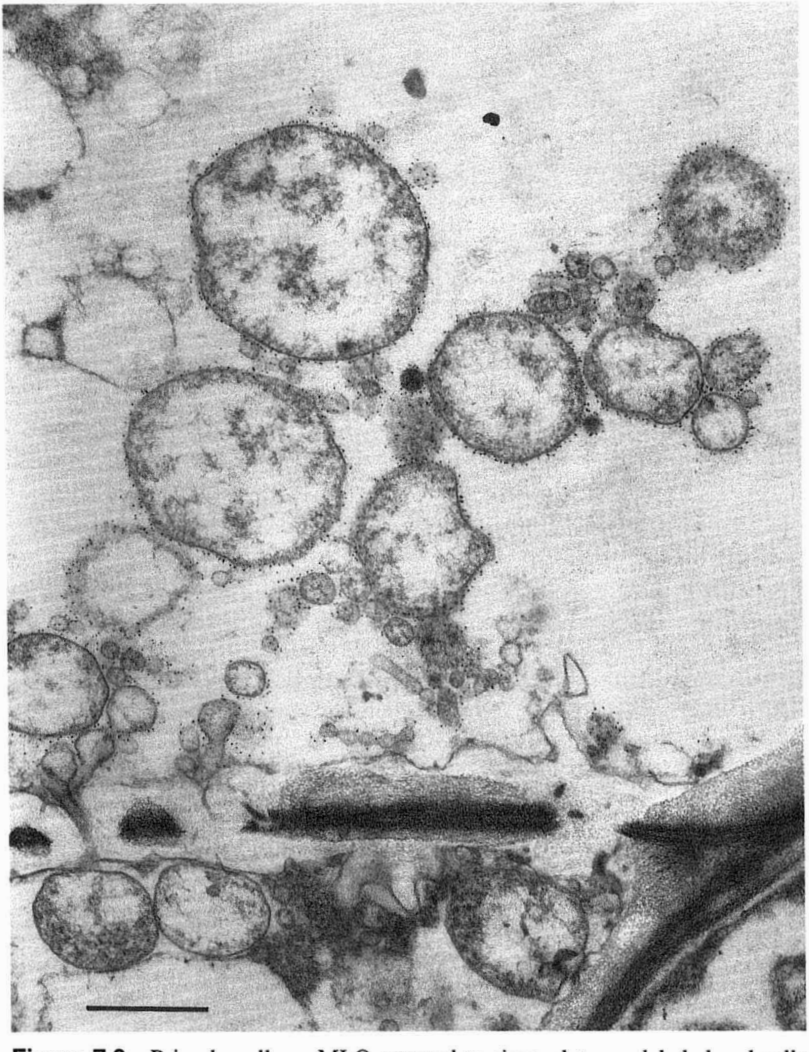


Figure 7.9 Primula yellows MLO external to sieve plate are labeled and cells below sieve plate that are not exposed to antibody remain unlabeled; bar represents 500 nm (Kluwer Academic Publishers Netherlands). (Courtesy of Milne et al., 1995. Reprinted by permission of Kluwer Academic Publishers.)

(1988) developed the immunogold labeling procedure to demonstrate the presence of lipopolysaccharide antigens on both the sheathed flagellum and cell surface of *Vibrio cholerae*. Li et al. (1993) employed the immunogold labeling technique to determine the location of protein epitopes in *Pseudomonas andropogonis*, using monoclonal antibodies conjugated with protein A–gold complex. Gold particles were observed around lysed cells and within the cytoplasm of thin sections of bacterial cells (Appendix 7 [iv]).

SUMMARY

The basic principle involved in electron microscopy, methods of preparing support film, calibration of the electron microscope, and negative staining and different stains employed are described. The standard electron microscopic techniques, such as metal shadowing, dip method, leaf-dip serology, immunosorbent electron microscopy, decoration, and gold label antibody decoration, have been employed for rapid detection of viruses and MLOs in plant materials.

APPENDIX 7(i): DETECTION OF MLOs IN CRUDE EXTRACTS OF PLANTS AND VECTOR INSECTS BY GOLD LABEL ANTIBODY DECORATION (GLAD) (VERA AND MILNE, 1994)

i) Coat carbon-fronted Formvar-filmed 400 mesh nickel grids with F(ab')2 of MLO-specific IgG at 6 μg/ml in 0.1 *M* maleate buffer, pH 6.8 (MB), for 15 min at room temperature.
ii) Extract the sap of infected/healthy plant stem in 0.3 *M* glycine buffer, pH 8, containing 20 m*M* MgCl (GMgB), at 200 μl/100 mg of tissue, using a roller press; in case of infective insects, anesthetize with CO_2 and homogenize individual insects in 50 μl of GMgB.
iii) Rinse coated grids in MB and float them on MLO extracts for 3 hr at room temperature and rinse the grids with MB.
iv) Float the grids on 1% glutaraldehyde in MB for 15 min and rinse the grids with MB.
v) Float the grids on intact anti-MLO or control IgG at 20 μg/ml in MB for 15 min and rinse with MB.
vi) Float the grids on goat antirabbit IgG bound to 5 nm or 15 nm diameter gold particles at 1:50 dilution (v/v) in MB for 15 min and rinse first with MB and then with distilled water.
vii) Negatively stain with 0.5% (w/v) ammonium molybdate, pH 6.5.

APPENDIX 7(ii): IDENTIFICATION OF MLOs BY IMMUNOGOLD LABELING OF THIN SECTIONS OR BULK TISSUES FROM INFECTED PLANTS (MILNE ET AL., 1995).

A. Post embedding procedures for thin section labeling
   i) Fix pieces of leaf in 4% formaldehyde (FA) (w/v), 0.1% glutaraldehyde (GA) (w/v), and 0.5 m*M* calcium chloride in 0.1 *M* potassium phosphate buffer, pH 7 (PB), at room temperature by vacuum infiltration for 1 hr; remove the edge tissue and treat the small veins with fresh fixative on ice in a shaker for another 1 hr.
   ii) Wash four times, 10 min each, in PB plus calcium chloride, containing 3.5% sucrose, and shake for 1 hr in the same solution plus 50 m*M* ammonium chloride.
   iii) Wash the tissues four times, 15 min each, in cold 0.1 *M* maleate buffer, pH 6.8 (MB), containing sucrose; change the maleate buffer and leave the tissues overnight in the buffer.
   iv) Soak the tissues for 2 hr in MB-sucrose containing 2% uranyl acetate (UA), pH 6.4.
   v) Transfer the tissues to 50% acetone (v/v) at 0°C and then to a rotor in a freezer at -20°C.
   vi) Perform the following steps at -20°C: transfer tissues to 90% acetone and infiltrate with LR gold resin (LRG); then transfer the tissues to LRG plus 0.5% Lowicryl initiator (w/v); embed in closed BEEM capsules and polymerize under indirect UV light.
   vii) Perform further processing at room temperature; block nonspecific sites for 15 min in NGS-TBS-BSA (normal goat serum (NGS) at 1/30 (v/v) dilution in 0.05 *M* Tris-HCl, 0.15 *M* NaCl (TBS) containing 0.2% bovine serum albumin (BSA) (w/v), pH 7.6).
   viii) Incubate with primary antibody (50–200 μg/ml) diluted in NGS–TBS–BSA overnight at 4°C and rinse five times, 3 min each, on NGS-TBS-BSA.
   ix) Incubate on GAR-G5 or GAM-G5 at 1/50 dilution in TBS, pH 8.2, containing 1% BSA; rinse on TBS, pH 8.2 five times, 3 min each, and rinse again on deionized water five times, 1 min each.
   x) Stain with 5% aqueous uranyl acetate (w/v) for 60 min; rinse on deionized water five times, 3 min each, and transfer the sections on filmed (Formvar) copper grids.

B. Preembedding procedures for labeling bulk tissues
   i) Chill young leaves infected with MLO on ice; isolate the veins and slice transversely into disks of 0.2–0.4 mm thickness.
   ii) Immerse a group of disks in 50–200 μl of primary antibody diluted

in PB placed in a well of a microtiter plate; agitate at 4°C for 60 min and rinse the disks in cold PB four times, 3 min each.

iii) Incubate the disks in 200 μl of GAR-G5 or GAM-G5 diluted in PB for 60 min and rinse them in cold PB four times, 3 min each.

iv) Fix the tissues in the cold on a shaker in 2.5% GA (w/v) in PB for 60 min; then fix in 0.1% osmium tetroxide (w/v) in PB for 60 min and embed in Epon, after proper orientation of the tissues for longitudinal sectioning.

v) Cut longitudinal sections through the entire disk of tissue, exposing both ends to antibody and strain in lead citrate, and observe under the electron microscope.

vi) Maintain controls in heterologous combinations.

APPENDIX 7(iii): DETECTION OF FUNGAL ANTIGENS BY PROTEIN A–GOLD LABELING (SVIRCEV ET AL., 1986)

A. Electron microscopy

i) Cut leaf disks (1 mm diameter) from inoculated and control leaves at predetermined intervals after inoculation.

ii) Fix in 4% glutaraldehyde in cacodylate buffer, pH 6.8, for 2 hr and rinse twice in buffer.

iii) Postfix with 2% osmium tetroxide for 1 hr and rinse twice in water.

iv) Stain in 5% uranyl magnesium acetate for 20 min at room temperature; dehydrate in a graded acetone series; infiltrate with Epon-Araldite; cut sections by using a diamond knife on an ultramicrotome; and mount the sections on coated grids.

B. Protein A–gold labeling

i) Add 4.0 ml of 1% aqueous sodium citrate to 100 ml of a boiling solution of 0.01% chloroauric acid; cool for 5 min until a wine red color develops and store the colloidal gold suspension in the dark.

ii) Adjust the pH of colloidal gold suspension (10 ml) to 6.9 with potassium carbonate; add the suspension to 0.3 mg of protein A in 0.2 ml of distilled water and centrifuge at 48,000 g at 4°C.

iii) Resuspend the dark red protein A–gold complex pellet in 10 ml of 0.01 *M* phosphate-buffered saline solution, pH 7.4, and store at 4°C.

iv) Place thin sections of test plant tissues on a saturated solution of sodium periodate for 2–3 min; wash the sections three times in distilled water and treat with 1% ovalbumin for 5 min to prevent nonspecific binding.

v) Float the sections on specific antiserum for 30 min at room temperature and wash the grids through a series of water droplets.

vi) Treat the sections with protein A–gold solution for 30 min; wash as before; stain with 3% uranyl acetate for 20 min and observe under the electron microscope.

APPENDIX 7(iv): IMMUNOGOLD LABELING OF BACTERIAL PATHOGENS (FUERST AND PERRY, 1988; LI ET AL., 1993)

A. Immunolabeling of whole cells
   i) Cover a copper grid with nitrocellulose and stabilize with carbon; place the grid on a bacterial cell suspension, carbon-side down, for 5 min in a plastic petri dish or parafilm sheet.
   ii) Place the grid successively on 1% glutaraldehyde in phosphate-buffered saline solution (PBS), pH 7.2, for 5 min; on PBS-20 m*M* glycine for 10 min; on PBS-1% bovine serum albumin (PBS-BSA) for 5 min; on MAB-culture supernatant fluid for 30 min; on 4 drops of PBS-BSA for 1 min each; on goat antimouse IgM and IgG gold at 1/20 dilution in PBS-BSA for 30 min; and finally on 3 drops of sterile distilled water (SDW) for 5 min each.
   iii) Blot excess water with filter paper, air-dry, and observe under the electron microscope without staining or stain first with 4% uranyl acetate for 1 min and then with citrate for 30 sec before examining the grids under the electron microscope.

B. Immunolabeling of thin sections
   i) Fix the pellet of bacterial cells, after centrifugation with 5% glutaraldehyde in 0.05 *M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 8.0, for 1 hr at room temperature.
   ii) Wash the cells with buffer, suspend in 3% agarose, and fix in 1% osmium tetroxide in HEPES buffer for 1 hr at room temperature.
   iii) Treat with 2% aqueous uranyl acetate for 30 min; dehydrate through two changes of 70% ethanol for 30 min each; infiltrate with LR white resin overnight at 4°C and embed in LR white resin by polymerization at 50°C for 24 hr.
   iv) Cut thin sections by using an ultramicrotome and collect them on carbon-stabilized nitrocellullose-filmed copper grids.
   v) Place the grids successively on PBS-BSA for 5 min on MAB diluted in PBS-BSA for 30 min, on 4 drops of PBS-BSA for 1 min each, of protein A–gold at 1/20 dilution in PBS-BSA for 30 min, and on 3 drops of SDW for 5 min each; remove excess fluid with filter paper and air-dry.
   vi) Stain the sections with uranyl acetate for 5 min and then with Reynolds lead citrate for 2 min and observe under the electron microscope.

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8
Serodiagnostic Methods

Early detection of pathogenic infection is essential to eliminate all infected seeds and planting materials which form primary sources of infection and to rogue out infected plants present in the field whenever possible. The detection and characterization of plant pathogens usually depend on isolation of the pathogen and recording of the symptoms it induces on susceptible host plants. This procedure is a time-consuming process and often cannot be used to distinguish reliably between closely related species and strains of the pathogen, necessitating the use of faster and more discriminating detection methods. The methods of detecting plant pathogens may be divided into two groups: a) specific methods which may be used to detect a particular species or group of pathogens after preliminary diagnosis suggesting the presence of the particular pathogen, b) nonspecific methods which may be employed in detection of unknown pathogens or when the presence of a number of pathogens is to be detected, as in plant quarantines (Chu et al., 1989). However, the use of the term *specific* or *nonspecific* to indicate the extent of the reliability of a method to be employed for the detection of pathogens will be more appropriate. Thus serological and nucleic acid hybridization methods can be considered as more specific and reliable than most of chemical methods, which are often found to be nonspecific and less reliable, though these methods are simpler.

8.1 DETECTION OF PLANT VIRUSES

Production of antiserum, either polyclonal or monoclonal, is the first requirement for performing any of the several serological assay techniques. Different antibody preparations are available for the detection of various plant viruses. Polyclonal antibodies can detect several isolates or strains of a virus, whereas

monoclonal antibodies can be used to differentiate between isolates or strains. The plant viruses for which monoclonal antibodies (MABs) have been produced are listed in Table 8.1 (Fig. 8.1). The choice of serological technique depends on specificity, sensitivity, and accuracy of results; ability to detect even at low concentrations of antigens; adaptability to field conditions; relatively inexpensive nature; and amenability to testing of a large number of samples within short periods, so that it can be employed in certification programs. Methods of production of antisera and various serological techniques have been described in detail by Matthews (1957), Bercks et al. (1972), Van Regenmortel (1982), Van Regenmortel and Dubs (1993), and Narayanasamy and Doraiswamy (1996). Currently, labeled antibody techniques and immunoelectron microscopy (Chapter 7) are widely employed and hence these methods are discussed.

8.1.1 Labeled Antibody Techniques

By attaching a label to either the antigen or antibody, the sensitivity of detection of antigen–antibody reactions can be substantially increased. Three types of markers, viz. enzymes, fluorescent dye, and radioactive materials, have been most commonly used for labeling the antibodies specific for the antigen to be detected.

8.1.1.1 Enzyme-Linked Immunosorbent Assay

Introduction of enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1971; Clark and Adams, 1977) has been an important landmark in serological detection and assay of plant viruses. It has become a preferred method, because of its sensitivity, economical use of antiserum, availability of quantifiable data, and the capacity for handling large numbers of samples rapidly. There are many variations in ELISA; the most common form is the double-antibody sandwich (DAS) method (Fig. 8.2). Other widely used variations are direct antigen coating (DAC)-ELISA, protein A (extracted from the cells of *Staphylococcus aureus*) coating (PAC)-ELISA, and indirect ELISA using virus antibody, in addition to labeled antiglobulin conjugate. The methods are described in Appendix 8 (i) A, B, C, and D. The DAS-ELISA method is highly strain-specific and requires the preparation of different antibody conjugate for each virus to be tested. On the other hand, the indirect ELISA procedure has an important advantage in that a single conjugate can be used for different viruses and the antirabbit globulin conjugate or antigoat globulin conjugate commercially available can be employed. All serotypes of a virus may be detected with an antiserum to one strain (Van Regenmortel and Burckard, 1980). However, Su and Wu (1989), using monoclonal antibody specific to

Table 8.1 Plant Viruses for Which MABs Have Been Produced

Virus group	Virus	References
1. Alfalfa mosaic virus	Alfalfa mosaic virus	Halk et al., 1984 Hajimorad et al., 1990
2. Carlavirus	Carnation latent virus	Jordan, 1989
	Potato virus M	Saarma et al., 1989
3. Carmovirus	Carnation mottle virus	Saarma et al., 1989
4. Caulimovirus	Carnation etched ring virus	Hsu and Lawson, 1985a,1985b
5. Closterovirus	Apple chlorotic leaf spot virus	Poul and Dunez, 1989
	Carnation necrotic fleck virus	Saarma et al., 1989
	Citrus tristeza virus	Vela et al., 1986 Permar et al., 1990
6. Comovirus	Bean pod mottle virus	Joison and Van Regenmortel, 1991
	Cowpea mosaic virus	Kalmar and Eastwell, 1989
	Cowpea severe mosaic virus	Kalmar and Eastwed, 1989
7. Cucumovirus	Cucumber mosaic virus	Haase et al., 1989 Maeda et al., 1988 Porta et al., 1989
8. Dianthovirus	Sweet clover necrotic mosaic virus	Hiruki et al., 1984
9. Furovirus	Soil-brone wheat mosaic virus	Bahrani et al., 1988
	Peanut clump virus	Huguenot et al., 1989
10. Geminivirus	African cassava mosaic virus	Thomas et al., 1986.
	Maize streak virus	Dekker et al., 1988
	Bean golden mosaic virus	Cancino et al., 1995
11. Ilarvirus	Apple mosaic virus	Halk et al.,1984
	Citrus variegation virus	Hsu et al., 1983
	Prune dwarf virus	Jordan, 1984
	Prunus necrotic ringspot virus	Halk et al., 1984
	Tobacco streak virus	Halk et al., 1984

(*continued*)

Table 8.1 Continued

Virus group	Virus	References
12. Luteovirus	Banana bunchy top virus	Wu and Su, 1990
	Barley yellow dwarf virus	Diaco et al., 1983 D'Arcy et al., 1990
	Beet western yellows virus	D'Arcy et al., 1989 Ohshima and Shikata, 1990
	Potato leaf roll virus	Martin and Stace-Smith, 1984; Ophshima et al., 1988; Ohshima and Shikata, 1990
	Subterranean clover red leaf virus	Hewish et al., 1983
	Tobacco necrotic dwarf virus	Ohshima et al., 1989
13. Nepovirus	Arabis mosaic virus	Dietzgen, 1983; Tirry et al., 1988
	Grapevine fanleaf virus	Huss et al., 1987
	Satsuma dwarf virus	Nozu et al., 1983, 1986
	Tomato ringspot virus	Powell and Marquez, 1983; Powell, 1990
14. Potexvirus	Potato virus X	Torrance et al., 1984; Lizarraga and Fernandez-Northote, 1989
15. Poty virus	Bean common mosaic virus	Wang et al., 1985
	Bean yellow mosaic virus	Scoft et al., 1989
	Clover yellow vein virus	Scoft et al., 1989
	Grapevine leaf roll virust type III	Hsu et al., 1989; Zimmerman et al., 1990
	Lettuce mosaic virus	Hill et al., 1984
	Maize dwarf mosaic virus	Hill et al., 1984
	Papaya ringspot virus	Baker and Purcifull, 1984
	Pea mosaic virus	Scoft et al., 1989
	Peanut mottle virus	Sherwood et al., 1985; Sherwood et al., 1987
	Peanut stripe virus	Culver and Sherwood, 1988
	Plum pox virus	Himmler et al., 1988
	Potato virus A	Gugerli, 1983; Bonnekamp et al., 1990
	Potato virus Y	Gugerli and Fries, 1983; Rose and Hubbard, 1986
	Soybean mosaic virus	Hill et al., 1984
	Sugarcane mosaic virus	Cheng et al., 1993

Table 8.1 Continued

Virus group	Virus	References
	Tobacco etch virus	Dougherty et al., 1985
	Tulip breaking virus	Hsu et al., 1984
	Watermelon mosaic virus II	Somowiyarjo et al., 1988
	Zucchini yellow mosaic virus	Somowiyarjo et al., 1988; Wisler et al., 1989
16. Reovirus	Rice dwarf virus	Harjosudarmo et al., 1990
	Rice ragged stunt virus	Ohshima et al., 1990
17. Sobemovirus	Southern bean mosaic virus	Tremaine and Ronald, 1983
18. Tenuivirus	Rice stripe virus	Omura et al., 1986
19. Tobamovirus	Beet necrotic yellow vein virus	Merten et al., 1985; Torrance et al., 1988
	Cucumber green mottle mosaic virus	Takahashi et al., 1989
	Odontoglossum ring spot virus	Dore et al., 1987
	Tobacco mosaic virus	Dietzgen and Sander, 1982; Briand et al., 1982
	Tomato mosaic virus	Dekker et al., 1987 Takahashi et al., 1989
20. Tospovirus	Tomato spotted wilt virus	Sherwood et al., 1989 Huguenot et al., 1990 Hsu et al., 1990 Adam et al., 1991

banana bunchy top virus (BBTV), reported that direct ELISA was 16 times more sensitive than indirect ELISA in detecting BBTV. When the PAC-ELISA procedure is followed, care should be taken to use the optimal concentration of protein A, as higher concentrations may cause nonspecific reactions, and lower concentrations may result in negative results. Under a short incubation period (1–2 h at 35°C) DAC-ELISA and PAC-ELISA were as sensitive as DAS-ELISA in detecting groundnut mottle and clump viruses (Hobbs et al., 1987). Lin et al. (1991) reported that avidin–biotin–peroxidase complex indirect sandwich ELISA was more sensitive than DAS-ELISA and protein A indirect sandwich ELISA in detecting cucumber mosaic virus.

Many signal enhancement techniques have been used in conjunction with the basic ELISA procedure.

a. Biotin–avidin. The protein avidin has very high affinity for biotin, which is chemically coupled to the IgG. Avidin is coupled to the enzyme used for

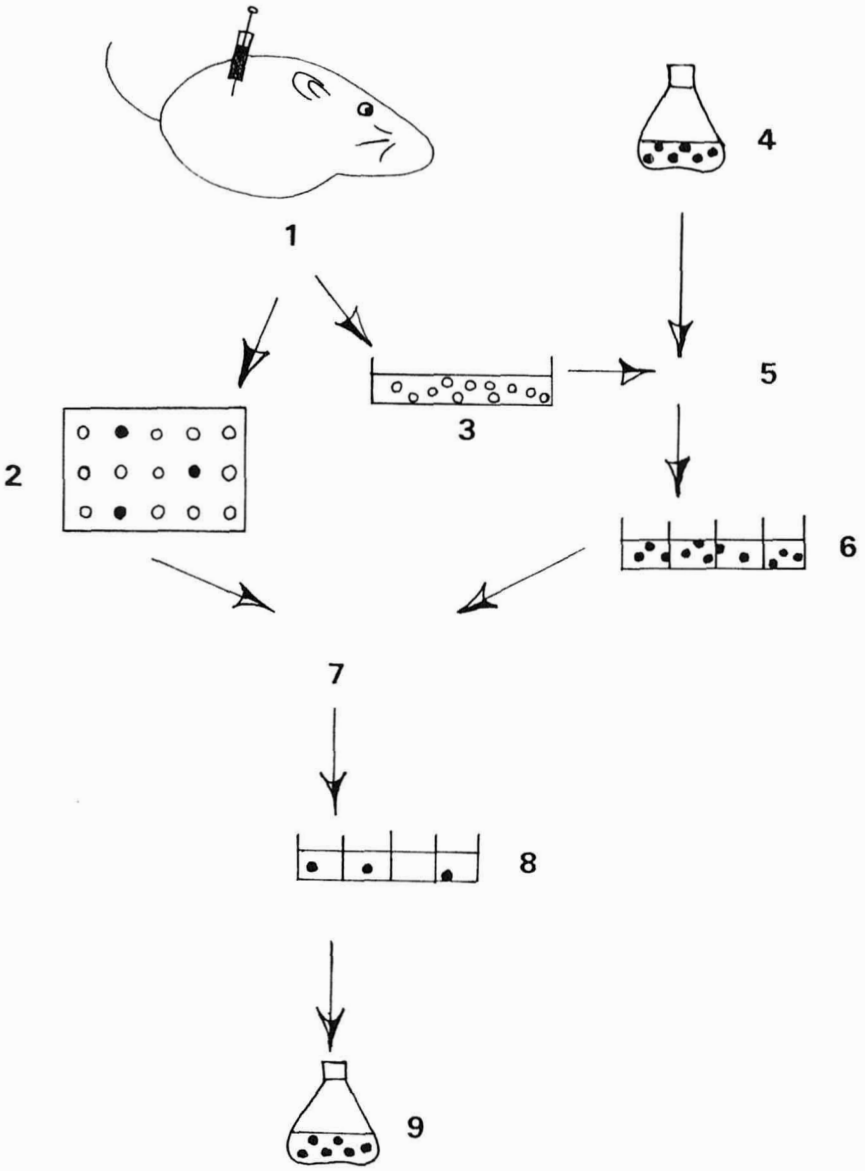


Figure 8.1 Steps in the production of monoclonal antibodies: 1, Immunization of mouse with antigen; 2, testing of screening methods with test bleed antisera; 3, collection of β-lymphocytes from spleen; 4, preparation of myeloma cell suspension; 5, fusion of myeloma cells and β-lymphocytes; 6, selection of hybridoma in multiwell plates; 7, determination of antibody contents secreted by selected hybridomas; 8, dilution of hybridomas to yield 1 cell/well; 9, cultivation of selected hybridoma cells and storing by freezing (CAB International, U.K.). (Adapted from Leach and White, 1991.)

detection. The procedure increases the number of enzyme molecules trapped on antigen, increasing the sensitivity (Zrein et al., 1986).

b. Fluorogenic substrate. A fluorogenic substrate, 4-methyl-umbelliferyl phosphate, for alkaline phosphate is used instead of the standard chromogenic substrate (Torrance and Jones, 1982).

c. Radioactive labels. In the radioimmune ELISA ^{125}I-labeled IgG is substituted for enzyme-linked IgG and the bound and labeled IgG is, after dissociation, assayed (Ghabrial and Shepherd, 1967).

d. Polystyrene beads. Polystyrene beads (6.5 mm diameter) are used as solid-phase instead of ELISA plates. Chen et al. (1982) found this procedure to be more sensitive in detecting the differences among isolates of soybean mosaic virus.

Van Regenmortel and Dubs (1993) have reviewed the merits of various other variations in ELISA suggested by different researchers.

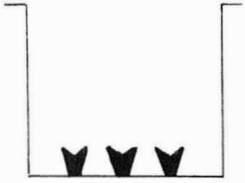
Modifications to improve sensitivity and to reduce assay time have been suggested by Goodwin and Banttari (1984) and Stobbs and Barker (1985). Wounding by removing a small piece of the bulbs just after lifting, followed by storage at 17°C or 20°C for 3 weeks, markedly increased the detectability of iris severe mosaic virus by ELISA (van der Vlugt et al., 1993). Attempts have been made to find less expensive chemicals to reduce the cost of testing without losing the sensitivity of the alkaline phosphatase (ALP) system. An ELISA test conducted with horseradish peroxidase (HRP) was as sensitive and specific as the ALP system for detection of turnip yellow mosaic virus, potato virus Y, and potato leaf roll virus in infected plants (Polak and Kristek, 1988). Neustroevx et al. (1989) reported that β-galactosidase isolated from *Escherichia coli* was more effective in labeling antibodies to potato virus X (PVX) than horseradish peroxidase, as it decreases background reaction because of the lack of this enzyme in plant sap. Penicillinase-(PNC)-based ELISA had similar sensitivity to ALP- and H RP-based ELISA for detecting maize mosaic virus, peanut mottle virus, and tomato spotted wilt virus. Penicillinase and penicillin are readily available and substantially less expensive (Sudharsana and Reddy, 1989).

It was found that DAS-ELISA with urease conjugate was superior to the ALP system, as the color change from yellow to violet could be more easily visually recognized than the change from colorless to yellow in the ALP system. The visual detection threshold was 0.5 ng/ml of TMV with urease and 500 ng/ml with phosphatase (Gerber and Sarker, 1988). Mizenina et al. (1991) developed the protocol for using the inorganic pyrophosphatase (PPase) from *Escherichia coli* conjugated with antibodies and tetrazolium pyrophosphate as the substrate in ELISA. The advantages of this method are high sensitivity, negligible level of background reactions in controls, bright blue-greenish color

A

B

1



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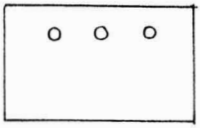

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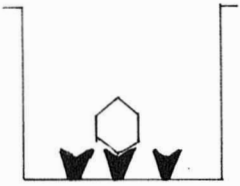

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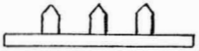

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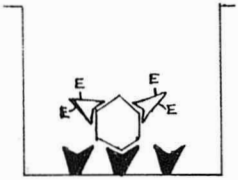

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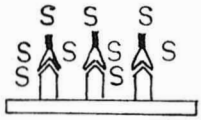

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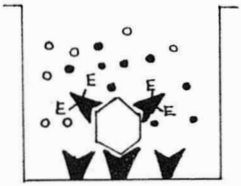

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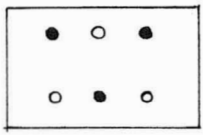

allowing visual detection of reaction, and high stability of PPase-conjugated antibodies. Twelve viruses, such as potato viruses X, Y, M, S, and leaf roll; carnation mottle virus; barley stripe mosaic virus; tobacco necrosis virus; soybean mosaic virus; and cucumber mosaic virus, could be detected by this method.

e. Applications of ELISA. Enzyme-linked immunosorbent assay tests have been performed to detect the viruses in different plant parts, seeds, and vectors which transmit the plant viruses even when the viruses are present in very low concentrations and in very early stages of disease development. Arabis mosaic virus could be detected at concentrations as low as 80 ng/ml and in extracts of infected leaves at dilutions up to 10^{-16}. Plum pox virus, an elongated virus, was detectable at 10 ng/ml in purified virus preparations and in leaf extracts at 10^{-4} dilution (Voller et al., 1976). Enzyme-linked immunosorbent assay has been used to detect several viruses, including citrus tristeza virus (Bar-Joseph et al., 1979); groundnut viruses (Nolt et al., 1983; Konate and Barro, 1993); squash mosaic virus in extracts from seed coats, papery layers, and distal halves of embryos from individual curcurbit seeds (Nolan and Campbell, 1984); rice tungro-associated viruses in rice plants (Bajet et al., 1985); strawberry latent ringspot virus in many plant species (Kristek and Polak, 1990); rice grassy stunt virus in infected rice plants and individual viruliferous planthopper (*Nilaparvata lugens*) (Iwasaki et al., 1985); and maize streak virus in infected maize plants and inoculative *Peregrinus maidis* (Falk et al., 1987) and in tomato leaf curl virus-infected tomato plants and viruliferous whiteflies (Ragupathi, 1995). Using DAS-ELISA, MacKenzie and Ellis (1992) observed that there was no systemic accumulation of TSWV in transgenic tobacco plants expressing viral nucleocapsid protein, since these plants were resistant to infection after mechanical inoculation with virus. Dahal et al. (1992) employed ELISA to determine the amount of viral coat protein of rice tungro-associated viruses in agroinfected rice plants and found that it was highly correlated with viral nucleic acid contents determined by DNA hybridization.

Using the polyclonal antiserum raised against African cassava mosaic virus (ACMV), eight geminiviruses were detected by ISEM in leaf extracts of

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Figure 8.2 Schematic representation of different steps in enzyme-linked immunosorbent assay (ELISA) and immunoblot assay (DIBA): ELISA: (A) 1, Adsorption of specific antibody to plate; 2, trapping of antigen by antibody; 3, addition of enzyme-labeled specific antibody; 4, addition of enzyme substrate; 5, color development indicating positive reaction. DIBA: (B) 1, Spotting of antigen sample onto paper; 2, addition of specific antibody; 3, addition of protein A–enzyme conjugate; 4, addition of enzyme substrate; 5, color development indicating positive reaction (CAB International, U.K.). (Adapted from Leach and White, 1991.)

plants infected by them, indicating their serological relationship with ACMV. In DAS-ELISA tests, two of the eight geminiviruses did not react with ACMV antiserum, whereas the strength of the reaction of other viruses varied widely (Table 8.2). Among the panel of 10 MABs, there was no reaction between 5 MABs and the 8 geminiviruses tested. The reaction strength with the rest of the MABs employed varied from 0 to 4, as determined by the A_{405} nm values after overnight incubation (Table 8.3) (Harrison et al., 1991).

The presence of viruses in vectors has been detected by ELISA tests. Tomato spotted wilt virus (TSWV) could be detected in 210 individuals of 340 *Frankliniella occidentalis* and in 24 of 120 of *F. schultzei* laboratory-grown adult thrips which had access to infected plants as larvae (Cho et al., 1988; Marchoux et al., 1991; Chamberlain et al., 1993). The accumulation of two proteins—the nucleo capsid (N) and a nonstructural (NSs) proteins—of TSWV in the larvae and adults of *F. occidentalis* after ingestion of the virus for short

Table 8.2 Detection of Geminiviruses by ISEM and DAS-ELISA in Leaf Extracts

Virus	Source plant	Technique	
		ISEM[a]	DAS-ELISA[b]
Bhendi yellow vein mosaic virus (BYVMV)	*Abelmoschus esculentus*	+	0
Croton yellow vein mosaic virus (CYVMV)	*Croton bonplandianum*	+++	+++
Dolichos yellow mosaic virus (DYMV)	*Lablab purpureus*	+	E
Horsegram yellow mosaic virus (HYMV)	*Phaseolus lunatus*	+	-
Horsegram yellow mosaic virus (HYMV)	*P. vulgaris*	++	0
Indian cassava mosaic virus (ICMV)	*Manihot esculenta*	+	+++
Malvastrum yellow vein mosaic virus (MYVMV)	*Malvastrum coromandelianum*	0	E
Tomato leaf curl virus (TomLCV)	*Lycopersicon esculentum*	+	+
Thailand mungbean yellow mosaic virus (TMYMV)	*P. vulgaris*	+++	+++

[a]Virus particles abundant (+++), common (++), few (+), or not found (0).
[b]Reaction strong (+++), moderate (++), weak (+), equivocal (E), not detected (0), or not tested (-).
Source: Harrison et al. (1991).

Table 8.3 Reactions of Geminiviruses with a Panel of MABs to African Cassava Mosaic Virus

Virus	Monoclonal antibodies (MABs)[a]				
	SCR 15	SCR 17	SCR 18	SCR 20	SCR 23
Bhendi yellow vein mosaic virus (BYVMV)	–	0	4	1	0
Croton yellow vein mosaic virus (CYVMV)	3	4	4	4	3
Dolichos yellow mosaic virus (DYMV)	0	4	3	3	4
Horsegram yellow mosaic virus (HYMV)	–	0	4	3	3
Indian cassava mosaic virus (ICMV)	1	0	4	4	0
Malvastrum yellow vein mosaic virus (MYYVMV)	–	0	1	1	0
Tomato leaf curl virus (TomLCV)	–	0	2	0	0
Thailand mungbean yellow mosaic virus (TMYMV)	0	4	2	4	–

[a]Reaction strengths classified on A_{405} nm values as 4 (> 1.8), 3 (1.2–1.8), 2 (0.6–1.2), 1 (0.3–0.6), and 0 (<0.3).
Source: Harrison et al. (1991).

periods on infected plants was detected by ELISA. Within 2 days the amounts of both proteins increased above the levels ingested, indicating the possible multiplication of TSWV in the thrips (WijKamp et al., 1993). Bandla et al. (1994) also reported that monoclonal antibodies specific to NSs proteins could be employed to detect TSWV in thrips vectors. Tospovirus subgroups I and II are considered distinct species and designated as species-tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV) on the basis of results obtained with DAS-ELISA (Vaira et al., 1993). Virus contents of individual aphids carrying sugar beet western yellows, barley yellow dwarf virus, and alfalfa mosaic virus could be determined by ELISA (Kastirr, 1990; Ahoonmanesh et al., 1990). Chickpea chlorotic dwarf geminivirus (CCDV) was detected in individual leafhoppers (*Orosius occidentalis*) by DAS-ELISA. The concentration of the virus decreased when the leafhoppers were fed on a nonhost for the virus, indicating the absence of virus multiplication in the leafhopper vector (Horn et al., 1994).

The availability of virus reservoirs in weed hosts is an important epidemiological factor. Cho et al. (1989) analyzed more than 9000 samples by

ELISA and found that 44 plant species in 16 families were infected with TSWV. Among these, 25 plant species were important reservoirs and 24 species were new hosts for TSWV. Enzyme-linked immunosorbent assay can be used for rapid field tests to screen a large number of samples, and it is of great benefit for epidemiological studies and for indexing of seeds and vegetatively propagated plant materials (Huguenot et al., 1990). Indirect ELISA using monoclonal antibody (MAB) gave accurate results for the detection of bean common mosaic virus in individual bean seeds. Klein et al. (1992), using indirect ELISA to test flour samples of seeds, reported that incidence of bean common mosaic virus–infected seedlings could be predicted in germplasm accessions. Konate and Barro (1993) also detected the peanut clump virus in 16.5% of seeds by ELISA. Pelargonium line pattern virus could be reliably detected by ELISA, if petioles of fully expanded leaves were tested. Virus distribution in infected pelargonium plants was studied by assaying the virus concentration by ELISA (Bouwen and Maat, 1992).

Routine indexing of fruit trees and asexually propagated plants in the field has been done for the presence of several viruses, such as apple mosaic virus (Clark et al., 1976), plum pox virus (Adams, 1978), prune necrotic ringspot virus (Barbara et al., 1978), and citrus tristeza virus (Bar-Joseph et al., 1979), and the testing of potato leaves, tubers, and sprouts for the presence of potato virus S (Richter et al., 1977), potato virus Y and potato virus A (Maat and DeBokx, 1978b), potato leaf roll virus (Maat and DeBokx, 1978a; Smith et al., 1993), and PVX (De Bokx et al., 1980). Tomato ringspot virus, naturally transmitted by nematode, was transmitted by dodder to *Cucumis sativa*, and the presence of the virus was detected by ELISA test (Welliver and Halbrendt, 1992). By using a specific MAB, bean golden mosaic, euphorbia mosaic, rhynchosia mosaic, squash leaf curl, soybean yellow mosaic, and tomato mottle geminiviruses were detected by Cancino et al. (1995).

Resistance to peanut stripe virus in different *Arachis* spp. was tested by detecting the virus by ELISA test. Certain lines of *A. diogi, A. halodes,* and *Arachis* spp. did not have detectable amounts of the virus (Culver et al., 1987). The effectiveness of elimination of peanut mottle virus, peanut stripe virus, and TSWV from vegetatively maintained groundnuts was tested by DAS-ELISA (Dunbar et al., 1993). Tiongco et al. (1993) employed the ELISA test or latex test to monitor the incidence of rice tungro disease. Rice tungro spherical virus (RTSV) was detected 1 week earlier than rice tungro bacilliform virus (RTBV). The infectivity of green leafhoppers collected from the field corresponded to disease development. Induction of resistance to rice tungro disease in susceptible cultivars by using the antiviral principles (AVPs) from the seed sprouts of pigeonpea and mungbean was demonstrated by determining the titers of RTBV and RTSV, after the application of AVPs (Muthulakshmi and Narayanasamy, 1996).

Though the ELISA test is found to be useful for detection of many plant viruses, Ramsdell et al. (1979) reported that it was less sensitive than infectivity tests on *Chenopodium quinoa* for indexing immature grape tissue for peach rosette mosaic virus. Hughes and Ollennu (1993) also found that ELISA was less sensitive for the detection of cocoa swollen shoot virus than the virobacterial agglutination (VBA) test involving *Staphylococcus aureus* cells for conjugation with antivirus antibodies. The VBA test alone detected the virus in all cocoa trees infected by swollen shoot virus and additionally identified infection in many symptomless trees. Moreover, the need for careful interpretation of the results obtained by ELISA is indicated by Mink et al. (1985), who observed that extracts from rapidly growing shoot tip leaves of apparently healthy apple rootstock and scion trees showed absorbance values A_{405} nm that were similar to those of tomato ringspot virus–infected samples. No virus could be detected in these tissues by bioassay or partial purification. The rapidly growing apple shoots appear to contain nonviral antigens capable of reacting with antibodies in several tomato ringspot virus antisera.

Using ELISA, serological differentiation and identification of plant viruses and strains have been performed. The serological relationships of five isolates of Indian peanut clump virus were established by ELISA (Nolt et al., 1988). Sherwood et al. (1989) reported that the monoclonal antibody (MAB) against TSWV reacted positively with isolated nucleocapsid proteins, but not with envelope-associated proteins in the ELISA test. Twenty isolates of TSWV were grouped into three serotypes based on their reactions to the antisera raised against virus nucleocapsid protein and glycoprotein (de Avila et al., 1990). On the basis of the serological reactivity and infectivity on *Capsella bursa-pastoris*, the common strain and a new strain of beet mild yellowing luteovirus could be distinguished (Table 8.4) (Stevens et al., 1994). The common strain reacts with all three MABs positively and also infects *C. bursa-pastoris*, whereas the new strain reacts positively only with two of the three MABs and does not infect *C. bursa-pastoris*. Hill et al. (1994) proposed a system of antigenic signature analysis for the rapid differentiation of soybean mosaic virus isolates by em-

Table 8.4 Differentiation of Beet Mild Yellowing Luteovirus Strains by ELISA Test and Infectivity[a]

Strain of BMYV	Infectivity	MABs MAFF24	MAFF24 BWYVV-BC-510H	BWYVV-BC-510H BYDV-PAV-IL-1
Common strain	+	+	+	+
New strain	-	+	+	-

[a] +, positive; –, negative.
Source: Stevens et al. (1994).

ploying a panel of nine MABs. The antisera may be prepared against disrupted virus particles or other proteins present in virus-infected plants such as helper components, and these antisera can also be used for detection and differentiation of plant viruses. Joisson et al. (1992) demonstrated that by using MABs prepared with proteolysed tobacco etch potyvirus and five other potyviruses, viz., PVY, pepper mottle virus, papaya ringspot virus, watermelon mosaic virus, and bean yellow mosaic virus, could be detected. Canto et al. (1995) reported that MABs and PABs raised against the helper component-protease (HC-Pro) purified from plants infected with a nonaphid transmissible strain of PVY can be used to differentiate strains of PVY.

The serological affinities of five cereal viruses transmitted by fungal vectors, viz., barley yellow mosaic (Ba YMV), barley mild mosaic (BaMMV), oat mosaic (OMV), wheat yellow mosaic (WYMV), and oat golden stripe (OGSV) viruses, were studied by using F(ab')2 and protein A ELISA tests. Within the group, BaYMV and WYMV were serologically related. Barley yellow mosaic virus reacted with antiserum against one isolate of bean yellow mosaic potyvirus (BYMV-G) among the antisera against 29 other elongated viruses tested. Oat golden stripe virus showed affinities with BYMV-G, potato virus M, red clover vein mosaic virus, and possibly hordeum mosaic virus. Barley mild mosaic virus, OMV, and WYMV did not exhibit any affinity with different viruses tested (Jianping and Adams, 1991). Reddy et al. (1992) reported that groundnut bud necrosis virus (BNV) did not react with antisera to TSWV obtained from different sources and in reciprocal tests TSWV antigen did not react with antiserum to BNV infecting groundnut in India. On the basis of the serological differences, BNV is considered distinct and this serotype appears to be restricted to Asia. Shalitin et al. (1994) identified two serogroups of Israeli citrus tristeza virus strains that could be differentiated by monoclonal antibodies, and these groups were correlated with groups differentiated by sequencing of their coat protein genes. Ellis and Wieczorek (1992), using selected monoclonal antibodies specific either to beet western yellows (BWYV) or to potato leaf roll virus (PLRV); isolates of beet mild yellowing virus; turnip yellows virus; and the *Rhopalosiphum padi*–transmitted (RPV) strain of barley yellow dwarf found that they were closely related to BWYV; *Solanum* yellows virus and four isolates from potato were identified as PLRV. Abutilon mosaic virus, tobacco leaf curl virus, and tomato yellow leaf curl virus, belonging to the geminivirus group, could be detected by selected MABs specific to African cassava mosaic virus or Indian cassava mosaic virus by indirect ELISA. These viruses could be differentiated by using two other MAB clones (MacIntosh et al., 1992). The geminiviruses African cassava mosaic (ACMV), okra leaf curl (OLCV), tobacco leaf curl (TobLCV), and tomato leaf curl (TYLCV) viruses could be distinguished by determining the epitope profiles by using panels of MABs to ACMV, OLCV, and Indian cassava mosaic virus. African

cassava mosaic virus and OLCV had similar, but distinguishable profiles (Konate et al., 1995).

Joisson et al. (1992) demonstrated that by using MABs prepared with proteolysed tobacco etch potyvirus, five potyviruses, viz., PVY, pepper mottle virus, papaya ringspot virus, watermelon mosaic virus, and bean yellow mosaic virus, could be detected. Richter et al. (1994) developed a polyclonal antiserum that showed high reactivity with 20 aphid-borne potyviruses as well as with the mite-borne ryegrass mosaic virus and the fungus-borne barley mild mosaic virus with indirect ELISA or ISEM and Western blotting tests. The antiserum raised against tomato leaf curl virus (TLCV) reacted only with TLCV, but not with Indian cassava mosaic (ICMV) and African cassava mosaic (ACMV) viruses, whereas the antiserum against ACMV reacted positively with ICMV, indicating the serological relationship between ICMV and ACMV (Ragupathi and Narayanasamy, 1996) (Fig. 8.3).

8.1.1.2 Dot immunobinding assay

Dot immunobinding assay (DIBA), in principle, is similar to ELISA; the polystyrene plates are replaced by nitrocellulose- or nylon-based membranes on

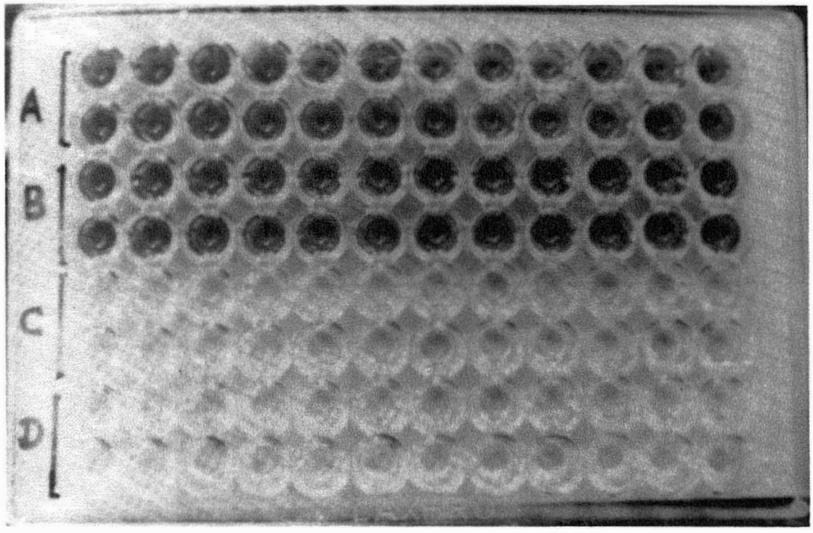


Figure 8.3 Cross-reactivity of antisera raised against tomato leaf curl virus (TLCV) and African cassava mosaic virus (ACMV) with TLCV, ACMV, and Indian cassava mosaic virus (ICMV), as determined by indirect ELISA: A rows, TLCV + TLCV antiserum; B rows, ICMV + ACMV antiserum; C rows, TLCV + ACMV antiserum; D rows, ICMV + TLCV antiserum. Note positive reactions in wells of A and B rows.

which the antigen is immobilized. As these membranes have high affinity for proteins, the free protein-binding sites present in the membranes necessarily have to be blocked by using bovine serum albumin (BSA) or nonfat dry milk powder or gelatin. Of the blocking agents, nonfat dry milk powder is less expensive, readily available, and as effective as other substances. The unconjugated virus-specific antibody is then allowed to react with the immobilized antigen. The trapped antibody is then probed with alkaline phosphatase, horseradish peroxidase–labeled protein A, anti-Fc, or anti-IgG. Appropriate substrate is provided to allow visual detection of a colored product (Appendix 8 [ii] A) (Fig. 8.2).

The DIBA technique could detect seed-borne infection of barley stripe mosaic virus in barley and bean common mosaic virus in French bean when single seed or flour was tested (Lange and Heide, 1986). The assay method is also useful for the detection of TMV, tobacco ringspot virus, and tomato ringspot virus in different plant species (Powell, 1987). The DIBA is satisfactory in detecting peanut mottle virus only when monoclonal antiserum is used, whereas ELISA is more effective when either monoclonal or polyclonal antiserum is used (Sherwood et al., 1987). Using electroblot immunoassay, Lenardon et al. (1993) showed that maize dwarf potyvirus strains A, D, E, and F could be serologically differentiated. These strains were differentiated earlier on the basis of their biological properties. Dot immunobinding assay has been employed to detect virus coat proteins in transgenic plants (Barker et al., 1992; Hibrand et al., 1992).

The direct tissue blotting technique, a variant of D1BA, was developed by Hsu and Lawson (1991) for detection of TSWV in infected leaves of *Nicotiana benthamiana* and leaf and stems of *Eustoma (Lisianthus)* and *Impatiens* plants showing viruslike symptoms (Appendix 8 [ii] B). In asymptomatic leaves or stems that produced negative results in ELISA tests, positive reactions could be observed by a dissecting microscope. This assay was found to be eight times more sensitive than ELISA for TSWV and five times more sensitive for purified potyviruses (Berger et al., 1985). Dot blot immunoassays can be performed by using a chemiluminescent substance (disodium 3-(4-methoxy spirol 1,2-dixoetane-3-2′-tricyclo-(3,3,1,1)decan-(4,4,1) phenylphosphate) or a chromogenic compound (nitroblue tetrazolium or 5-bromo-1-chloro-3-indobutyl phosphate) as substrate to detect TSWV, bean yellow mosaic virus, and lily symptomless virus in infected leaf extracts or purified virus preparations (Mansky et al., 1990; Chahal and Nassuth, 1992; Makkouk et al., 1993).

Dot immunobinding assay has the following advantages over ELISA: a) It detects viruses in extremely small volumes, as in insect or plant extracts; b) the membranes can be easily stored and transported as required during disease surveys; c) membranes can be more easily processed than plates; d) DIBA

requires a shorter period than ELISA; e) direct tissue blotting does not require any sample preparation: the sensitivity can be increased appreciably by limiting the area of application and/or by applying a higher concentration of antigen on the membrane; f) quantitative measurements are possible by using a reflectance densitometer (Banttari and Goodwin, 1985). g) DIBA has been further simplified by Lange et al. (1991), who showed that immunoblots could be prepared by using plain paper, which was as effective as nitrocellulose membrane. Five seed-borne viruses, viz. pea seed-borne mosaic virus, pea early browning virus, bean common mosaic virus, barley stripe mosaic virus, and squash mosaic virus, were detected by DIBA on paper.

8.1.1.3 Filter paper seroassay

Filter paper seroassay (FiPSA), developed by Haber and Knapen (1989), is simpler, faster, and cheaper than ELISA. In this test, ELISA plates are replaced by no. 1 filter paper disks (Whatman) as solid phase and it seems to be suitable only for viruses which are present in high concentrations, since the filter paper disks retain lower quantities of proteins than membranes (Goodwin and Nassuth, 1993). Barley stripe mosaic virus was detected in infected barley leaves, barley embryos from infected seeds, and partially purified sucrose gradient fractions of the virus suspension. This technique can detect viral antigens in 1–2 µl samples containing more than 2 ng, and the results may be obtained within 2–3 hr of spotting the samples. Another advantage of the method is that it can be used for the detection of plant pathogenic bacteria and seed-borne fungi with suitable modifications (Appendix 8 [iii]).

8.1.1.4 Rapid immuno–filter paper assay

The Rapid immuno–filter paper assay (RIPA) was developed by Tsuda et al. (1992, 1993) for the detection of plant viruses using white and colored latex beads coated with antibodies. Quantitative assays can be made by using a chromatoscanner. This technique is simpler, less time-consuming, and less expensive than ELISA, but it is as sensitive as ELISA and can be used as easily as pH test paper (Cabauatan et al., 1994) (Appendix 8 [iv]) (Fig. 8.4).

Using RIPA, purified preparations of TMV and CMV could be detected at 5 ng/ml and 50 ng/ml, respectively, and in extracts of infected tissues at dilutions of 10^{-7} and 10^{-5}, respectively, with the naked eye. In 13 species in six families CMV was detected and it could be detected, using chromatoscanner, at 10 to 100 times lower concentrations than detected with the naked eye (Tsuda et al., 1992). Simultaneous detection of several viruses, viz. TMV, CMV, PVY, and turnip mosaic virus, using different colors of sensitized beads, was

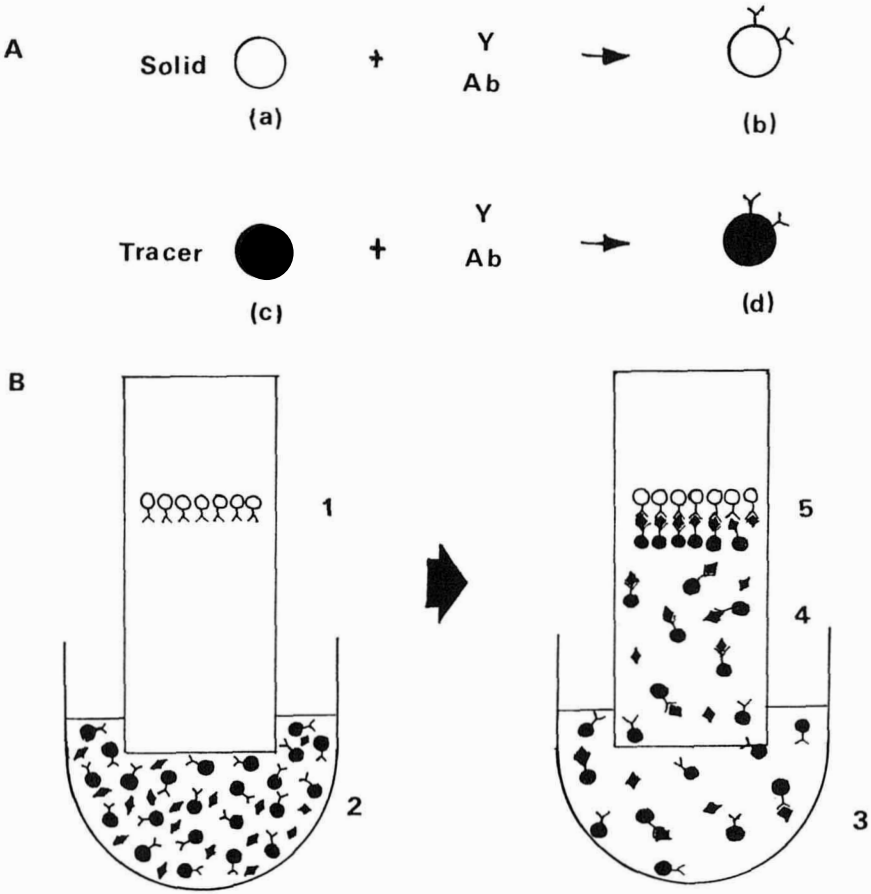


Figure 8.4 Rapid immuno filter paper assay (RIPA): A, White latex particle (a) as solid phase coated with antibody (b); pink latex particle (c) as tracer coated with antibody (d); B, 1, Immobilized solid phase as a strip on filter paper strip; 2, bottom end of the filter paper strip dipped into a mixture of dyed latex particles (tracer) coated with antibody and sample extract or purified virus; 3, reaction between virus and antibody linked to tracer; 4, movement of virus–antibody–tracer complex by capillary action; 5, virus particles on tracer sandwiched with solid phase, forming a pink line (American Phytopathological Society, Minnesota, USA). (Courtesy of Tsuda et al., 1992.)

reported by Tsuda et al. (1993). The rice viruses rice tungro bacilliform virus (RTBV), tungro spherical virus (RTSV), grassy stunt virus (RGSV), stripe virus (RStV), gall dwarf virus (RGDV), black streaked dwarf virus (RBSDV), ragged stunt virus (RRSV), dwarf virus (RDV), and transitory yellowing virus (RTYV) were detected by Cabauatan et al., (1994).

8.1.1.5 Enzyme-linked fluorescent assay

The enzyme-linked fluoroescent assay (ELFA) using polyclonal antiserum is more sensitive than the standard DAS-ELISA and has had sensitivity levels equal to those of biotin–avidin-ELISA. The test can detect lettuce mosaic virus (LMV) in seed lots containing even 1 infected seed of 500 seeds (Diaco et al., 1985; Dolores-Talens et al., 1986). Hill and Durand (1986) also reported that ELFA was more sensitive in detecting soybean mosaic virus than standard DAS-ELISA using polyclonal antiserum (Appendix 8 [v]).

8.1.1.6 Fluorescent antibody techniques

Tests involving the use of antibodies labeled with fluorescent dyes, ferritin, and radioactive iodine have been employed to study the intracellular location and distribution of plant viruses in infected plants and in insect vectors and vector cell monolayers. The labeling compounds used as specific tracers form a valuable tool for histochemical and cytochemical studies designed to elicit details of virus replication. The labeled antibodies present in the tissues are detected by fluorescent microscopy, electron microscopy, and radioautography (Appendix 8 [vi] A, B, and C).

The distribution and synthesis of TMV in various plant tissues have been studied by Schramm and Rottger (1959), Nagaraj (1965), and Schonbeck and Spengler (1979). Otsuki and Takebe (1969) employed the immunofluorescence test to determine the multiplication of TMV in tobacco protoplasts. Multiplication of wound tumor virus in the leafhopper vector was demonstrated by using fluorescent antibodies (Nagaraj et al., 1961; Sinha, 1965; Chiu and Black, 1969). The development of WTV in vector cell monolayers was studied by Peters and Black (1970), Reddy and Black (1972), and Hsu (1978). By conjugating globulin with ferritin, which is a small protein with high iron content, the specific reaction between antigen and labeled antibody can be detected in the electron microscope as ferritin itself acts as an electron-dense stain. The distribution of TMV protein in tomato leaf cells has been studied, and it is possible to detect even a single virus particle by using this method (Shalla and Amici, 1967). By fluorescent antibody labeling, southern bean mosaic virus (SBMV) and bean pod mottle virus were detected in veins leading from the feeding wound caused by beetles (*Epilachna varivestis*) fed on purified virus preparation and primary infection sites in mesophyll cells at 2–3 days post feeding on plants (Field et al., 1994). The presence of potato leaf roll virus in intestinal epithelium was revealed by immunofluorescence technique (Garret et al., 1993). Southern bean mosaic virus and the cowpea strain of TMV were detected in the gut lumen and epithelial cells of the beetle vector (*Diabrotica*

undecimpunctata howardii) by using immunofluorescent- and electron microscopy (Wang et al., 1994).

8.1.1.7 Time-resolved fluoroimmunoassay

The time-resolved fluoroimmunoassay (TR-FIA) was developed by Hemmila et al. (1984) and Halonen et al. (1986) and modified by Siitari et al. (1986) for the detection of plant viruses. This technique is based on the determination of fluorescence of antibody–lanthanide (europium) conjugates. Using lanthanide molecules such as europium tags, TR-FIA provides higher specific activity than conventional radioactive labels, but the drawbacks of radioactivity and hazards are eliminated and it also entirely eliminates the nonspecific background fluorescence originating from the sample. The lanthanide molecules, when excited, have much larger decay times (100–1000 μs) than background fluorescence (< 1 μs) and hence show a larger shift between the excitation and emission wavelengths. By determining the emission, after a delay period and at a higher wavelength, the background due to autofluorescence may be reduced (Appendix 8[vii]).

Using the TR-FIA procedure potato viruses M, S, X, and Y and potato leaf roll virus have been detected in leaf and tuber extracts (Siitari and Kruppa, 1987; Sinijarv et al., 1988). With purified PVX, TR-FIA was found to be 5–100 times more sensitive than DAS-ELISA (Siitari and Kruppa, 1987). In the case of potato virus M, 0.5 ng/ml of the virus could be detected by TR-FIA; ELISA could detect the virus only at 10 ng/ml (Jarvekulg et al., 1989). By using monoclonal antibodies labeled with europium and samarium, two potato viruses were detected simultaneously (Saarma et al., 1989).

8.1.1.8 Virobacterial agglutination test

Different types of serological agglutination tests have been employed to detect and identify plant viruses. Although these tests are simple, they require antisera of relatively high titer. This limitation may be overcome by using the bacterium *Staphylococcus aureus* for sensitization with the antiserum raised against the test virus(es). Protein A, which occurs in high concentration and is naturally covalently linked to the bacterial cell wall, forms the binding sites for immunoglobulins, especially the IgG type. The Fc portion of IgG is linked to the binding sites, leaving Fab arms for trapping antigens.

Walkey et al. (1992) reported that by using the VBA test (Appendix 8[viii]) seven potyviruses—bean common mosaic virus, lettuce mosaic virus, maize dwarf mosaic virus, papaya ringspot virus, potato virus Y, turnip mosaic virus, and zucchini yellow mosaic virus—could be differentiated in both

homologous and heterologous reactions. However, the VBA test did not differentiate strains of bean yellow mosaic virus. The test was found to be as sensitive as ISEM and local lesion assay, but less sensitive than the direct ELISA test. On the other hand, Hughes and Ollennu (1993) found the VBA test more useful in detecting cocoa swollen shoot virus (CSSV) than ELISA, which failed to detect latent infection of CSSV in cocoa. The relationships among seven isolates of CSSV that cause a range of mild to severe symptoms could be established. A field study for comparing the efficacy of the VBA and ELISA (direct and indirect) tests showed that the VBA test alone could detect CSSV in all cocoa trees known to be infected and in many symptomless trees, indicating its usefulness in the rapid detection of CSSV in cocoa.

8.2 DETECTION OF MYCOPLASMA-LIKE ORGANISMS

The necessity of developing sensitive detection methods is probably greater for plant pathogenic mollicutes than for any other kind of plant pathogens, since only three helical spiroplasmas have been cultured and adequately characterized. All the mycoplasma-like organisms (MLOs) resemble true mycoplasmas, but they cannot be classified because of lack of information on other properties. Characterization based on type of symptoms caused, host range, and vector transmission characterisitcs is found to be unreliable. Serological assays employing polyclonal or monoclonal antibodies have been found to be more reliable and sensitive in detecting and establishing relationships among MLOs (Lin and Chen, 1985; Clark et al., 1989; Lin et al., 1993). Jiang et al. (1988) reported that by using an affinity column consisting of protein A covalently linked to Sepharose matrix and coupled with MABs specific to MLO, the aster yellows MLO could be purified and intact, undamaged cells were observed under the electron microscope.

8.2.1 Growth Inhibition Test

Twofold dilutions of the polyclonal antiserum and normal serum are prepared and a drop of spiroplasma culture is added. The dilutions are incubated at 32°C for 4 days. The lowest dilution that permits acid production representing the growth of spiroplasma is recorded. The acid production is detected by using phenol-red, which turns yellow, as indicator. The correlation between acid production and growth can be established by examination under a microscope (Markham et al., 1974). A growth inhibition test showed that the spiroplasma causing brittle root symptoms in horseradish was a strain of *Sprioplasma citri* (Davis and Fletcher, 1983).

8.2.2 Rapid Slide Test

McIntosh et al. (1974) developed the rapid side test on the basis of the reduction of spiral forms to oblong and round forms in the presence of specific antibodies. The antiserum prepared against *Spiroplasma citri* (California strain) and the 72-hr-old *S. citri* broth culture (diluted to 1:10 with growth medium) are mixed. A drop of this mixture is placed on a microscope slide and covered with a cover slip and observed under the dark field of a microscope. If the reduction in spiral forms is 50% or more, the reaction is considered positive. The antiserum against California strain reacted positively with Morocco strain. It was also shown that *S. citri* was antigenically related to corn stunt spiroplasma, but not to aster yellows agent.

8.2.3 Enzyme-Linked Immunosorbent Assay

Monoclonal (MABs) and polyclonal (PABs) antibodies have been prepared against several spiroplasmas and MLOs (Lin and Chen, 1985, 1986). Production of monospecific polyclonal antibodies capable of reacting with MLO-associated protein was reported by Errampalli and Fletcher (1993). Using MABs specific for *S. citri* in indirect ELISA, the specific reaction of nine clones of hybridoma cell lines with different strains of *S. citri* was demonstrated. The MABs are able to distinguish *S. citri* from corn stunt spiroplasma, whereas PABs could not distinguish them (Lin and Chen, 1985). The relative sensitivities of PABs and MABs against aster yellows (AY) MLO were tested by indirect ELISA. Monoclonal antibody reacted specifically with AY MLO–infected plants and differentiated AY MLO from other MLOs.

The spiroplasma that causes brittle root symptoms in horseradish plants was identified as a strain of *Spiroplasma citri* by using PAB in ELISA tests (Davis and Fletcher, 1983). A polyclonal antiserum prepared against groundnut witches'-broom MLO was used to detect the MLO infection by employing a protein A indirect ELISA procedure. The MLO could be detected in crude extracts of leaves, stems, and pegs of infected groundnut plants. The extracts of tissues infected with eggplant (brinjal) little leaf, *Catharanthus roseus* witches'-broom, and *Datura* witches'-broom diseases of presumed MLO origin did not react with the groundnut witches'-broom MLO antiserum (Hobbs et al., 1987). The MLO associated with faba bean (*Vicia faba*) was detected by indirect ELISA by coating directly either the whole antigen or $F(ab')_2$ fragments of the IgG (Saeed et al., 1993). Sesamum phyllody disease MLO, could be detected in infected sesamum plants and inoculative leafhoppers by indirect ELISA. Vector indexing carried out for 14 months by using ELISA showed that the percentage of leafhoppers remaining inoculative varied from 16% to 60% and that there was a significant positive correlation between percentage of in-

oculative leafhoppers and phyllody incidence at 45 days after sampling (Srinivasulu and Narayanasamy, 1995 a,b). Using standard DAS-ELISA and DAC-ELISA, the grassy shoot disease–associated MLO was detected in infected sugarcane (Viswanathan and Alexander, 1995).

In partially purified preparations from AY MLO–infected plants, proteins associated with MLO were detected by Western blotting. The PABs produced against AY-MLO recognized a specific protein (23 kDa) in infected but not in healthy plants. The antibodies specific for MLO-associated protein were purified by trapping them on AY-MLO protein obtained by electrophoresis of infected plant extracts, and they were then transferred to a nitrocellulose membrane. The monospecefic antibodies eluted from nitrocellulose reacted specifically with the AY MLO–associated proteins. The monospecific PABs reacted positively with AY isolates from carrots and lettuce, but not with other MLOs and spiroplasma tested (Errampalli and Fletcher, 1993).

8.2.4 Immunofluorescent Technique

The immunofluorescent technique can be used for in situ detection of AY MLO in the midribs of AY-infected lettuce plants. The acetone fixed sections are stained with fluorescein isothiocyanate conjugated antimouse IgG. When MABs are used, they are bound specifically to AY MLO in the sieve tubes of diseased plants, whereas use of PABs results in fluorescence throughout the sections in both healthy and diseased plants, indicating the nonspecific binding of PABs to the cell wall and membrane (Lin and Chen, 1985).

8.2.5 Immunosorbent Electron Microscopy and Gold-Labeled Antibody Decoration

The aster yellows (AY) MLO bodies are trapped from the extracts of infected periwinkle plants or infected leafhoppers (*Macrosteles quadripunctulatus*) by using electron microscope grids coated with the (Fab′)2 portion of specific rabbit IgG. The MLO bodies are then decorated with intact IgG-labeled goat antirabbit IgG conjugated with gold particles (5 or 15 nm diameter). The grids are negatively stained with 0.5% ammonium molybdate. The European AY MLO could be distinguished from serologically unrelated tomato big bud MLO, which is morphologically indistinguishable from AY MLO (Vera and Milne, 1994).

8.3 DETECTION OF PLANT PATHOGENIC BACTERIA

As the bacterial antigens are complex and have not been well characterized, much difficulty is experienced in producing antisera against bacterial pathogens.

Polyclonal antisera have been raised against some important bacteria (Mushin et al., 1959; Choi et al., 1980; Zeigler et al., 1987; Quimio, 1989; Reddy and Reddy, 1989). Reproducibility of the reaction, loss of reactivity of a particular strain after subculturing, and colony-type variants with different serological properties are some of the problems associated with PABs. Development of monoclonal antibody technology helped to overcome these problems. MABs have been produced for many important genera of bacterial pathogens (Alvarez et al., 1985; De Boer and Mc Naughton, 1987; Magee et al., 1986; Alvarez et al., 1989; Benedict et al., 1989). Using a modified ELISA, the loss of bacterial cells from plates is prevented by drying the bacteria on the walls of microtiter plates (Benedict et al., 1989).

The MABs produced by using whole cells of *Xanthomonas campestris* pv. *campestris* (Xcc) were specific at the genus, pathovar, and strain levels (Alvarez et al., 1985). The MABs specific to certain cellular or extracellular fractions used as immunogens have been employed to differentiate serogroups of *Erwinia carotovora* subsp. *atroseptica* or *E. chrysanthemi* (De Boer and McNaughton, 1987) and *Corynebacterium sependonicum* (DeBoper and Wieczorek, 1984). The MABs generated after immunization with formalinized bacteria are found to be specific to antigens located on the surface of *Xanthomonas orzyae* pv. *oryzae*. A pathovar-specific MAB thus developed was used in an ELISA test to identify the pathogen in an outbreak of bacterial blight in the United States (Benedict et al., 1989; Jones et al., 1989). A highly specific MAB was used to detect latent ring-rot infections in potato by immunofluorescence assay (DeBoer and McNaughton, 1986).

8.3.1 Agglutination Test

The agglutination test was used to distinguish different species or strains of *Xanthomonas* spp. (Fang et al., 1950; Patel et al., 1951). The eight species studied were differentiated into five serogroups based on serological relationships (Patel et al., 1951). *Xanthomonas translucens* that causes bacterial stripe blight in cereals and grasses was differentiated into five special forms (strains) by Fang et al. (1950). Lyons and Taylor (1990) developed a rapid slide agglutination test that uses polyclonal antisera conjugated to *Staphylococcus aureus* cells which have high concentrations of protein A on their surface. This test could be used for the detection of *Pseudomonas syringae* pv. *phaseolicola* and *P s* pv *pisi* in lesions on bean and pea, respectively. *Pseudomonas gladioli* pv. *alliicola* and *Lactobacillus* sp. were detected in rotted onion bulbs. The presence of specific strains of *Rhizobium phaseoli* in bean root nodules could also be detected (Appendix 8[viii]). Using a monospecific antiserum (MSA) raised against a specific protein associated with the bacterium causing blood disease

in banana, an agglutination test and a colony blot test were performed. The MSA reacted specifically with all virulent strains of blood disease bacterium, but not or only weakly with *Pseudomonas solanacearum* or other bacterial species tested (Baharuddin et al., 1994).

8.3.2 Gel Diffusion Test

The Ouchterlony gel diffustion test can be used to distinguish pathovars; *Pseudomonas syringae* pv. *syringae* and *P. syringae* pv. *pisi* may be reliably distinguished by inoculating them on susceptible cultivars. Mazarei and Kerr (1990) developed a more rapid and convenient serological test for distinguishing these pathovars. The antiserum raised by using glutaraldehyde-fixed bacterial cells showed a high level of specificity in the Ouchterlony gel double-diffusion test. The antiserum against *P. syringae* pv. *pisi* may be used to detect the pathogen in pea seeds. Bragard and Verhoyen (1993), using phenol-treated cells, reported that the specificity of PABs could be improved in double-diffusion tests for differentiating pathovars of *X. campestris*. By using MABs, the pathovars *X. c. undulosa, X. c. translucens, X. c. hordei, X. c. cerealis,* and *X. c. secalis* were positively distinguished.

8.3.3 Enzyme-Linked Immunosorbent Assay

The ELISA tests have been widely employed for the detection and differentiation of pathogenic bacteria by using PABs and MABs. Zhu et al. (1988) detected *Xanthomonas campestris* pv. *oryzae* in leaves and seeds of 60 rice accessions. The MAB (Xco-1) specific for *X. c. oryzae* reacted positively with all 178 tested strains of *X. c. oryzae* from diverse geographical locations, but not with *X. c. oryzicola* or other xanthomonads in modified ELISA tests (Benedict et al., 1989). Using a specific MAB generated against a Florida citrus nursery strain of *X. campestris* in ELISA and microfiltration enzyme immunosorbent assay, the existence of at least two serologically distinct populations of the bacteria could be observed (Permar and Gottwald, 1989). The monoclonal antibody specific for *Pseudomonas andropogonis,* which causes leaf spot disease, could be employed to check the presence of the pathogen in carnation cuttings by indirect ELISA and immunofluorescence assay (Li et al., 1993) (Appendix 8[ix]).

The types of antigens recognized by different MABs generated by *E. amylovora* were determined. Six of the MABs reacted with protein antigens, as determined by loss of reactivity in indirect ELISA, after treatment of sonicated bacterial cells with proteinase K, whereas two MABs reacted with purified polysaccharide from *E. amylovora*. This indicates that the MABs may be

bound to different epitopes (McLaughlin et al., 1989). Lipp et al. (1992), by employing 6 MABs, identified 12 major serogroups in *X. c.* pv. *diffenbachiae.*

Benedict et al. (1989) showed that 178 strains of *X. o. oryzae* could be differentiated and classified into groups I, II, III, and IV on the basis of their reaction with four monoclonal antibodies (Table 8.5). Two of these MABs reacted positively with *X. c. oxyzicola*, but there was no reciprocal reaction between the MAB specific for *X. c. oryzicola* and any of the four groups of *X. o. oryzae* strains. One of the four MABs (X1) positively reacted with other xanthomonads in addition to *X. c. oryzicola.*

On the basis of the serological reactivity of MABs and PABs, 63 strains of *X. c.* pv. *oryzae* were grouped into 9 reaction types consisting of 4 serovars and 7 subserovars (Huang et al., 1993). Gnanamanickam et al. (1994) showed that pathovar-specific MABs could be used for the identification of *X. o.* pv. *oryzae* in rice seeds which were contaminated by other seed-borne microflora, and the reactivity with MABs was found to be correlated with pathogenicity tests. Alvarez et al. (1994) reported that *X. campestris* pv. *campestris* and *X. c.* pv. *armoraciae* could be rapidly identified in field and seed assays by employing specific panels of MABs.

The sensitivity of the ELISA test can be enhanced by using a semiselective enrichment broth (SSEB) (Appendix 8[x]). The lower limit of dettection of *Xanthomonas campestris* pv. *undulosa*, which is seed-borne in wheat, was 5 $\times$ 10^3 colony-forming units (Cfu)/ml. By enrichment, *X. c. undulosa* could be detected in samples that originally had less than 5 $\times$ 10^2 Cfu/ml. Percentages of seed infection determined by SSEB-ELISA were highly correlated with potential seed infection (PSI), determined by greenhouse tests (Table 8.6). The SSEB-ELISA method may be a convenient tool for rapid initial screening of

Table 8.5 Grouping of Strains on *Xanthomonas oryzae* pv. *oryzae* with Monoclonal Antibodies

	X. o. oryzae group						
MAB	I	II	III	IV	*X. c. oryzicola*	Other xanthomonads	Nonxanthomonads
X1	+	+	+	+	+	+	–
Xco-1	+	+	+	+	–	–	–
Xco-2	+	–	+	–	–	–	–
Xco-5	–	–	+	+	+	–	–
Xccola	–	–	–	–	+	–	–
Total No.	140	9	15	14	8	130	89

Source: Benedict et al. (1989).

Table 8.6 Comparative Efficacy of Determination of Wheat Seed Infection with *X. campestris* pv. *undulosa* by Three Different Laboratory Methods and Seedling Infection Under Optimal Greenhouse Conditions

Seed lot	Percentage of seedling infection	Percentage of infected seeds		
		XTS	ELISA-ST	SSEB-ELISA
1	12.0	5.2	2.72	17.93
2	14.0	6.5	2.73	16.30
3	1.0	0.1	7.07	3.26
4	8.75	8.0	15.78	10.30
5	2.5	6.6	14.72	5.00
6	14.0	5.8	1.10	18.29
7	6.5	3.6	2.10	8.54
8	8.75	4.5	2.45	10.97
9	6.5	8.0	2.48	6.10
10	12.0	3.6	1.22	7.60
11	9.0	4.1	2.28	12.19
12	6.5	2.6	4.88	8.50

Source: Frommel and Pazos (1994).

wheat seed lots in wheat certification programs. The antiserum raised against somatic antigens of *X. c. undulosa* can also be used to detect other pathovars: *X. c. cerealis, X. c. translucens,* and *X. c. phleipratensis* (Frommel and Pazos, 1994) (Fig. 8.5).

8.3.4 Immunofluorescence Tests

Using the PABs and MABs either individually or as a mixture, Franken (1992) reported that *X. c.* pv. *campestris* could be detected in crucifer seeds. However, Franken et al. (1992) found taht all six MABs tested cross-reacted with other pathovars of *X. campestris*, such as *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *amoraciae*. Bragard and Verhoyen (1993) successfully applied the indirect immunofluorescence test for the detection of *X. c.* pv. *undulosa* in infected wheat seed lots. Gnanamanickam et al. (1994) employed the immunofluorescence test to identify *X. o.* pv. *oryzae* in highly contaminated rice seeds. Immunofluorescence tests indicated that the MABs Xco-1 and Xco-2 detected surface antigen in *X. c.* pv. *oryzae* and their epitopes were heat-sensitive and heat-resistant, respectively. The Xco-2 epitope was present in the lipopolysaccharide fraction (Benedict et al., 1989). Three serovars were distinguished among 215 strains of *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease, by immunofluorescence assay. Serovar I is the largest, includ-

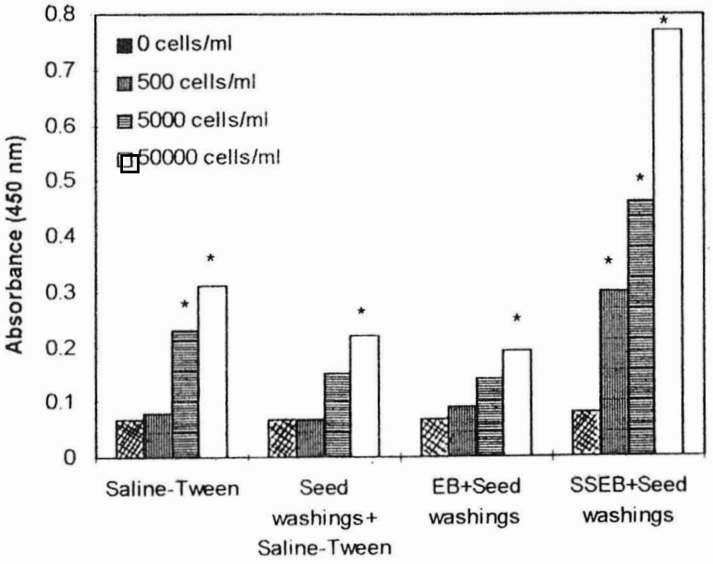


Figure 8.5 Effect of semiselective enrichment (4 hr incubation period) on the detection of *Xanthomonas campestris* pv. *undulosa* strain LP_2 by ELISA (DAS), as affected by seed bacterial saprophytes (Blackwell Science Ltd. and British Society for Plant Pathology, U.K.). (Courtesy of Frommel and Pazos, 1994.)

ing strains from Australia, the United States, Guadeloupe, India, Mauritius, South Africa. Serovar II consists of strains from Africa—Burkino Faso, Cameroon, Kenya, and Ivory Coast—whereas serovar III, the smallest group, has strains from Caribbean islands, Oceania (Fiji), and Asia (Sri Lanka) (Rott et al., 1994).

8.3.5 Immunosorbent Electron Microscopy

Immunosorbent electron microscopy, along with the immunofluorescence test, was used for the characterization of MABs generated against *X. c.* pv. *oryzae* (Benedit et al., 1989). The immunogold staining technique was found to be a highly specific and rapid method of detecting the bacterium associated with citrus greening disease (Ariovich and Garnett, 1989).

8.4 DETECTION OF FUNGAL PATHOGENS

Fungal pathogens, in general, are relatively easily diagnosed by the symptoms induced in infected plants and characteristics of spores and mycelium. In cer-

tain cases alternative methods are required for their detection and identification. Contamination by fast-growing saprophytes poses a difficult problem in detecting the slow-growing seed-borne fungi in many crops, for example, rice. The fungi are complex antigens, and the lack of characterization of antigens is a major problem in the application of serological techniques for the early detection and identification of fungal pathogens. However, many researchers have attempted to make suitable modifications to improve the sensitivity and reliability of the tests.

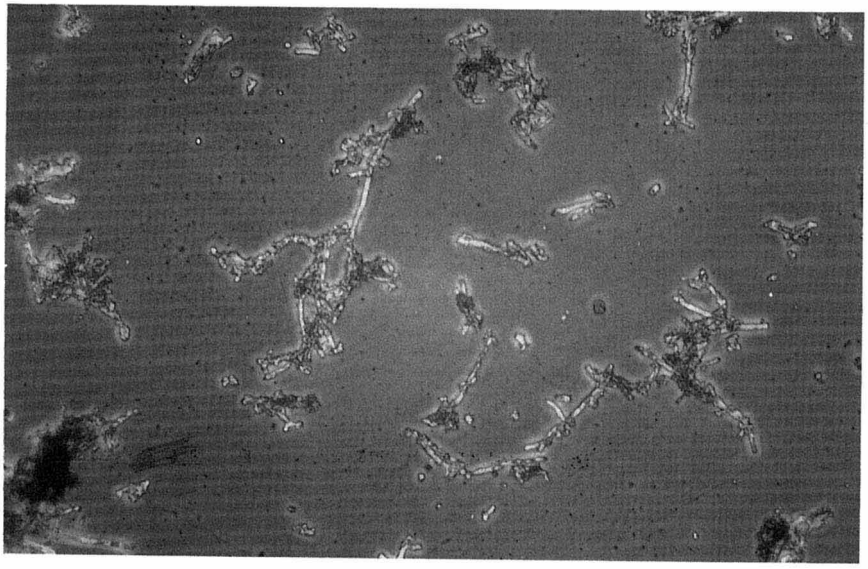
Tempel (1959) used the gel diffusion test to differentiate *formae speciales* of *Fusarium oxysporum*. Later more sensitive techniques were employed for the detection of fungal pathogens (Holland and Choo, 1970; Nachmias et al., 1979; Fitzell et al., 1980; Savage and Sall, 1981; Nachmias et al., 1982; Gerik et al., 1987). Polyclonal antisera using culture filtrate, cell fractions, whole cells, cell walls, and extracellular components, as immunogens have been prepared, and production of highly specific monoconal antibodies has been found to be difficult in many fungi. Species-specific and subspecies-specific monoclonal antibodies have been generated for pathogens such as *Phytophthora cinnamomi* (Hardham et al. 1986), and inclusion of glutaraldehyde in the fixative improves the specificity of reaction (Hardham et al., 1991). Specific detection of *Humicola languinosa* and *Penicillium islandicum* causing discoloration of stored rice is possible by using monoclonal antibody probes (Dewey et al., 1989, 1990). *Penicillium islandicum* is known to produce mycotoxins that cause liver lesions, cirrhosis, and primary liver cancer; hence its rapid detection is very important to eliminate the affected grains in storage.

Currently, simple diagnostic kits are being developed to conduct plant "side testing." Turf diseases due to *Pythium* sp., *Rhizoctonia solani*, and *Sclerotina homoecarpa* can be diagnosed by using visible immunodiagnostic assay kits developed commercially by Agri-Diagnostics, Cinnaminson, USA (Rittenberg et al., 1988).

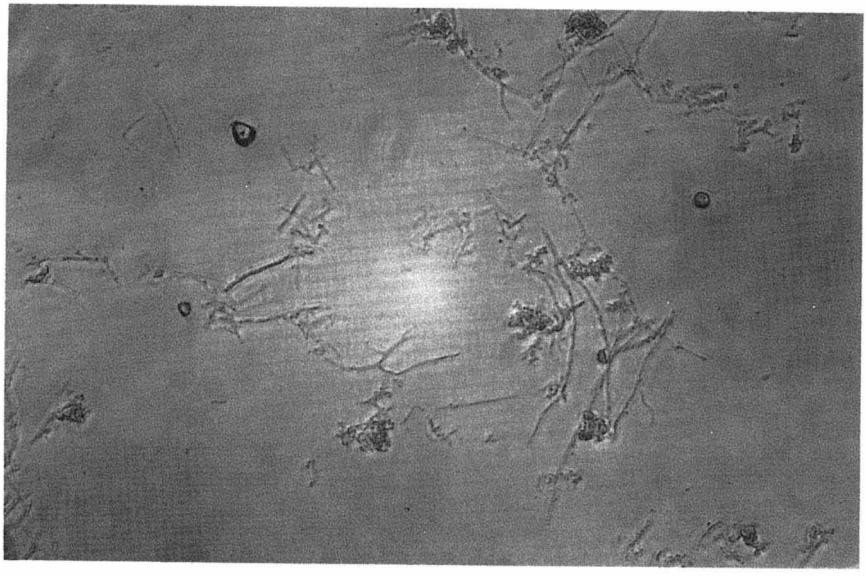
Pathogen propagules present in soil or other complex substrates have not been quantified satisfactorily. Immunoassays have the potential to detect and quantify the soil-borne pathogens more accurately. Seroassays when used in conjunction with baiting assay can give results rapidly (El-Nashaar et al., 1986).

8.4.1 Enzyme-Linked Immunosorbent Assay

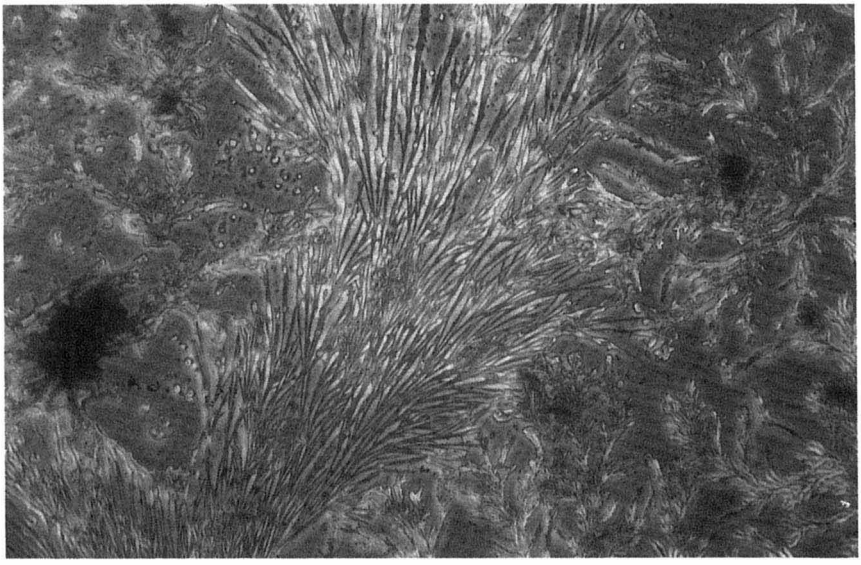
Standard ELISA and its variants have been employed to detect and quantify the pathogenic fungi and to assay their metabolic products. Banks and Cox (1992) developed a method to immobilize fungal hyphae onto microplate walls by precoating the walls with poly-L-lysine and glutaraldehyde, then attaching the hyphae to walls by overnight drying. The attached hyphae were uniformly coated and remained reactive. The plates could be stored at -20°C (Fig. 8.6 A, B, and C) (Appendix 8[xi]).



(A)



(B)



(C)

Figure 8.6 Detection of *Penicillium aurantiogriseum* var. *melanoconidium* by ELISA: A, *P. aurantiogriseum* var. *melanoconidum* hyphae after drying overnight on poly-L-lysine-bound to glutaraldehyde-pretreated microplate well; B, before addition of enzyme substrate; C, *P. aurantiogriseum* var. *melanoconidium* dried on a well not treated with poly-L-lysine or glutaraldehyde (Kluwer Academic Publishers, Netherlands). (Courtesy of Banks and Cox, 1992.) Reprinted by permission of Kluwer Academic Publishers.)

Using the antiserum produced with β-D-galactosidase-labeled antirabbit IgG as the secondary antibody and cell fragments of the strain of *Fusarium oxysporum* f. sp. *cucumerianum* attached to balls (Amino Dylark) as the solid-phase antigen, a highly specific and sensitive ELISA test was developed for the detection of homologous strains (Kitagawa et al., 1989). The PABs generated against purified mycelial proteins from *Verticillium dahliae* reacted positively with 11 of 12 *V. dahliae* isolates from potato, cotton, and soil, but not with the isolate from tomato. Using DAS-ELISA, *V. dahliae* and *V. albo-atrum* in infected roots and stems of potato could be detected (Sundaram et al., 1991). Sugarcane red rot pathogen (*Colletotrichum falcatum)* was detected by employing polyclonal antibodies in the DAC-ELISA test (Viswanathan, personal communication). Infection of young petals of rapeseed by *Sclerotinia sclerotiorum* was detected by the DAS-ELISA test by Jamaux and Spire (1994). The pres-

ence of *Pseudocercosporella herpotrichoides* could be detected by using a specific MAB in plants infected by the pathogen based on the absorbance value (Fig. 8.7). *Rhizoctonia solani* and other *Rhizoctonia* spp. in poinsettia cuttings were rapidly detected by ELISA (Benson, 1992). Lyons and White (1992) employed PAB to detect *Pythium violae* in cavities developed in field-grown carrots. Bossi and Dewey (1992) reported that MABs specific for *Botrytis cinerea* could be used to detect the pathogen in strawberries (Fig. 8.8). The MABs recognized mycelial fragments, saline extracts of mycelia, and germinating conidia by both ELISA and immunofluorescence. Thornton et al. (1993) developed four lines that produced species-specific monoclonal antibodies capable of recognizing the antigen from *Rhizoctonia solani* by the ELISA technique. This assay technique can be used to detect live propagules of the pathogen present in the soil. Hardham et al. (1986) showed that MABs raised against components on the surface of glutaraldehyde-fixed zoospores and cysts of an isolate of *Phytophthora cinnamomi* could be used as isolate-specific, species-

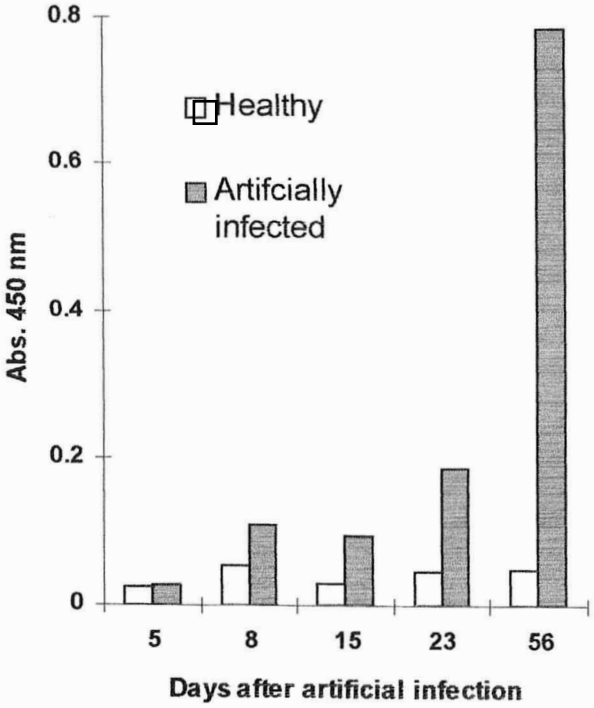


Figure 8.7 Absorbance values from DAS-ELISA tests of extracts from stem bases of healthy wheat plants and wheat plants 5, 8, 15, 23 and 56 days after infection. (British Society for Plant Pathology, U. K.) (Courtesy of Priestley and Dewey, 1993.)

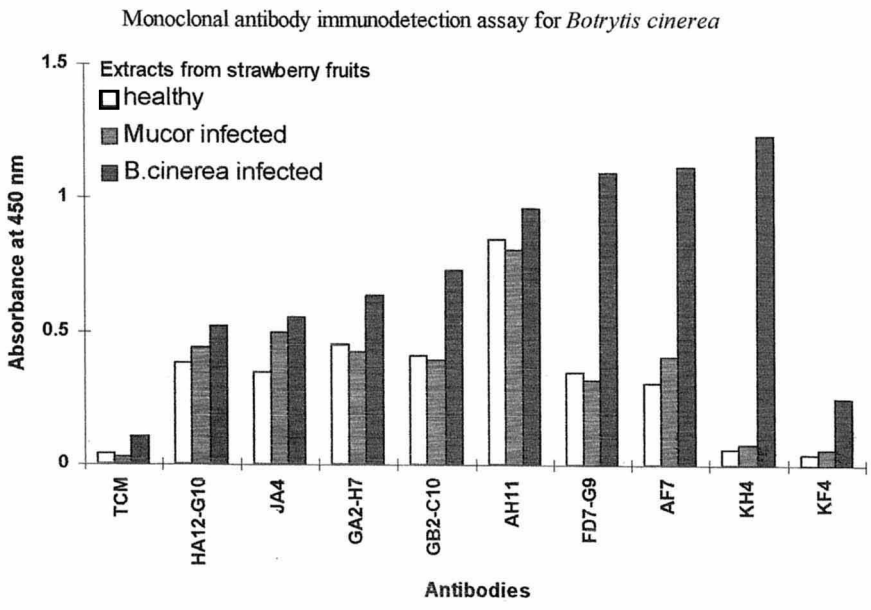


Figure 8.8 Antibodies tested for specificity by ELISA against extracts from healthy strawberries and strawberries infected by *Botyrtis cinerea*. (Association of Applied Biologists, U.K.) (Courtesy of Bossi and Dewey, 1992.)

specific, and genus-specific markers without any ambiguity. These MABs have a valuable spectrum of taxonomic specificities. Stace-Smith et al. (1993) using specific MABs to distinguish highly virulent and less virulent strains of *Leptosphaeria maculans* that cause black leg of canola. Polyclonal antibodies raised against *Phomopsis longicolla* from soybeans using culture filtrate (cf) and mycelial extact (me) were tested by DAS-ELISA and indirect ELISA. The DAS-ELISA test was found to be more specific and 100 times more sensitive in detecting the fungus in the *Diaporthe–Phomopsis* complex, and the variability in specificity was less when DAS-ELISA was employed (Brill et al., 1994). An MAB specific to *Pythium ultimum* was highly reactive to 21 isolates of *P. ultimum* and did not react with any of the 16 species of *Pythium* tested by ELISA. The test was effective in detecting the pathogen in sugar beet seedling roots, with more than two infections/10 cm of root (Yuen et al., 1993). A dipstick immunoassay based on detection (Azodye) of monoclonal antibody–labeled cysts attached to a nylon membrane was used for the rapid detection of *Phytophthora cinnamomi* in a wide range of soil samples collected from beneath a diverse range of host species (Cahill and Hardham, 1994).

In ELISA tests, using PABs prepared against crude cell wall fractions of *Pythium aquatile* or *P. coloratum* associated with root rot of tomato, specific reactions with closely related isolates were observed (Rafin et al., 1994). A fungal capture sandwich ELISA was developed by using the polyclonal antiserum raised against soluble protein extracts of chlamydospores and mycelium of *Thielaviopsis basicola* and the IgG was labeled with biotin. The test detected both brown and gray cultural types of *T. basicola* and the antibodies showed negligible cross-reactivity with other soil-borne fungi found in cotton field soil. The minimum detection limit was between 1 and 20 ng of *T. basicola* protein, and the pathogen could be detected in cotton roots at 2 days after inoculation when initial symptoms were not apparent (Holtz et al., 1994). Using the PAB produced by employing a homogenate of spore balls (cystosori) of *Spongospora subterranea*, the presence of the pathogen could be detected in dilute tuber extract containing the equivalent of as little as 0.08 spore balls/ml (Harrison et al., 1993) (Fig. 8.9).

Commercial immunoassay kits have been developed for the detection of fungal pathogens in plant tissues as well as in soil. Miller et al. (1994) compared the efficacy of the Albert *Phytophthora* "flow through" immunoassay and multiwell ELISA kits (Agri-Screen) for the detection of *Phytophthora capsici*

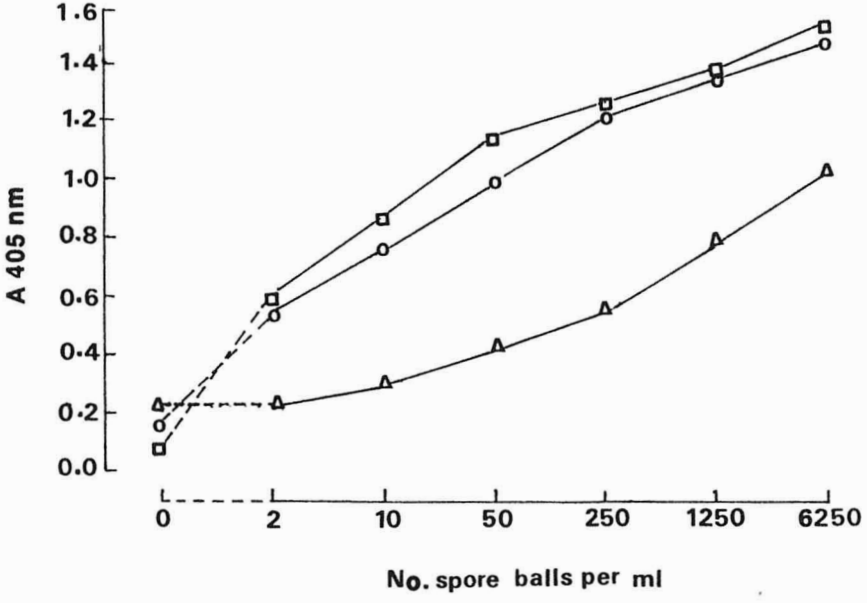


Figure 8.9 Effect of potato tuber sap concentration on detection of spore balls of *Spongospora subterranea* by PTA-ELISA. (British Society for Plant Pathology, U.K.) (Courtesy of Harrison *et al.*, 1993.)

and *P. cactorum*. The former was easy to perform and rapidly (within 10 minutes) detected *P. capsici* in pepper and cucurbit crops; the latter was effective in detecting the pathogen in pepper tissues but had higher absorbance values for healthy samples. There was excellent agreement between results of ELISA tests and isolation of pathogens in semiselective medium. The presence of *Phytophthora* in soils also could be detected by the kits. More specific detection and characterization of *P. capsici* are possible by using MABs. Kimishima and Kobayashi (1994) produced three MABs (PC1A6, PC1B2, and PC1C5) against mycelial suspension of *P. capsici*, which were tested by ELISA and Western blot analysis. The study suggested that among the three MABs, PC1C5 could recognize one epitope common to all isolates tested and PC1A6 recognized a carbohydrate epitope, whereas PC1B2 and PC1C5 were able to recognize protein epitopes.

The ELISA tests are useful to detect the production of different kinds of metabolites of the fungi. A competitive inhibition ELISA was developed to detect picograms/gram of ergot alkaloids in seeds and flour by Shelby and Kelley (1992). A monoclonal antibody generated against ergonovine was sensitive and specific to detect alkaloids of *Claviceps purpurea* when sclerotia were diluted to 10^{-5} by weight in whole wheat flour or about one sclerotium in 20 kg of wheat. Nemec et al. (1991) reported that naphthazarin toxins produced by *Fusarium solani* could be detected by competitive ELISA tests in xylem fluid of citrus roots infected by *F. solani* and symptomless scaffold roots and branches of apparently healthy and diseased citrus trees.

The ELISA test was used to quantify fungal antigen in resistant and susceptible cultivars after inoculation with the pathogen. The polyclonal antiserum developed by using mycelia and zoospores of *Aphanomyces euteichus* as immunogens reacted positively with *A. euteichus*, but not with *Phytophthora*, *Fusarium,* and *Pythium*. When the roots of resistant lines were exposed to 100 zoospores/ml, the buildup of *A. euteichus* was slower when compared to that of susceptible lines as determined by ELISA test using the antiserum, indicating inhibition of the growth of the pathogen within the inoculated tissues (Kraft and Boge, 1994). Commercial ELISA kits have been developed to detect fungal pathogens such as *Phytophthora citrophthora* in plant roots and soil samples to estimate propagule densities (Timmer et al., 1993).

8.4.2 Dip Stick Immunoassay

A recent significant development in diagnostic assays are the visible immunodiagnostic assay methods in which the samples can be handled easily under field conditions. The 96-well titer plate is replaced by dip-stick formats (Dewey et al., 1989; Cahill and Hardham, 1994a). The dipstick immunoassay is based on the phenomena of chemotaxis and electrotaxis to attract the zoospores to a

membrane on which they encyst and are then detected by immunoasasy. The zoospores are attracted by a variety of chemicals, such as amino acids, alcohols, phenols and isovaleraldehyde, pectin, and the phytohormone abscissic acid. Positively charged nylon membranes strongly attract the zoospores. The dipstick assay can detect as few as 40 zoospores/ml within 45 min. Immunolabeled cysts attached to the membrane can be seen with the naked eye or observed under low-power magnification after silver enhancement of a gold-labeled secondary probe. By using MABs *Phytophthora cinnamomi* can be rapidly detected (Cahill and Hardham, 1994b).

8.4.3 Dot Immunobinding Assay

Immunoblotting assays have been found to be useful to overcome problems with nonspecific interference in ELISA procedures. Gleason et al. (1987) developed the seed immunoblot assay (SIBA) to detect *Phomopsis longicolla* in infected soybean seeds (Appendix 8[xii]). The mycelium of *P. longicolla* growing onto a nitrocellulose sheet from infected soybean seeds forms a conspicuous colored blotch when assayed by the SIBA test. As SIBA detects only viable *P. longicolla*, this technique may be preferable to ELISA, which does not differentiate live and dead fungus (Fig. 8.10).

Dot immunobinding assay (DIBA) was used to detect *Phomopsis phaseoli*, cause of pod and stem blight, and *P. longicolla*, cause of seed decay of soybean in asymptomatic soybean tissues. Polyclonal antiserum was raised against *P. longicolla* and the PABs reacted strongly with all *Phomopsis* spp. and *Colletotrichum truncatum*. The culture filtrates and mycelial extracts of *Phomopsis* spp. showed similar reactions with PABs. A method of quantification of antigen based on the "antigen unit," instead of absorption values, was developed (Velicheti et al., 1993).

8.4.4 Immunofluorescence Assay

Aldehyde-fixed zoospores and cysts of *Phytophthora cinnamomi* were used to generate 24 MAB clones. Using the immunofluorescence assay (IFA), 11 MABs were found to be species-specific, reacting specifically with zoospores and cysts of *P. cinnamomi* only, but not with other species of fungi, such as *Phytophthora* spp., *Pythium* spp., *Saprolegnia* spp., *Fusarium* spp., *Verticillium* spp., *Rhizoctonia* spp., and *Schizophyllum* spp. One MAB was genus-specific, reacting with other *Phytophthora* spp. One MAB capable of reacting only with *P. cinnamomi* in both IFA and ELISA was identified (Gabor et al., 1993). For the detection of *Botrytis cinerea*, whole conidia, their extracellular material, and a putative cut in esterase isolated from conidia were used as antigens to prepare MABs. Three MABs capable of recognizing conidia of 43 isolates of *B. cinerea* from

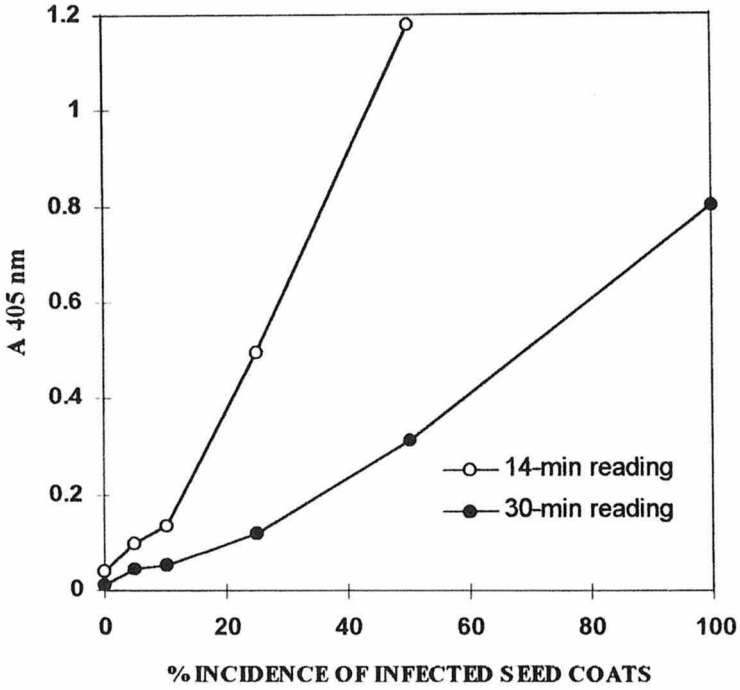


Figure 8.10 Effect of dilution of seed coats infected by *P. longicolla* with uninfected seed coats on detection of *P. longicolla* by indirect ELISA. (British Society for Plant Pathology, U.K.) (Courtesy of Gleason *et al.*, 1987.)

different hosts representing six countries were identified by Salinas and Schots (1994). These MABs could be used to detect *B. cinerea* in flowers of gerbera by immunofluorescence test. The presence of *Thielaviopsis basicola* in cotton roots was detected by immunofluorescence assay by Holtz et al. (1994).

8.5 DETECTION OF VIROIDS

It has been demonstrated that viroids do not possess any messenger activity and that the viroid is not translated in the *Xenopus larvis* oocyte system (Davies et al., 1974; Semancik et al., 1977), leading to the absence of any viroid-specific protein in the infected plants. However, production of enhanced levels of host-specific proteins has been reported in tomato infected by potato spindle tuber viroid (PSTVd) and *Gynura aurantica* infected by citrus exocortis viroid (CEVd) (Zaitlin and Hariharasubramanian, 1972; Conejero et al., 1979).

Diener et al. (1985) reported that a host protein with a molecular weight of 70,000 accumulated in tomatoes after inoculation with planta macho viroid and this protein was isolated after density gradient centrifugation. The antiserum raised against the host-specific protein designated PM antigen could be used for the detection of tomato planta macho viroid (TPMVd) infection, using the double-diffusion test. However, stimulation of PM antigen synthesis does not appear to be specific to TPMVd infection. The presence of PM antigen in tomato plants infected by other viroids, such as PSTVd, chrysanthemum stunt viroid (CSVd), tomato apical stunt viroid (TASVd), columnea viroid (CVd), and the cucumber mosaic virus, along with CARNA5, has been detected (Diener et al., 1985). This reveals that use of serological tests for viroid detection and identification may not be very specific.

SUMMARY

Serological techniques have been employed for the detection of viruses, MLOs, bacteria, and fungi present in infected seeds, seed materials, plants, soil, and water. The sensitivity and reliability of these techniques are greater than those of infectivity tests and chemodiagnostic methods in the case of many pathogens. Bacterial, mycoplasmal, and fungal pathogens are complex antigens and much difficulty is experienced in producing antisera against these pathogens. Polyclonal and monoclonal antisera have been prepared against these pathogens, and by using panels of monoclonal antibodies, the sensitivity and reliability of detection have been markedly enhanced. Serological techniques have been extensively used for the detection, differentiation, and assay of plant viruses. The ELISA test and its variants, the dot immunobinding assay (DIBA), filter paper seroassay (FiPSA), rapid immuno-filter paper assay (RIPA), enzyme-linked fluorescent assay (ELFA), and time-resolved fluoroimmunoassay (TR-FIA), have been the preferred methods. Only very low concentrations of viruses and very small volumes of antigen and antisera are required for these tests, and the results are obtained in a few hours.

APPENDIX 8(i)

A. *Double-antibody sandwich (DAS) ELISA* (Clark and Adams, 1977)
   i. Precipitate globulins from the antiserum using 36% sodium sulfate; wash the precipitate with 18% sodium sulfate and store at –70°C. Conjugate a portion of globulin with alkaline phosphatase using glutaraldehyde as the coupling agent.

ii. Dilute the globulin fraction (unlabeled) with 0.05 *M* carbonate buffer at pH 9.6 to yield a concentration of 10 μg protein/ml. Add 200 μl of antibody solution to each well in polystyrene ELISA plates and incubate at 37°C for 3–5 hr. Empty the wells; wash thrice with 0.15 *M* phosphate-buffered saline solution at pH 7.2 containing 0.05% Tween 20 (PBS-Tween) and dry.

iii. Add samples (purified antigen or extracts of infected tissues) in 200 μl quantities in PBS-Tween; incubate at 4°C overnight or for 18 hr and wash the wells as before.

iv. Add aliquots of 200 μl of enzyme-labeled antibody conjugate to each well; incubate for 4 hr at 37°C and wash the wells as before.

v. Add enzyme substrate p-nitrophenyl phosphate at a concentration of 1 mg/ml in diethanolamine buffer at pH 9.8 at room temperature. Stop the reaction after 30 min by adding 3 *M* NaOH at 50 μl/well.

vi. Determine the color intensity (OD) at 405 nm in an ELISA reader.

B. Direct antigen coating (DAC)-ELISA

i. Add samples at 200 μl to each well in the ELISA plate; incubate at 37°C for 1 hr and wash the wells with PBS-Tween.

ii. Add antiserum at suitable dilution at 200 μl/well; incubate for 1 hr at 37°C and wash the wells with PBS-Tween.

iii. Add enzyme-labeled antirabbit IgG at 200 μl to each well; incubate for 1 hr at 37°C and wash the wells with PBS-Tween.

iv. Follow steps (v) and (vi) as in DAS-ELISA.

C. Protein A-coating (PAC)-ELISA

i. Dissolve protein A (1–10 mg/ml) in carbonate buffer; dispense 200 μl/well in ELISA plate; incubate for 1 hr at 37°C and wash with PBS-Tween.

ii. Dispense antiserum (at suitable dilution) at 200 μl/well; incubate for 1 hr at 37°C and wash with PBS-Tween.

iii. Dispense 200 μl of samples (purified antigen/extracts of tissues at suitable dilution); incubate at 37°C for 1 hr and wash the wells with PBS-Tween.

iv. Dispense 200 μl of antiserum and proceed as in step (ii).

v. Dispense enzyme-labeled antirabbit IgG or Fc at 200 μl/well; incubate for 1 hr at 37°C and wash with PBS-Tween.

vi. Follow steps (v) and (vi) as in DAS-ELISA.

D. Indirect DAS-ELISA

i. Dispense goat or chicken antivirus globulins (1–10 μg/ml) at 200 μl/well; incubate at 37°C for 1 hr and wash with PBS-Tween.

ii. Dispense 200 μl of suitably diluted samples in each well; incubate at 37°C for 1–3 hr and wash with PBS-Tween.

iii. Dispense 200 µl of antivirus rabbit globulin/well; incubate at 37°C for 1–3 hr and wash with PBS-Tween.
iv. Dispense 200 µl of antirabbit globulin conjugate/well; incubate for 1 hr at 37°C.
v. Follow steps (v) and (vi) as in DAS-ELISA.

APPENDIX 8(ii)

A. Dot immunobinding assay (DIBA)
   i. Dip a nitrocellulose membrane grid (1.0 or 2.5 cm squares) in Tris buffer (0.02 *M* Tris-CL, 0.5 *M* NaCl, pH 7.5) (TBS) and dry it on filter paper for 5 min.
   ii. Spot the samples (1 µl) prepared in TBS in the center of the grid and dry. Place the grid in blocking solution (3% gelatin, 2% Triton X-100 in TBS) in a petri dish and agitate for 1 hr.
   iii. Dip the grid into distilled water; transfer to 50 ml antiserum. (1 mg protein/ml) and 1% gelatin in a petri dish and agitate for 1 hr.
   iv. Dip the grid into distilled water; then wash twice by agitation for 10 min in TBS containing 0.05% Tween 20 (TTBS).
   v. Dip the grid into distilled water; transfer to horseradish peroxide conjugated IgG (1/1000 dilution) and 1% gelatin kept in a petri dish; agitate for 1 hr.
   vi. Dip the grid into distilled water; wash in TTBS and TBS successively for 10 min.
   vii. Transfer to substrate solution (dissolve 0.06 g 4-chloro-1-naphthol in 20 ml of 4-C-methyl alcohol, then add 100 ml of TBS and 0.06 ml of 30% hydrogen peroxide); incubate for 10–30 min in darkness.

B. Direct tissue blotting technique (Lawson, 1991)
   i. Prepare tissue blots by pressing a newly cut leaf, flower, or insects on a nitrocellulose membrane (0.45 µm pore size); immerse in PBS containing 1% bovine serum albumin (BSA) for 60 min with gentle agitation.
   ii. Incubate with alkaline phosphatase–labeled antibodies in PBS for direct detection; wash the blots thrice in PBS-Tween.
   iii. For indirect detection, incubate blots with virus-specific primary antibodies in PBS for 60 min; wash thrice in PBS-Tween; incubate with enzyme-labeled species-specific secondary antibodies for 60 min; wash three times in PBS-Tween.
   iv. Immerse in solution containing nitroblue tetrazolium (14 mg) and 5-bromo-4-chloro-3-indolyl phosphate (7 mg) in 40 ml substrate buffer

consisting of 0.1 *M* Tris, 0.1 *M* NaCl, and 5 *mM* $MgCl_2$, pH 9.5). Purple color in blots indicates a positive result.

APPENDIX 8(iii): FILTER PAPER SEROASSAY (FiPSA)

i. Heat filter paper disks (Whatman No. 1) (7 mm) at 150°C for 1 hr; preblock the binding sites with rabbit serum albumin; suction dry and store the disks in a desiccator.
ii. Extract the antigen in a few drops of spotting buffer containing 0.05 *M* Tris-HCl, pH 7.4; 0.2 *M* NaCl; and 20% sucrose with 0.02% phenol red as spotting aid. Transfer to plastic microvial (400–1500 µl size); centrifuge at 5000 rpm for 10 min; dilute the supernatant suitably.
iii. Spot a 2 µl sample in the center of the disk and dry; transfer to individual cylindrical vials (10–15 mm diameter) and dry the disks at 40°C for 10 min.
iv. Incubate the disks in vials containing 150 µl of antiserum/normal serum at suitable dilutions for 30–60 min; remove the sera by aspiration from the wells; wash with 1 ml of TBS (spotting buffer without sucrose) for 10 min by shaking the vials; remove the excess buffer.
v. Immerse the disks in protein A–peroxidase conjugate (2 µg/ml) in TBS and incubate with gentle shaking for 15–30 min; wash the disks.
vi. Incubate the disks in substrate solution containing 5 parts of TBS + 1 part of 4-chloro-1-naphthol at 3 mg/ml in methanol + 0.018 part of 3% hydrogen peroxide for 1 min in darkness; remove the disks and immerse in water for 10 min; dry at 40°C. Development of violet–blue color indicates a positive reaction.

APPENDIX 8(iv): RAPID IMMUNO–FILTER PAPER ASSAY (RIPA)

i. White latex beads, used as solid phase, and pink latex beads, used as tracer, in TBS are mixed with antibody (100 µg/ml) at pH 7.2 for coating the beads; incubate for 2 hr at room temperature; centrifuge at 15,000 rpm for 10–15 min with TBS-BSA; suspend the sediments in TBS-BSA.
ii. Apply 5 µl of coated white latex beads at 1.5 cm from the lower end of a glass filter paper GF/A (8 × 0.5 cm) (Whatman) strip, and air-

dry; store the strips in a desiccator till use; keep sensitized pink latex in suspension at 4°C.

iii. Dilute pink latex to 0.025% (v/v) with TBS and mix with an equal volume of purified virus solution or tissue extract in flat-bottomed tubes (Eppendorf). Add Tween 20 to the mixture to yield a final concentration of 0.3% (v/v).

iv. Dip the filter paper strip with immobilized white latex into the solution containing coated pink latex to immerse 0.5 cm of the filter paper.

v. Observe the appearance of a pink band on the immobilized white latex. Use a chromatoscanner to determine color intensity at 700 nm (visible light).

APPENDIX 8(v): ENZYME-LINKED FLUORESCENT ASSAY (ELFA)

i. Sensitize microtiter plates or polystyrene beads (6 mm diameter) with IgG in 0.05 *M* sodium carbonate, pH 9.6; block nonspecific reactions with 2% ovalbumin or 0.2% ovalbumin and 2% polyvinyl pyrrolidone in 0.02 *M* $NaPO_4$, pH 7.2, containing Tween 20 and 0.85% NaCl (PBS-Tween); wash with PBS-Tween thrice.

ii. Use aliquots of 200 µl for microtiter plates; 0.5 ml of antibody and 1.0 ml of antigen samples for beads.

iii. Apply the virus antigen to the solid phase; incubate for 12 hr at 4°C.

iv. Apply the antibody-labeled biotin, diluted with PBS-Tween or labeled with alkaline phosphatase diluted in PBS-Tween containing 2% polyethylene glycol; wash with PBS-Tween.

v. Add 4-methyl-umbelliferyl phosphate (MUP) (0.1 m*M*) in 1.0 m*M* diethanolamine, pH 9.8, containing 0.01 m*M* $MgCl_2$; terminate the reactions by adding NaOH (to a final concentration of 0.27 *M*) for colorimetric assay or Na_2HFO_4 adjusted to pH 10.4 with KOH to a final concentration of 0.2 *M* for fluorescent assays.

vi. Determine color intensity/fluorescence at 405 nm spectrphotmetrically or in a fluorometer.

APPENDIX 8(vi): FLUORESCENT ANTIBODY TECHNIQUES

A. Preparation of conjugated antibodies

i. All steps are at 4°C. Precipitate globulins by using equal volumes of neutralized saturated ammonium sulfate; dialyze against phosphate-

buffered saline solution at pH 7.0, using a 0.025 *M* sodium carbonate buffer, pH 9.8.

ii. Adjust the protein concentration of antibody to 1%; dialyze against 10 volumes of 0.01% fluorescein isothiocyanate in the same buffer for 24 hr.

iii. Dialyze the globulin solution against phosphate-buffered saline solution, pH 7.0, till unconjugated dye is washed out.

iv. Centrifuge the conjugated antibody at low speed and store at -40°C.

B. Fluorescence test with leaf tissues

i. Place leaf segments (2 × 5 mm) in small aluminum foil boats containing 20% gelatin in 5% glycerol; freeze the samples with dry ice at -20°C; remove the aluminum foil.

ii. Cut sections (10–20 μm) at room temperature; mount the sections on slides smeared with gelatin-glycerol adhesive.

iii. Place drops of conjugated antibody solution on sections and incubate in a moist chamber 45–60 min in the dark.

iv. Wash the sections for 10–15 min with phosphate-buffered saline solution, pH 7.2, to remove excess dye; mount in a glycerol–phosphate mixture (1 ml acid-free glycerol + 9 ml phosphate buffer, pH 7.2).

v. Observe under a fluorescent microscope.

C. Fluorescence test with insect vectors

i. Crush individual leafhoppers on a microscope slide; extract with buffered saline solution.

ii. Centrifuge the extract at low speed; perform the ring interface test with supernatant solution.

iii. Flood the insect tissue on the slide with conjugated antibody solution; wash to remove excess unreacted conjugate.

iv. Observe under a fluorescent microscope.

APPENDIX 8(vii): TIME-RESOLVED FLUOROIMMUNOASSAY (TR-FIA) (SIITARI ET AL., 1986)

A. Sample preparation

i. Dilute the leaf or tuber extract to 10^{-6} with buffer.

B. Conjugation of antibodies with europium

i. Purify IgG using a Sepharose column.

ii. Conjugate IgG with Eu^{3+}–N^1 (p-*iso*-thiocyanatobenzyl)-diethylene triamine-N^1, N^2, N^3-tetraacetate for 18 hr at 0°C in 0.1 *M* carbonate buffer, pH 9.3; separate the Eu–IgG complex from free Eu re-

agent by gel filtration and store at 4°C in 0.05 *M* Tris HC1 buffer, pH 7.7, containing 0.9% NaCl, 0.5% gelatin, 20 μ*M* diethylene-triamine N^1, N^1, N^2, N^3-pentaacetic acid, 0.01% Tween 40, and 0.05% NaN_3 in 0.05 *M* Tris HCl buffer, pH 7.7.

C. One-step incubation procedure
   i. Wash the wells in polystyrene microtitration strips twice with TBS-T containing 20 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.5, with 0.05% Tween 20; coat the wells with antibodies (IgG or MABs) for 18 hr at 37°C in carbonate buffer, pH 9.6, and block with BSA as in ELISA.
   ii. Incubate antigen (50 μl) and europium conjugate already coated with IgG or MABs for 1 hr at 37°C; wash with 20 *mM* Tris-HCl buffer, pH 7.5, containing 150 *mM* NaCl; add the enhancement solution containing 15 μ*M* 2-naphtoyl trifluoroacetone, 50 μ*M* tri-n-octyl-phosphine oxide, and 0.1% Triton X-100 in 0.1 *M* acetate phthalate buffer, pH 3.2; shake for 10 min.
   iii. Determine the fluorescence in a fluorometer.

D. Two-step incubation procedure
   i. Coat the wells with antibodies (IgG or MABs) as in the one-step incubation procedure.
   ii. Incubate the antigen (100 μl) for 1 hr at 37°C; wash the wells with TBS-T.
   iii. Incubate the Eu conjugate for 1 hr at 37°C. Further steps as in the one-step incubation procedure.

APPENDIX 8(viii): VIROBACTERIAL AGGLUTINATION (VBA) TEST

A. Preparation of *Staphylococcus aureus* reagents (Lyons and Taylor, 1990)
   i. Cultivate the authentic culture of *S. aureus* (Cowan strain [NCTC, 8530; ATCC, 12598) on nutrient agar for 24–48 hr at 37°C; prepare the suspension of bacterial cells (10^8–10^9) in glycerol broth containing nutrient broth (Difco) (1.6 g), glycerol (30 ml), and distilled water (170 ml); transfer in 1–2 ml aliquots to sterile vials and equal volume of sterile 3 mm hollow glass beads; store at -80°C.
   ii. Thaw the container when required; transfer the contents to nutrient broth (10 ml); distribute the culture after shaking well to 40 nutrient agar plates; incubate overnight at 37°C.
   iii. Flood the plates with phosphate buffered saline (PBS) solution that contains 0.02% sodium azide; scrape the surface of the plates gently with a glass streaker; centrifuge the bacterial suspension at 300 g for 30 min.

iv. Resuspend the pellet in 20 ml of 1.5% aqueous formaldehyde; shake the suspension well for 30 min; heat at 80°C for 30 min; rapidly cool to room temperature.

v. Wash twice by centrifugation and resuspension with PBS containing 0.05% sodium azide added at the rate of 9 volumes of PBS to 1 volume of pellet.

B. Virobacterial agglutination (Walkey et al., 1992)

i. Prepare appropriate dilutions of the antiserum against the test antigen in PBS containing $Na_2HPO_4 \cdot 12\ H_2O$ (2.9 g), KH_2PO_4 (0.2 g), NaCl (8.0 g), and KCl (0.2 g) in distilled water (11), pH 7.2; add sodium azide (NaN_3) at 0.2 g/l.

ii. Mix *S. aureus* cells with diluted antiserum at a 1:5 ratio for conjugation; add ethanol saturated basic fuchsin to color the conjugate for easy recognition of bacterial agglutination; store at 4°C; stir the conjugate before use; check with a hand lens to detect autoagglutination, if any.

iii. Prepare the extracts from healthy and infected leaves using a small quantity of K_2HPO_4 (10 g/l) solution.

iv. Dispense a 4 µl aliquot of the bacterium–antiserum conjugate and 2 µl of extract on a multitest slide (Flow Laboratories Ltd., UK); mix the reactants well; maintain suitable controls in the same slide; shake the slide gently by hand as the reaction proceeds; observe the reaction with a hand lens.

v. Record agglutination, which indicates a positive reaction, after 30 sec to 5 min.

APPENDIX 8(ix): INDIRECT IMMUNOFLUORESCENCE MICROSCOPY FOR DETECTION OF BACTERIAL PATHOGEN (LI ET AL., 1993)

i. Cut infected leaves and healthy parts of diseased leaves and leaves artificially inoculated by wounding into 2–3 mm pieces and immerse in 1 ml of sterile distilled water (SDW) in Eppendorf tubes for 10 min.

ii. Remove leaf pieces; centrifuge at 13,000 rpm for 5 min; resuspend the pellets in 0.1 ml SDW.

iii. Heat-fix drops of suspensions on glass slides; expose to a few drops of 95% ethanol for 10 min.

iv. Cover the slide with a drop of specific MAB supernatant for 15 min and wash five times with SDW.

v. Keep the slides in darkness and cover with a drop of goat antimouse

IgG fluorescent conjugate (1:700 dilution) for 15 min; wash five times with SDW.

vi. Air-dry the slides in the dark; place a drop of 50% glycerol; cover with a cover slip; examine under fluorescent microscope fitted with a UG-1 ultraviolet excitation filter.

vii. Determine the number of bacterial cells by the following formula:

$$\text{Number of bacterial cells} = \frac{N \times A}{a}$$

where N = average number of cells/field, A = circle area (mm^2) on the glass slide, and a = area of microscope field = $(9.1 \times 0.01)^2$ mm^2.

APPENDIX 8(x): DETECTION OF BACTERIAL PATHOGENS (FROMMEL AND PAZOS, 1994)

A. Antigen preparation

i. Grow xanthomonads on yeast-dextrose-chalk (YDC) agar, pseudomonads on King's B medium, and other bacteria on Clark's medium or nutrient yeast dextrose agar for 48 hr at 25°C.

ii. Suspend the cells in 10 ml of 0.85% sterile saline solution (SSS); pour the suspension into a flask containing 200 ml of YDC broth and incubate for 48 hr at 26°C on a horizontal shaker (350 rpm).

iii. Centrifuge at 10,000 rpm for 15 min; wash the pellets once in SSS and then in 0.1% sarcosyl solution for 10 min; resuspend in SSS.

iv. Adjust the concentration of cells to 3×10^7 cells/ml; expose the cells to 100°C for 20 min to eliminate flagellar antigens.

B. Preparation of polyclonal antibodies (PABs)

i. Mix the immunogen prepared as above in equal quantities with Freund's incomplete adjuvant.

ii. Inject a New Zealand male rabbit intradermally, at 12 sites along the back of the rabbit, at the rate of 100 µl/site; repeat injections after 7, 21, and 28 days and inject 1 ml intramuscularly as a final dose at 60 days after the primary injection.

iii. Bleed the rabbit at 0, 30, 50, and 70 days after the first injection; assess the antibody titer of different fractions by indirect ELISA; preserve the fractions with titers above 1/5120 with 0.01% sodium azide and store at -20°C.

iv. Precipitate globulins in the antiserum by using saturated ammonium sulfate; dialyse against three changes of 0.1 *M* phosphate buffer, pH 7.4 (PB).

v. Fractionate gamma globulins on a DEAE-Sephadex A 50 column; place globulins to be used immediately at 4°C and store the rest at -70°C.

vi. Adjust the protein concentration to 1 mg/ml by using a spectrophotometer; conjugate with alkaline phosphatase; store unconjugated IgG at -70°C in PB with bovine serum albumin (BSA).

C. Production of monoclonal antibodies (MABs) (Alvarez et al., 1985)

i. Streak the culture on yeast–glycerol agar (YGA); incubate for 48–72 hours at 28°C; collect the cells in 0.01 *M* phosphate-buffered saline (PBS) solution, pH 7.4, for immediate use or store in PBS containing 0.5% formalin at 6°C.

ii. Inject BALB/C mice intraperitoneally with 10^8 living cells of bacterium and repeat after 14 days; give a booster injection 2–3 days before hybridization.

iii. Remove the spleen and collect 5×10^7 cells; mix them with 5×10^7 myeloma cells; centrifuge and fuse, using 45% polyethylene glycol (molecular weight [MW] 1300–1600).

iv. Wash the cells; resuspend in Dublecco's modified Eagle's medium with 10% fetal calf serum, 1 m*M* sodium pyruvate, 0.1 m*M* hypoxanthine, 0.0004 m*M* aminopterin, 0.016 m*M* thymidine, and 1% nonessential amino acid solution (Gibco Laboratories, Grand Island, NY), and 10% NCTC 109 lymphocyte growth medium, dispense in culture plates.

v. Coat the culture plates with peritoneal macrophage "feeder" cells 1 day prior to transfer of fused cells.

vi. Screen the supernatant fluids from the well with healthy hybridomas for antibody titer by radioimmunoassay or ELISA; select stable and efficient clones.

vii. Inject selected stable clones into pristane-primed BALB/C mice intraperitoneally with about 10^6 hybridoma cells; collect the ascitic fluid; centrifuge; store the MABs at -20°C.

D. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

i. Adjust the concentration of primary antibody at 8 μg/ml in coating buffer consisting of 0.13 *M* $Na_2CO_3H_2O$ + 0.035 *M* $NaHCO_3$, pH 9.6.

ii. Dispense the sample at 200 μl/well and incubate overnight at 4°C; wash three times with PBS, pH 7.2; block with 1% BSA for 1 hr at 37°C.

iii. Add IgG–alkaline phosphatase conjugate at a concentration of 3.2 μg/ml in PBS containing 1% BSA; incubate.

iv. Add p-nitrophenyl phosphate as substrate at a concentration of 1 mg/ml.
v. Perform other steps as in Appendix 8 [i].

E. Semiselective enrichment procedure (Frommel and Pazos, 1994)
i. Prepare the semiselective enrichment broth (SSEB) consisting of gentamycin (5 μg/ml) cephalexin (6 μg/ml), tyrothricin (150 μg/ml), ampicillin (5.6 μg/ml), cycloheximide (200 μg/ml), benomyl (80 μg/ml), and Tween 20 (0.02%), pH 7.0, added to enrichment broth (EB) (basal medium of Schaad and Forster, 1985) consisting of nutrient broth (8 g/l), glucose (5 g/l, pH 7.0) or saline-Tween buffer (STB) (containing NaCl 8.5 g/l, Tween 20, 2 ml/l, pH 7.0).
ii. Dispense 120 ml of either SSEB or EB to groups of four Erlenmeyer flasks; seed with cells of bacteria at different concentrations for assay standardization.
iii. Place 400 wheat seeds-into each flask; incubate at 26°C in a shaker at 350 rpm.
iv. Draw 2 ml samples from flasks at 2, 4, 6, and 8 hours of incubation; assay by DAS-ELISA as in section D.

F. Semiselective enrichment broth (SSEB)-ELISA (Frommel and Pazos, 1994)
i. Place individual seeds in each well of acid-washed 96 well microtiter plates.
ii. Dispense SSEB, STB, or NB to 80 wells at 300 μl/well; remaining 16 wells serve as negative controls and bacterial standards; seal the plates with cellulose tape and incubate for 4 hr at 26°C in a wrist action shaker at 300 rpm.
iii. Transfer aliquots of 200 μl from each well to microtiter plate wells precoated with specific antiserum at 8 μg/ml in carbonate buffer.
iv. Follow the DAS-ELISA procedure as in section D.

APPENDIX 8(xi): PRODUCTION ANTISERUM AGAINST FUNGAL PATHOGENS (BANKS ET AL., 1992)

A. Antigen preparation
i. Prepare spore suspensions in 0.01% Tween 80; wash thrice by centrifugation; inoculate 1 ml of spore suspension (10^6 spores/ml) into 100 ml liquid medium supplemented with NaCl (100 g/l); incubate at 25°C for 7 days in the dark by placing the flask on a rotary shaker.
ii. Transfer the mycelium by filtering into a sintered glass filter; wash with sterile water and then with sterile phosphate-buffered saline (PBS); freeze overnight at -20°C; thaw and transfer to centrifuges; dry in a vacuum dryer.

iii. Collect the mycelium and add 50 ml of liquid nitrogen; mince the mycelium in a blender for 1 min and grind in a mortar with pestle to have a fine powder.
iv. Suspend the mycelial powder in PBS (200 mg in 10 ml); centrifuge at 4500 rpm (3000 G) for 10 min at 4°C; divide the supernatant containing soluble antigen into 0.5 ml aliquots and store at -20°C.
v. Estimate the total protein content of antigen preparation.
iv. Add 10 ml of warm serum-free RPMI 1640 medium (Gibco) over the next 60 sec with gentle stirring. Add another 20 ml of warm RPMI; centrifuge at 400 g for 3 min at room temperature.
v. Suspend the pellet of cells in 50 ml of growth medium (RPMI 1640 with 20% [v/v] of Myoclone fetal calf serum [FCS]; dispense cell suspension into five 96 well microplates at 100 µl/well.
vi. After 24 hr, add 100 µl of hypoxanthine-aminopterin-thymidine (HAT) medium (diluted to 1:50 in growth medium to each well in the fusion plates.
vii. Add growth medium + HAT on 2, 4, 7, and 10 days by removing 100 µl of the medium and replacing with 100 µl of fresh medium.
viii. Screen the hybridoma cells for antibody production by indirect ELISA.
ix. Clone healthy growing hybridomas twice by limiting dilution in non-selective medium; preserve by freezing slowly in 7.5% (v/v) dimethyl sulfoxide (DMSO) and store in liquid nitrogen.

B. Production of polyclonal antiserum
i. Mix soluble antigen preparation with equal quantities of Freund's complete adjuvant (Difco) to produce a final protein concentration of the mixture at 1 mg/ml.
ii. Inject rabbits intramuscularly with 1 ml of the mixture at predetermined intervals.
iii. Bleed the animal at 4 weeks after the first injection and subsequently at 14, 16, and 18 weeks.
iv. Take the serum after clotting and centrifugation.

C. Production of monoclonal antibodies
i. Mix soluble antigen preparation with an equal quantity of Freund's complete adjuvant to yield a final protein concentration of 1 mg/ml.
ii. Inject a BALB/C mouse, after anaesthetization, with 0.1 ml of the immunogen intraperitoneally and subsequently at 2, 4, and 6 weeks and at 8 weeks after the first injection with PBS; remove the spleen after sacrificing the animal by cervical dislocation.
iii. Carry out fusion of splenocytes with myeloma cell line P3-NS 1-Ag4 at a ratio of 1×10^8:5×10^7 by gentle addition of 2 ml of 30% (w/v) of polyethylene glycol (PEG) over 60 sec.

D. Solid-phase attachment of fungal hyphae in ELISA plates (Banks and Cox, 1992)
   i. Coat 96 well microplates with 0.005% poly-L-lysine (PLL) at 50 µl/well; incubate for 45 min at 25°C.
   ii. Wash four times with PBS with 0.05% Tween 20 (PBST) at 250 µl/well; blot dry; treat with 2% glutaraldehyde at 50 µl/well; incubate for 15 min at 25°C and wash as before.
   iii. Add antigen (at a concentration of 3.4 µg/ml) at 50 µl/well pretreated with PLL and glutaraldehyde or untreated wells (to serve as control); incubate at 25°C overnight to allow the antigen to dry onto the bottom of the wells.
   iv. Wash the wells with PBST four times; add 3% bovine serum albumin (BSA) at 250 µl/well; incubate at 25°C for 60 min; wash and use immediately or store at -20°C after washing thrice before storage and once before using.

E. Indirect enzyme-linked immunosorbent assay (ELISA) to screen antifungal antibodies (Banks and Cox, 1992)
   i. Add antiserum at appropriate dilution to microplate wells prepared by the procedure in Appendix 8 (x) D, at 50 µl/well; incubate for 60 min at 25°C; wash four times with PBST.
   ii. Add horseradish peroxidase conjugated goat antimouse immunoglobulins at 200 µl/well; incubate for 60 min at 25°C; wash as before.
   iii. Add *O*-phenylenediamine dihydrochloride (0.4 g in 100 ml phosphate citrate buffer [PCB], diluted to 1:10 in PCB with 0.012% hydrogen peroxide) as substrate at 200 µl/well; allow the reaction in darkness for 10 min at 25°C.
   iv. Add 2.5 *M* sulfuric acid at 50 µl/well to stop the reaction; determine color intensity at 490 nm in a microplate reader.

APPENDIX 8(xii): SEED IMMUNOBLOT ASSAY (SIBA) (GLEASON ET AL., 1987)

   i. Surface-sterilize the seeds in 0.5% sodium hypochlorite for 30 sec; rinse in deionized water three times; blot-dry the seeds with paper towels.
   ii. Place the seeds 2 cm apart on a nitrocellulose sheet; transfer the nitrocellulose sheet to a plastic tray (18 × 29 × 5 cm) containing three layers of moist germination towels; place three additional layers of moist paper towels above the seeds; cover the tray with aluminum foil to maintain high humidity and incubate for 2–3 days at 25°C.

iii. Remove the seeds carefully; assay the antigens adsorbed to the nitrocellulose sheet; agitate the nitrocellulose sheet in a blocking solution containing Tris-buffered saline solution (50 m*M* Tris-HCl, 200 m*M* NaCl, pH 7.5) and 5% nonfat dry milk (TBS-milk) for 1 hr at room temperature.

iv. Replace the blocking solution with IgG preparation (10 μg/ml) in TBS-milk and agitate for 1 hr.

v. Rinse nitrocellulose sheet three times for 10 min each in TBS-milk; incubate with goat antirabbit IgG conjugated to horseradish peroxidase in TBS-milk in a shaker for 1 hr.

vi. Rinse the nitrocellulose sheets twice for 10 min each with TBS-milk and then with TBS.

vii. Add chloro-1-naphthol in hydrogen peroxide as substrate; observe for 15–20 min for the development of blue color, indicating a positive reaction; rinse in deionized water; dry under a heat lamp; store the blots.

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9
Nucleic Acid–Based Techniques

It is generally considered that closely related organisms share a greater nucleotide sequence similarity than those that are distantly related. A highly specific nucleotide sequence present in an isolate or strain of virus or other pathogen, but absent from other strains or species, may be used to test for the presence of that virus or pathogen. Detection of plant pathogens by hybridization is based on the production of nucleic acids by specific hybridization between the single-stranded target nucleic acid sequence (denatured DNA or RNA) and a complementary single-stranded nucleic acid probe. Probes for plant viruses are mostly cDNA, as the genomes of most plant viruses are RNA. Either RNA or DNA sequences may be used as probes. Transcription vectors to produce RNA probes in vitro can be developed to yield RNA:RNA or RNA:DNA hybrids, which are more stable than DNA:DNA hybrids.

Nucleic acid hybridization has several advantages over immunological assays. The antigenic determinants of the viral coat proteins represent only about 2% to 5% of the nucleic acid of viral genomes, and hence the differences in characteristics of the virus governed by major portions of the viral genome cannot be determined by serological assays. The bacteria and fungi are complex antigens, the nature of which may vary, depending on the stage of their development, and so the antisera produced against one type of spore or mycelium at one stage may not react positively with spores or mycelium produced at all stages in the life cycle. But the nature of genomic elements is constant in all stages of the pathogen, making it possible to detect, differentiate, and establish relationships between strains or related species. For the detection of viroids, serological techniques cannot be employed as they do not have any protein, and necessarily hybridization methods have to be employed for their detection and characterization. The cloned probes with varying specifications can be produced to suit requirements for different assays and in unlimited

quantities. Moreover, the sensitivity can be increased by amplification of desired sequences by using polymerase chain reaction (PCR).

9.1 HYBRIDIZATION METHODS

9.1.1 Probe Preparation and Labeling

DNA probes have been commonly used in experiments with DNA or RNA viruses, although labeled viral RNA itself has been used as a probe in certain cases. The probes may be labeled with either radioactive markers, such as ^{32}P or ^{3}H, or nonradioactive markers, such as biotin. For differentiation of viruses in a group or strains of a virus, the cDNA probes with appropriate common or specific sequences of nucleotides can be prepared and labeled in different ways.

9.1.1.1 Reverse transcription

The cDNA copies of the viral RNA are formed by using a retrovirus reverse transcriptase and labeled radioactively. These probes can be used to identify the virus or its strains.

9.1.1.2 Cloned probes

Double-stranded cDNA, after cloning in the bacterium, is labeled by nick translation (some nucleotides are replaced after excision with enzymes and insertion with labeled nucleotides). In another procedure, the double-stranded cDNA is separated by heating and randomly primed with short synthetic oligomers. Complete new double-stranded (ds) molecules are produced by using DNA polymerase and then labeled. Cloned cDNA probes have been used to detect viruses such as plum pox virus (Variveri et al., 1988), peanut mottle and stripe viruses (Bijaisoradat and Kuhn, 1988), and potato leaf roll virus (Robinson and Romerio, 1991, Smith et al., 1993). The probe representing a portion of the potato leaf roll virus particle protein gene could also detect beet western yellows luteovirus (BWYV) and the English strain of the RPV form of barley yellow dwarf luteovirus, but it reacted weakly with extracts from plants infected by groundnut rosette assistor luteovirus and carrot red leaf luteovirus (Robinson and Romerio, 1991). An increase in sensitivity of virus detection, as compared to ELISA, up to 250 times, has been reported (Variveri et al., 1988; Bijaisoradat and Kuhn, 1988).

9.1.1.3 Synthetic probes

Oligonucleotides (15–20 bases) representing the desired segment of viral genes can be chemically synthesized, if the nucleotide sequence of part or all of the viral genome is known. This method is useful because a) it is possible to produce required amounts of single-stranded probes which can be end-labeled with a ^{32}P-labeled nucleotide by using a polynucleotide kinase; b) a library of probes specifically designed to detect different segments of viral genome may be prepared; c) several oligonucleotides may be ligated in tandem and cloned; and d) probes specific to one strand of the viral genome may also be produced. Synthetic oligonucleotide probes have been used for the detection of viruses such as potato virus X (Rouhiainen et al., 1991), tulip breaking potyvirus (Langeveld et al., 1991), beet western yellows luteovirus (Jones et al., 1991), bean yellow mosaic geminivirus (Castro et al., 1993), and cymbidium mosaic potexvirus (Lim et al., 1993).

9.1.1.4 Nonradioactive probes

Nonradioactive probes are preferred by many researchers because of the short half-life of the isotope ^{32}P and the difficulty in handling it. Biotin has been shown to be a highly effective nonradioactive label, and the tests using biotin chemically linked to UTP and introduced into the probe by nick translation have revealed its usefulness as an alternative to radioactive markers. The biotin has a strong affinity for the bacterial protein streptavidin, which is conjugated with an enzyme. Photobiotin, an analog of biotin, has also been found to be a useful label, but it requires highly purified probe nucleic acid, since it can react with any organic material. Cloned photobiotin-labeled cDNA was used for the routine diagnosis of barley yellow dwarf luteovirus (BYDV) in nucleic acid extracts from field samples using the dot-blot method (Habili et al., 1987) and the sensitivity was comparable to that of tests employing ^{32}P-labeled probes. Similar sensitivity levels were reported in the case of biotin-labeled probe for papaya mosaic potyvirus (Roy et al., 1988). Welnicki and Hiruki (1992) reported that digoxigenin-labeled DNA probes were highly sensitive in detecting potato spindle tuber viroid, and that as little as 25 pg of the viroid RNA could be detected.

The hybridization reaction may be of three types: a) solution hybridization performed in solutions (Young and Anderson, 1985), b) in situ hybridization performed in cells or tissues (Pardue, 1985), and c) filter hybridization conducted on solid filter supports (Anderson and Young, 1985). For the detection of plant pathogens, filter hybridization and in situ hybridization methods have been most commonly used.

9.1.2 Filter Hybridization Methods

In filter hybridization denatured DNA or RNA is immobilized on an inert support such as nitrocellulose- or nylon-based membrane. In the case of plant samples, a small amount of sap is placed on nitrocellulose sheet and baked to bind the nucleic acid to the support. Prehybridization solution containing bovine serum albumin and small single-stranded fragments of an unrelated DNA, and salt is used to block nonspecific binding sites on the nitrocellulose membrane. Hybridization with labeled phosphorus (usually ^{32}P) is carried out, followed by extensive washing of the membrane to remove unreacted probe. The amount of probe bound to the target nucleic acid is estimated by autoradiography. Variants of filter hybridization are colony and dot-blot hybridization, and Southern and Northern blotting.

9.1.3 In Situ Hybridization Methods

With the in situ hybridization technique, it is possible to observe precise localization of target sequences at the organelle, cellular, or tissue level. Exposure of fixed tomato protoplasts to 0.2 *N* HCl at room temperature followed by heating at 70°C for 30 min and digestion with 10 μg/ml proteinase K resulted in localization of PSTVd-RNA and TMV-RNA by in situ hybridization without alteration in cellular structures (Yokoyama et al., 1990; Uehara and Hosokawa, 1994). Nucleic acids of pathogens present in only a small number of cells or at low concentrations in cells can be detected. This technique is most useful for detecting the latent infections, tissue- or organ-restricted diseases, and seed-borne pathogens (Chu et al., 1989). The hybridizations may be analyzed by either light microscope or electron microscope. Nonradioactive probes are increasingly preferred for in situ hybridization methods (Lewis et al., 1987).

9.1.4 Solution Hybridization Methods

Hybridization between target nucleic acid and probe is performed in solution, and the hybrids are analyzed by gel electrophoresis or by liquid scintillation counting. This procedure is useful for quantitative estimation of potato spindle tuber viroid concentration in purified RNA preparations (Owens et al., 1978) and for indexing of avocados for the presence of avocado sunblotch viroid (Palukaitis et al., 1981). This procedure has been used to detect potato virus X in crude leaf extracts by using both radioactive and nonradioactive labeled probes (Rouhiainen et al., 1991).

The nucleic acid hybridization methods are highly sensitive and require only very small samples containing about 40–100 pg per gram of leaf tissue. They are particularly useful for viroids which do not have any protein. All

genome sequences can be cloned simultaneously, and cloned probes can be prepared in unlimited supply. Detection and differentiation of viruses and other pathogens are possible with probes prepared for sequences which do not code for proteins. This provides a distinct advantage over serological methods, which can detect the presence of proteins which are coded by segments of viral genomes accounting for only about 10% of genomic content. Thus the serological methods cannot recognize the differences in the major portions of viral genomes. Hybridization methods can have greater applications and increased sensitivity when amplification techniques such as polymerase chain reaction (PCR) are combined (Chu et al., 1989).

9.2 DETECTION OF PLANT VIRUSES

Dot-blot hybridizations are extensively used for the detection of plant pathogens, especially viroids. These tests generally do not distinguish among different types and sizes of nucleic acids hybridizing to the probes. However, they can be very useful for qualitative detection, since this method can discriminate between closely related but different target sequences (Beltz et al., 1983). Diagnosis of diseases caused by RNA viruses using the dot-blot technique has been suggested by Palukaitis (1984). Banana bunchy top virus (BBTV) was detected by using BBTV-specific clones and radioactive or nonradioactive probes in a dot-blot hybridization assay, and this assay was as sensitive as ELISA (Xie and Hu, 1995). Tobacco mosaic tobamovirus was detected in tobacco and parsley by using radioactively labeled probe (Ogras et al., 1994). A variant of dot-blot hybridization termed squash blot was used to assay maize streak geminivirus in an individual leafhopper vector at different periods after acquisition feeding (Boulton and Markham, 1986). Navot et al. (1989) detected the tomato yellow leaf curl virus in squash blots of tomato leaves, roots, stems, flowers, and fruits, and in single whiteflies fed on infected tomato plants. Other viruses detected by this method are tobacco mosaic virus and potato virus Y. The presence of potato leaf roll virus in leaf and aphid vector extracts could be detected by dot-blot hybridization, which was equal to ELISA in sensitivity. Use of formaldehyde, instead of formamide, for denaturing leaf tissue extracts increased the effectiveness 32-fold (Smith et al., 1993).

Southern blot analysis is reported to be useful for the detection of tomato yellow leaf curl geminivirus (TYLCV) in whitefly vectors at different periods after the acquisition access period. In 15% of whitelies tested TYLCV-DNA could be detected after a period of 30 min and in all whiteflies tested after an 8-hr access period. The virus could be detected earlier (within 2 hr) in insects fed on young leaves which contained higher concentrations of TYLCV. It was

estimated that a single whitefly could acquire no more than 600 million viral genomes (1 mg viral DNA) (Zeidan and Czosnek, 1991).

9.3 DETECTION OF VIROIDS

The nucleic acid spot hybridization (NASH) technique was first developed for the detection of potato spindle tuber viroid (PSTVd) by Owens and Diener (1981), using ^{32}P-labeled cDNA probes. This method is based on hybridization of highly radioactive cDNA probe with the viroid RNA, which is bound to a solid matrix nitrocellulose; this method permits the detection of PSTVd in a large number of potato tubers (Fig. 9.1). Later, with the development of plasmid transcription vectors containing promoters for SP6, T3, and T7 bacterial polymerases, it was possible to prepare riboprobes (Mc Innes and Symons, 1989; Melton et al., 1984). The single-stranded cRNA probes are more sensitive than similar cDNA probes and can be prepared more easily and uniformly labeled (Lakshmanan et al., 1986; Varveri et al., 1988; Candresse et al., 1990).

The usefulness of spot (dot-blot) hybridization for the early detection and assay of several viroids, which cause potato spindle tuber, avocado sunblotch,

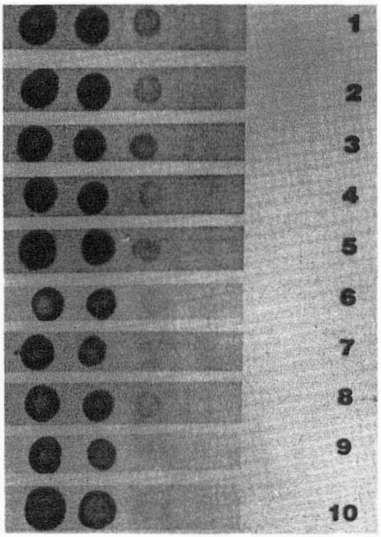

Figure 9.1 Sequential dot-blot hybridization of serially diluted sap extracts from PSTVd-infected potato leaves and stem with a digoxigenin-labeled PSTVd cRNA probe. 1–10, sequence in which membranes were hybridized (Elsevier Science B.V., Netherlands). (Courtesy of Podleckis et al., 1993.)

and coconut cadang-cadang diseases, has been reported (Owens and Diener, 1984). Latent infection of avocado by potato spindle tuber viroid was revealed by dot-blot hybridization (Querci et al., 1995). Employing a cDNA probe in a liquid–liquid system, coconut cadang-cadang RNA up to 1 ng/ml could be detected after a 96 hr hybridization time (Randles, 1985). A new viroid that causes fruit crinkle in apple could be differentiated from apple scar skin viroid by the hybridization technique (Ito et al., 1993). A spot hybridization test using purified cucumber pale fruit viroid (CPFVd) and hop stunt viroid (HSVd) showed that the sequence homology between CPFVd and HSVd was very high (Shikata, 1985), indicating the extent of relatedness.

The need for the development of alternative methods for detection arose because of the safety risks involved in the use of radioactive probes and short half-life of such probes. McInnes et al. (1989) reported that avocado sunblotch, coconut cadang-cadang, chrysanthemum stunt, and potato spindle tuber viroids could be detected in plant extracts by dot-blot hybridization, using nonradioactive photobiotin-labeled nucleic acid probes which were viroid-specific. The sensitivity of detection was similar to that of ^{32}P-labeled probe. Later chemiluminescent probes such as cRNA probe labeled with the steroid hapten digoxigenin were found to be suitable alternatives to the radioactive probes. In this system, the hybridized probe is detected by an antidigoxigenin polyclonal antibody conjugated to alkaline phosphatase. The conjugate probe complex can then be visualized by adding an enzyme substrate that, on dephosphorylation by alkaline phosphatase, yields sufficient light to expose film (Podleckis et al., 1993). The sensitivities of chemiluminescent digoxigenin-labeled cRNA probe with ^{32}P-labeled cDNA probe for the detection of PSTVd and apple scar skin group viroids (ASSVd) were compared. Dot-blot hybridization of purified viroids and sap extracts from infected plants showed that digoxigenin-labeled probes were as sensitive as ^{32}P-labeled probes. Positive detection was possible with 2.0–2.5 pg of purified viroid or 0.4 ng of total nucleic acid extract from infected tissue or in sap extracts diluted to 10^{-3} with healthy leaf extracts (Podleckis et al., 1993) (Fig. 9.1).

9.4 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a simple, sensitive, and versatile technique which can provide results rapidly. The procedure essentially involves heat denaturation of the target ds DNA and hybridization of a pair of synthetic oligonucleotide primers to both strands of the target DNA, one to the 5′ end of the sense strand and one to the 5′ end of the antisense strand by an annealing step. Then by using a thermostable Taq DNA polymerase enzyme from *Thermus aquaticus* (*Taq*), new DNA is synthesized on templates to produce twice

the number of target DNAs. Newly synthesized DNA strands are used as targets for subsequent DNA synthesis, and the steps discussed are repeated up to 50 times (Fig. 9.2). There is an exponential increase in the number of target DNA molecules. In this procedure DNA sequences between the primers are reproduced with high fidelity and an efficiency of up to 85% per cycle (Weier and Gray, 1988); the procedure can be automated if required. *Taq* DNA polymerase is heat-stable and can be used at temperatures between 60°C and 85°C.

The PCR products can be used a) as a target for hybridization; b) for direct sequencing of the DNA to determine strain variations, and c) as a specific probe. The PCR has many advantages over traditional methods of disease diagnosis. The pathogens need not be cultured before detection; is enough if the pathogen DNA is extracted. With PCR, it is possible to detect a single pathogen or many members of a group of related pathogens, as in serological methods, but serological methods are more expensive and time-consuming. Hundreds of different PCR primers may be synthesized at costs comparable to those of methods for developing only a few monoclonal anitbodies (Henson and French, 1993). Levy et al. (1994) reported that the commercial product Gene Releaser could be used to produce plant extracts suitable for PCR amplification without use of organic solvents, alcohol precipitation, or additional nucleic acid purification techniques. This procedure will be useful for the detection of viruses, viroids, and MLOs that infect woody hosts, and the samples can be prepared in 1–2 hr, as against the 1–3 days required for other extraction methods (Appendix 9[i]). The simplicity and sensitivity of PCR make this a potential technique for routine and large-scale detection of pathogens in difficult activities such as seed certification.

Specific primers are made from sequences of either amplified or cloned DNA (cDNA) or RNA from the virus or other pathogens to be detected, whereas any DNA can be amplified by PCR by the method described elsewhere (Appendix 9[ii]). Amplification of single- or double-stranded viral RNAs requires some modification. Usually RNA is reverse-transcribed before amplification using *Taq* polymerase. In the direct RNA amplification procedure, the manganese-dependent transcriptase activity of *Thermus thermophilus* (Tth) polymerase is used.

9.4.1 Detection of Plant Viruses

The reverse-transcription-polymerase chain reaction (RT-PCR) procedure has been used for the detection and assay of many plant viruses (Table 9.1). Potato leaf roll virus (PLRV) was detected by PCR in dormant tubers from field-grown plants and in vitro–propagated microtubers, whereas the presence of PLRV in tubers of field-grown plants could not be detected by ELISA (Spiegel

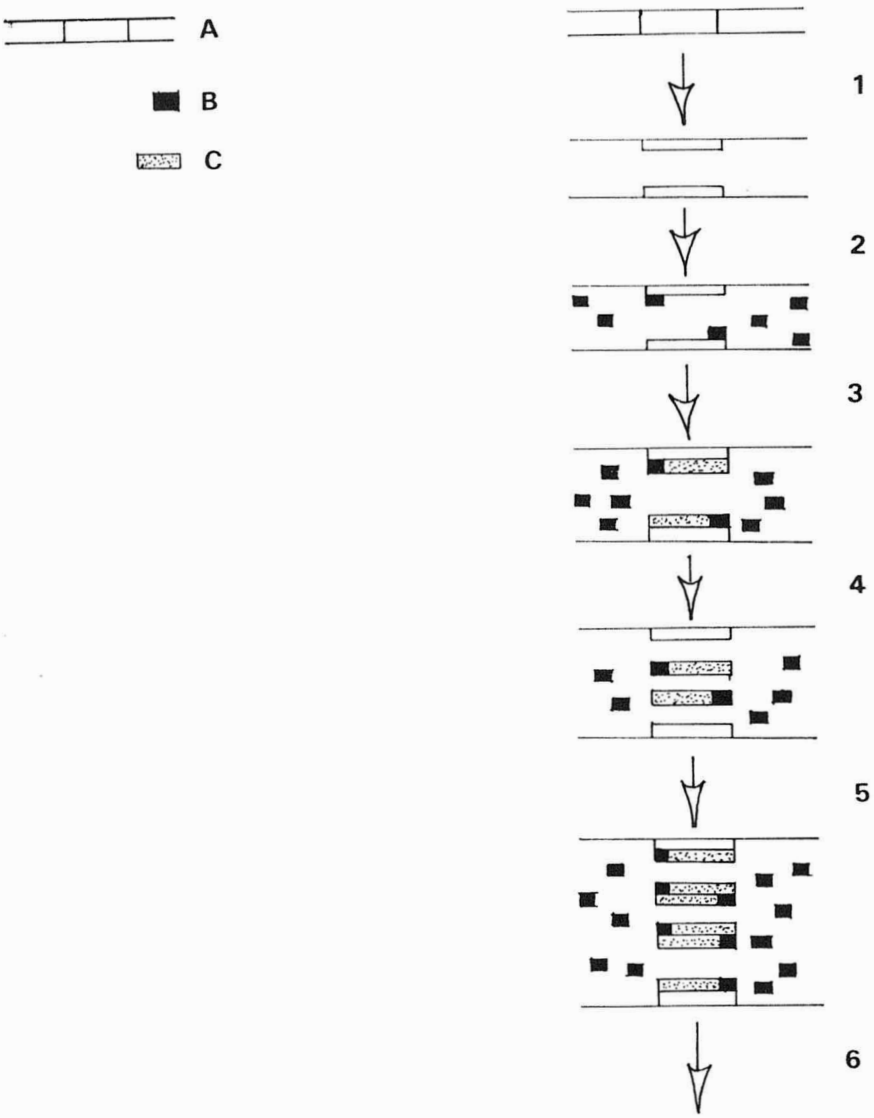

Figure 9.2 Amplification of pathogen DNA by polymerase chain reaction (A, target DNA; B, primer; C, new DNA): 1, Denaturation of DNA; 2, addition of primer and annealment; 3, synthesis; 4, denaturation; 5, synthesis; 6, repetition of denaturation and synthesis cycles (CAB International, U.K.). (Adapted from Leach and White, 1991.)

Table 9.1 Target Sequences of Plant Viruses Amplified by PCR

Virus	Target sequence	Reference
Banana bunchy top virus	Virus-specific sequnces; plant extract	Xie and Hu, 1995
Bean golden mosaic virus (BGMV)	BGMV sequence DNA from infected plant	Gilbertson et al., 1991.
Bean yellow mosaic virus (BYMV)	BYMV sequence; plant extracts containing nucleic acids	Vunsh et al., 1990, 1991
Beet pseudo yellows virus (BPYV)	BPYV sequence; plant extract	Coffin and Coutts, 1992
Beet western yellows (BWYV) and beet mild yellowing viruses	Virus-specific sequences and plant extracts	Jones et al., 1991
Cauliflower mosaic virus (CaMV) aphid	CaMV sequence; DNA extracted from aphid	Lopez-Moya et al,. 1992
Cherry leaf roll virus (CLRV)	Virus-specific sequences; plant extract	Borja and Ponz, 1992
Fiji disease virus (FDV)	Virus-specific sequences; plant extracts	Smith et al., 1992
Grapevine fan leaf virus	Virus-specific sequences; nematode extracts	Esmenjaud et al., 1994
Luteoviruses	Virus-specific sequences; plant extracts	Robertson et al., 1991
Maize streak virus (MSV)	Conserved virus sequences; plant extracts	Rybicki and Hughes, 1990
Pea seed-borne mosaic virus (PSbMV)	Virus-specific sequences; plant or seed extracts	Kohnen et al., 1992
Plum pox virus (PPV)	Virus-specific sequences; plant extracts	Korschineck et al., 1991; Wetzel et al., 1992
Potato leaf roll virus	Virus-specific sequences	Nolasco et al., 1993
Potyviruses	Conserved virus sequences; plant extracts	Langeveld et al., 1991
Rice tungro bacilliform virus (RTBV)	Virus-specific sequences; leaf-hopper extract	Venkitesh and Koganezawa, 1995
Tobacco mosaic virus (TMV)	MP gene sequences; plant extracts	Drygin et al., 1992
Tomato yellow leaf curl virus (TYLCV)	Virus-specific sequences; plant and whitefly extracts	Navot et al., 1992
Tomato spotted wilt virus	Virus-specific (sRNA) sequences; insect extracts	Tsuda et al., 1994; Mumford et al., 1994

and Martin, 1993). The RNA1 and RNA2 of wheat soil-borne mosaic virus (WSBMV) were individually detected in *Triticum aestivum*; RNA2 could be observed in root samples of both susceptible and resistant cultivars up to 7 weeks before the virus could be detected by ELISA (Pennington et al., 1993). A partially characterized virus isolated from *Gloriosa rothschidiana* could be positively identified as a potyvirus by specific amplification and subsequent sequence analysis of an amplified DNA fragment (Langeveld et al., 1991). Zerbini et al. (1995), using PCR, detected as much as 12% divergence in the coat protein–hypervariable region of the genome of lettuce mosaic potyvirus isolates; this variability does not lead to any change in the biological properties. The luteoviruses potato leaf roll virus, beet western yellows virus, and New York barley yellow dwarf virus (BYDV) were easily distinguished by restriction enzyme analysis of the amplified DNA products; this test could simultaneously detect all five BYDV serotypes (Robertson et al., 1991).

Two subgroups of cucumber mosaic cucumovirus (CMV) were distinguished by using two CMV specific primers that the flank CMV capsid protein gene and amplifying the cDNA fragment. Restriction enzyme analysis of this DNA fragment offers distinct restriction patterns that help to classify the CMV isolates accurately in the respective subgroups. Xie and Hu (1995) developed a PCR assay for the detection of banana bunchy top virus in banana plants and aphid vectors, and the assay was 1000 times more sensitive than dot-blot hybridization and ELISA tests. The PCR-based procedure provides a simple alternative to the serological assays used for classifying CMV isolates (Rizos et al., 1992). The RT-PCR procedure has been employed for the detection of several other viruses, such as cymbidium mosaic virus (Lim et al., 1993), strawberry mild yellow edge virus (Hadidi et al., 1993), sweet potato feathery mottle virus (Colinet et al., 1994), tomato spotted wilt virus (Mumford et al,. 1994), and viruses that infect woody plants (Rowhani et al., 1995). Using PCR, the cauliflower mosaic virus (CaMV) was detected in a single viruliferous aphid, and this method could detect about 10 pg/ml of purified CaMV and was more sensitive than serological methods (Lopez-Moya et al., 1992). Takahashi et al. (1993) detected the presence of rice tungro bacilliform badnavirus (RTBV) in tungro disease–affected rice leaves, but not in the leafhopper vector, by PCR, which was found to be 1000 to 10,000 times more sensitive than ELISA. However, Venkitesh and Koganezawa (1995) showed that a small fragment (569 bp) of RTBV-DNA could be amplified by PCR from the total nucleic acid extract of a single viruliferous leafhopper and detected on agarose gel. The RT-PCR method was performed directly in crude extracts of CMV-infected plants using primers complementary to conserved sequences of cucumber mosaic virus RNA 3 for broad-spectrum detection of isolates belonging to subgroups I and II from different geographical locations (Blasde et al., 1994). The other

plant viruses detected by the RT-PCR procedure have been listed by Henson and French (1993). This technique requires only very small amounts of infected leaf tissue (1 mm^2) or viral nucleic acid (10 fg), and the results are obtained in about 5 hr (Lin et al., 1993).

The RT-PCR technique can also be used to detect specific virus sequences or components of viral nucleic acid. The pathotypes P1 and P4 of pea seed-borne mosaic virus could be differentiated by employing sequence-specific RT-PCR (Kohnen et al., 1995). A region of coat protein gene of grapevine fan leaf virus was detected by RT-PCR in nematode vectors by Esmenjaud et al. (1994). The virus-specific SRNA of tomato spotted wilt virus could be detected in thrips vectors by the RT-PCR technique (Tsuda et al., 1994), and closely related furoviruses, beet necrotic yellow vein virus, and beet soil-borne mosaic virus could also be differentiated (Rush et al., 1994). Further, studies by Kruse et al. (1994) showed that by determining restriction fragment length polymorphism (RFLP) patterns of the RT-PCR products of different regions of viral genome, two major strain groups, designated A and B, of BNYVV could be differentiated. Hataya et al. (1994) developed the PCR–microplate hybridization method for detection of plant viruses. In this method a cDNA fragment from coat protein region of the PVY-RNA genome amplified by RT-PCR was detected by a digoxigenin-labeled cDNA probe. Detection of 10 fg of PVY genomic RNA was possible, and it was 10,000 times more sensitive than ELISA (Figs. 9.3 and 9.4) (Appendix 9[iii]).

The direct RNA amplification procedure was used to amplify the transport protein gene of TMV in addition to the RT-PCR method. Both amplified cDNA products were restricted with NCo I or Hae III endonucleases and identical restriction fragments were formed. Hence Drygin et al. (1992) suggested that direct RNA-PCR may be adopted for the detection of RNA viruses. Direct RNA-PCR can be used to amplify the target fragment in total RNA extracts from leaves infected by viruses such as beet western yellows virus. The PCR products are then detected by staining with ethidium bromide after agarose gel electrophoresis. The limit of detection may be further increased by Southern blotting. This procedure is far more sensitive than ELISA and dot-blot hybridization and is able to distinguish beet western yellows virus and beet mild yellowing virus (Jones et al., 1991).

Detection of viral pathogens becomes more sensitive when antibody binding and PCR are combined. These methods not only detect the presence of viruses but also may indicate their viability. In immunocapture PCR, as applied to plum pox virus, the virus is concentrated by using specific antibody; then the specific RNA sequence of the "captured" virus particles is amplified by PCR. The sensitivity of detection is 250 times that of direct PCR (Wetzel et al., 1992). A similar procedure was employed for the detection of bean yellow mosaic, cherry leaf roll, cucumber mosaic, citrus tristeza, grapevine

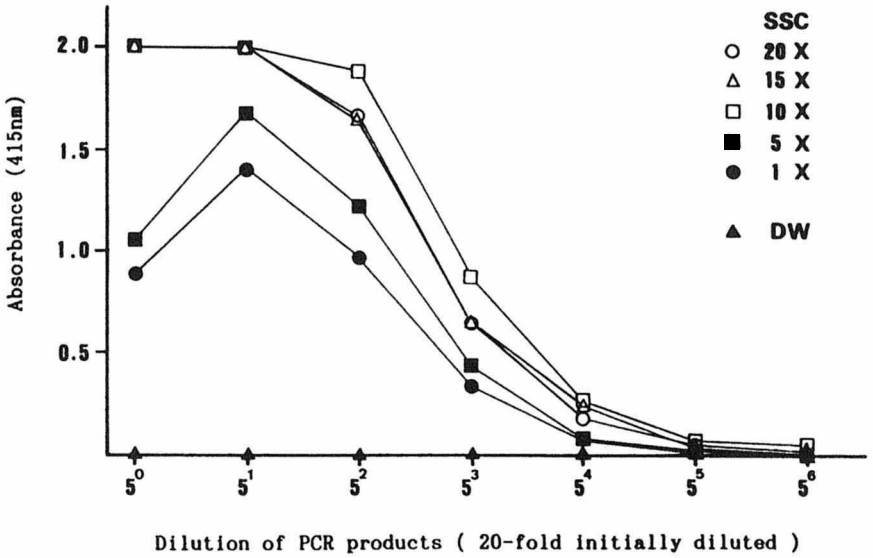

Figure 9.3 Effect of standard saline citrate (SSC) concentrations on adsorption of DNA to microplate wells. Absorbance values are determined after hydrolysis of substrate for 1 hr (Elsevier Science B.V., Netherlands). (Courtesy of Hataya et al., 1994.)

fanleaf, potato leaf roll, pepper mild mottle, and tomato spotted wilt viruses and satellite RNA of CMV and PSTVd by Nolasco et al. (1993). Immuno-PCR is another highly sensitive procedure in which a DNA fragment is linked to an antigen–antibody complex, using protein A (linking to antibody) and streptavidin (attached to DNA). Protein A and streptavidin have strong affinity. This complex is then bound to a biotin-labeled DNA sequence, which is subsequently amplified by PCR (Appendix 9[iv]). The immuno-PCR technique is 10^5 times more sensitive than ELISA (Sano et al., 1992). Only antigen-specific antibody is required for immuno-PCR, whereas nucleic acid sequence information is also needed for the immunocapture technique in addition to specific antibody. These techniques have great potential for the rapid and reliable detection of pathogens.

9.4.1.1 Single-strand conformation polymorphism analysis

Orita et al. (1989) reported that single-strand conformation polymorphisms (SSCP) could be used as the reliable alternative for the detection of differences in the genomic DNA. The + and – strands of a ds DNA, if separated, become metastable sequence-specific folded structures with distinct electrophoretic mobilities in nondenaturating polyacrylamide gels. Under such conditions, it is possible to detect even single nucleotide exchanges. Koening et al. (1995)

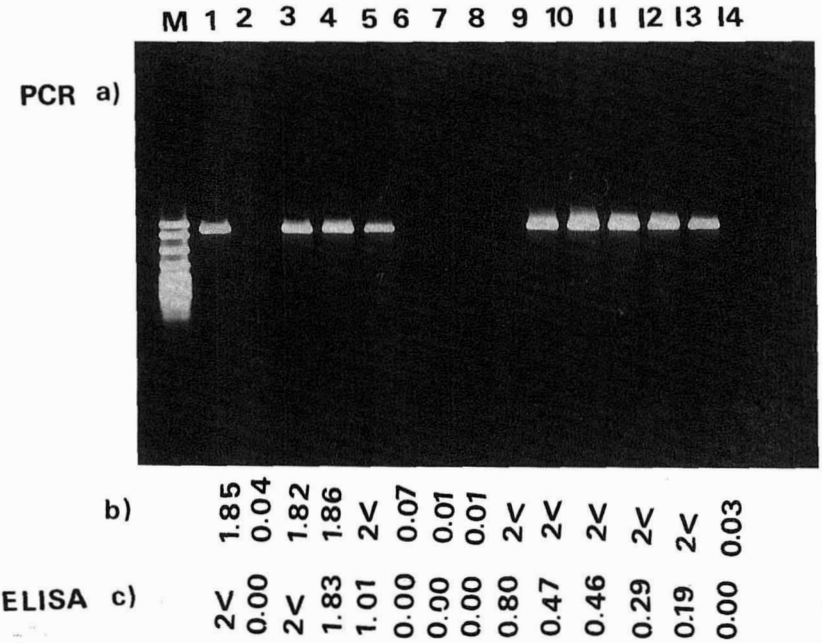

Figure 9.4 Detection of PVY in samples of potatoes from the field: a, by RT-PCR; b), by microplate hybridization; c, by ELISA, b and c, absorbance values at 415 nm after substrate hydrolysis for 2 hr (Elsevier Science B.V., Netherlands). (Courtesy of Hataya et al., 1994.)

employed SSCP analysis as a tool for rapidly assigning large numbers of beet necrotic yellow vein virus (BNYVV) isolates to strain groups A, B, or P and for detecting mixed infections, minor variants, or new strain groups (Table 9.2). The SSCP analyses are much less time-consuming than RFLP analysis and may be particularly useful for differentiating serologically indistinguishable strains of viruses.

9.4.2 Detection of Viroids

The viroids that cause plant diseases can be readily detected by the RT-PCR procedure. The viroids that infect citrus, exocortis, cachexia, and citrus viroid II A, can be detected and identified by assaying the nucleic acid extracts of infected sweet orange or Etrog citron. The RT-PCR assay is more sensitive than other available detecting methods, which require large samples or molecular hybridization (Yang et al., 1992). By using the RT-PCR technique, the hop

Table 9.2 Differentiation of Beet Necrotic Yellow Vein Virus Strains by SSCP Analysis

BNYVV RNA	Amplified region (nt)	Size of PCR product	No. nt exchanges between published sequences for A- and B-types	Differentiation between A- and B-types
1	6150–6651	501	Unknown	Excellent[a]
2 (Triple gene	2480–3241	761	30	Excellent
block region	2711–3398	687	23	Excellent
	2811–3108	297	12	Excellent
	2811–3241	430	19	Excellent[a]
	2811–3398	587	21	Excellent
	2950–3398	448	15	Excellent
3	409–1268	859	Unknown	Excellent[a]
	911–1268	357	Unknown	Excellent
4	699–1301	602	Unknown	Excellent[a]

[a]Recommended for screening tests.
Source: Koenig et al. (1995).

stunt viroid (Puchta and Sanger, 1989), apple scar skin and pear rusty skin viroid (Hadidi and Yang, 1990), and grape viroids (Rezaian et al., 1992) have been detected.

9.5 DETECTION OF MYCOPLASMA-LIKE ORGANISMS

9.5.1 Dot-Blot Hybridization Assay

Mycoplasma-specific DNA probes have been prepared from chromosomal or plasmid (extrachromosomal) DNA of the MLO pathogens. These probes are usually labeled with either radioactive ^{32}P or nonradioactive biotin. Recently another nonradioactive substance, digoxigenin, has been used to label probes specific for sweet potato witches'-broom MLO (Ko and Lin, 1994). Specific tissues such as phloem sieve tube elements or hemolymph or salivary glands of vector insects may be used to extract the MLO DNA, since the concentration of MLOs is higher in such tissues (Kirkpatrick et al., 1987; Davis et al., 1988; Kirkpatrick, 1989). The MLOs are usually detected by extracting the DNA from infected plants or inoculative insects and using a specific DNA probe in a dot-blot hybridization.

Dot-blot hybridization is useful to detect, differentiate, and quantify nonculturable MLOs infecting plants (Appendix 9[v]). Kirkpatrick et al. (1987) employed cloned DNA probes successfully in dot-blot assays to detect western

X MLO in infected plants and leaf hoppers (Appendix 9[vi]). The MLOs that cause maize bushy stunt disease (Davis et al., 1988), aster yellows disease (Lee and Davis, 1988; Kulske and Kirkpatrick, 1992), apple proliferation disease (Bonnet et al., 1990), periwinkle little leaf disease (Davis et al., 1990), chrysanthemum yellows disease (Bertaccini et al., 1990), clover proliferation disease (Deng and Hiruki, 1990), and palms lethal yellowing disease (Harrison et al., 1992) were also detected by this technique (Fig. 9.5). Nakashima et al., (1993) reported that rice yellow dwarf MLO could be differentiated from rice orange leaf MLO by using specific chromosomal and extra chromosomal DNA probes. Davis et al. (1988) reported that biotinylated DNA probes could be used to detect alfalfa witches'-broom, clover yellow edge, X-disease, clover phyllody, eastern and western aster yellows MLOs in different host plants, and aster yellows in the leafhopper vector *Macrosteles fascifrons*. By the dot-blot hybridization procedure, the presence of ash yellows MLO was detected in the innermost phloem at the trunk base, roots, twigs, and leaves of white ash trees. The DNA probe detected MLOs as consistently as the fluorescence dye DAPI (4,6-diamidino-2, phenyl indole 2 HCl) fluorescence test (Sinclair et al., 1992;

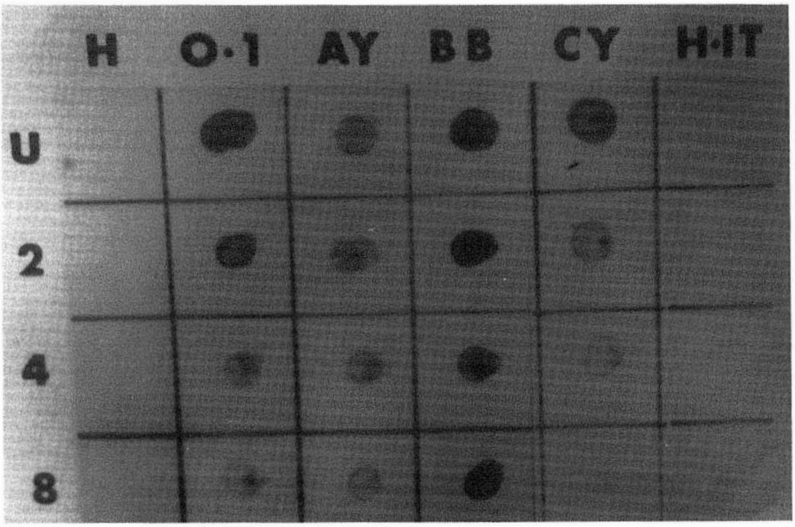

Figure 9.5 Dot hybridization of biotinylated cloned DNA probe to nucleic acid preparations extracted from healthy and MLO-infected *Catharanthus roseus* plants: H, healthy; H-IT, healthy Italy; CY, chrysanthemum yellows; 0–1, periwinkle little leaf; AY, aster yellows; BB, tomato big bud; U, undiluted; 2, 4, and 8, reciprocals of dilution (American Phytopathological Society, Minnesota, USA). (Courtesy of Bertaccini et al., 1990.)

Davis et al., 1992). The presence of MLOs in plant tissues micropropagated in vitro could be detected by dot hybridization (Bertaccini et al., 1992).

Mycoplasma-like organism–specific probes may be used to determine the host range of the MLO, and latent infections may also be recognized. The presence of MLO causing lethal yellowing (LY) was detected in true date (*Phoenix dactylifera*), cliff date (*P. rupicola*), chinese fan (*Livistona chinensis*), and five coconut palm cultivars. *Caryota rumphiana* and *L. rotundifolia*, two palm species, which were not known to be infected by lethal yellowing, also revealed the presence of LY MLO (Harrison et al., 1992).

The distribution and multiplication of the MLO in the infected plant can be studied by using labeled MLO-specific DNA probes, as in the case of aster yellows MLO in periwinkle plants. The MLO moved from grafted shoots into ungrafted shoots and then systemically throughout the plant. Distribution and concentration of the MLO were directly correlated with expression of virescence and proliferation symptoms, and the concentration was maximum in symptomatic, actively growing shoots (Kulske and Kirkpatrick, 1992). Clover proliferation (CP) and potato witches'-broom (PWB) MLOs were first detected in the external primary pholem tissues of periwinkle and then in the secondary phloem elements. The MLOs later spread into the internal phloem tissue (Hiruki and Deng, 1992).

The extent of relationship between MLOs can be reliably determined by dot-blot hybridization based on the amount of probes hybridizing with the target DNA. Probes specific to sweet potato witches'-broom MLO hybridized with serologically related peanut witches'-broom MLO. On the basis of differences in the band patterns formed in Southern blots, sweet potato witches'-broom MLO could be differentiated from peanut witches'-broom MLO (Ko and Lin, 1994). The relationship between the MLOs affecting six species of ash (*Fraxinus*) and lilac (*Syringa*) was studied by Griffiths et al. (1994). Dot-blot hybridization with clover proliferation MLO DNA probes showed that there was cross-hybridization with nucleic acid from potato witches'-broom–MLO-infected periwinkle, but not with the nucleic acid from plants infected by either western aster yellows MLO or clover phyllody MLO. This result indicates that MLOs that cause clover phyllody and proliferation are distinct and unrelated (Deng and Hiruki, 1990a).

Patterns of hybridization using DNA probes labeled with ^{32}P or biotin indicated that aster yellows MLO, orchard MLO, tomato big bud MLO, and blueberry stunt disease MLO belonged to the cluster of strains that share greater nucleotide sequence homology with one another than with other MLOs tested. Kulske et al. (1991a) observed that the native ^{32}P-labeled plasmid isolated from the severe western aster yellows (SAY) strain MLO hybridized with small extrachromosomal DNA molecules present in many virescence-inducing MLOs

and the maize bushy stunt (MBS) MLO but not with DNA from decline-inducing MLOs or spiroplasmas.

Lee and Davis (1988) constructed a ^{32}P-labeled single-stranded RNA probe (riboprobe) with plasmid vector pS64. This riboprobe was more sensitive and reliable than cDNA probe in detecting western X MLO. At higher concentrations of cDNA probe, a nonspecific hybridization signal was observed with nucleic acid from healthy plants and from plants infected by other MLOs. On the other hand, sensitivity of detection with complementary riboprobe was increased at higher concentration. Lee and Davis (1988) and Davis et al. (1988, 1990a), using ^{32}P-labeled riboprobes, showed that aster yellows (AY)–related MLO strain cluster could be recognized and that these probes distinguished the strains of this cluster.

As the MLOs are usually found in low concentrations in infected plants, the sensitivity of detection has to be improved. Many MLOs contain extrachromosomal DNA (Denes and Sinha, 1991), and the probes for extrachromosomal DNA usually give stronger hybridization signals than chromosomal DNA probes. It is possible that the higher sensitivity may be due to the presence of multiple copies of extrachromosomal DNA in MLO cells. The amount of plant tissue required for detecting maize bushy stunt MLO using extrachromosomal DNA probe was only 0.02 g of plant tissue, whereas 0.3 g of tissue was necessary for detection when chromosomal DNA probe was used (Davis et al., 1988). Depending on the type of host tissue, about 15–30 ng of MLO DNA may be required for detection of MLOs by dot-blot hybridization (Goodwin and Nassuth, 1993).

Use of nonradioactive labels has distinct advantages, as in serological assays (Chapter 8). Davis et al. (1990a, 1990b) employed biotinylated cloned DNA probes for detecting aster yellows (AY) MLO in infected plants and in the leafhopper vector *Macrosteles fascifrons*. Sinclair et al. (1992) found the hybridization signals, when biotin-labeled cloned DNA probe for ash yellows MLO was used, to be most consistent and intense with samples from innermost phloem at the trunk base of infected plants, indicating the possibly high concentration of the MLO in that tissue. Davis et al. (1992) showed that ash yellows MLO could be detected in leaves, twigs, trunk phloem, and roots of white ash trees and that this MLO represented a distinct strain cluster. Using digoxigenin-labeled DNA probes, the sweet potato witches'-broom MLO was detected in infected sweet potato and periwinkle plants. The majority of the probes tested hybridized with serologically related peanut witches'-broom MLO. The probes could detect sweet potato witches'-broom MLO DNA at 10 ng and 0.39 ng of DNA from periwinkle and sweet potato, respectively (Ko and Lin, 1994), indicating that the MLO may reach a higher concentration in sweet potato than in periwinkle.

9.5.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) can be successfully used to detect and differentiate MLOs even when they are in very low concentrations. It can be employed to develop a specific assay for MLO detection. A cloned fragment of a plasmid from the MLO is sequenced to identify oligonucleotide primers for PCR. Amplified DNA fragments of the predicted size are then obtained from the DNA extracted from plants or insects infected by the MLO, whereas no amplification occurs in healthy plant or insect DNA. The PCR-based assays are over 500 times more sensitive than a hybridization-based assay, as observed in the case of aster yellows MLO (Goodwin et al., 1994).

Deng and Hiruki (1990a) synthesized two PCR primer pairs to amplify specifically the two clover proliferation (CP) MLO DNA fragments from crude nucleic acids containing CP MLO DNA and host plant DNA. Using 5′ end-labeled sequence-specific internal probes, PCR products were identified by liquid hybridization. To detect CP MLO, only about 2.5×10^{-8} to 2×10^{-5} ng nucleic acid was required, whereas 2.5 ng nucleic acid was necessary when PCR was not used. Several other MLOs also have been detected in infected plants by the PCR technique (Deng and Hiruki, 1990; Ahrens and Seemuller, 1992; Schaff et al., 1992; Lee et al., 1992a; Bertaccini et al., 1992; Harrison et al., 1994).

Employing specific oligonucleotide primers synthesized by using sequence data of the 16S ribosomal RNA (rRNA) gene from three plant pathogenic MLO groups, the PCR technique was found to be effective for detecting MLO 16S rRNA genes from diseased plants and inoculative insect vectors (Namba et al., 1993). The MLOs that cause pear quick decline, apple proliferation, and sweet potato little leaf MLO (Phytoplasma) were detected by amplifying the 16S ribosomal RNA (rRNA) gene by using the PCR technique (Giunchedi et al., 1993; Firrao et al., 1994; Gibb et al., 1995).

The PCR is very useful in determining the phylogenetic relationships among MLOs that cause various plant diseases (Lee et al., 1992b). The faba bean phyllody MLO could be detected by employing a specific primer pair and DNA amplification by PCR (Saeed et al., 1994). Lee et al. (1994) reported that a MLO group-specific primer pair allowed sensitive detection and simultaneous classification of MLO strains and that the sensitivity of detection may be further increased by employing nested-PCR assays using the universal primer pair R 16 F2/R2 and a group-specific primer pair. The recycled PCR (R-PCR), a modified method, may be used for both diagnosing MLO diseases and developing a phylogenetically based MLO taxonomy. In this method, several different PCR reactions may occur simultaneously in one tube by addition of a second primer to the tube containing the first PCR products. Recycled PCR helps

in detection of mollicute-specific DNA fragments and MLO-specific or group-specific DNA fragments as multiple bands, and each MLO can be reliably identified (Namba et al., 1993).

A primer pair based on an MLO 16S rRNA gene was used to amplify regions representing about 80% of the 16S rDNA sequences of 40 MLO strains from North America, Asia, and Europe. The partial 16S rDNA sequences amplified from different MLOs were compared by RFLP analyses, and similarity coefficients were determined. The MLOs were classified into 9 distinct 16S ribosomal RNA (16Sr) groups and 14 subgroups with type MLO strains as follows (Lee et al., 1993):

16Sr IA	Tomato big bud
16Sr IB	Maryland aster yellows
16Sr IC	Clover phyllody
16Sr ID	*Paulownia* witches'-broom
16Sr IE	Blueberry stunt
16Sr II	Peanut witches'-broom
16Sr IIIA	Canada peach X
16Sr IIIB	Clover yellow edge
16Sr IV	Palm lethal yellowing
16Sr V	Elm yellows
16Sr VI	Clover proliferation
16Sr VII	Ash yellows
16Sr VIII	Loofat witches'-broom
16Sr IX	Pigeonpea witches'-broom

However, in certain cases, it may not be possible to detect and differentiate strains of MLO, as in apple proliferation and pear decline MLOs (phytoplasmas) using pathogen-specific primers (Lorenz et al., 1995).

9.6 DETECTION OF FUNGAL PATHOGENS

Detection of fungal pathogens by using nucleic acid–based techniques has several advantages. Serological methods have been found to be more difficult, since the fungal antigens are complex and variable at different growth stages. The presence or absence of spore-bearing structures and slow-growing nature of certain fungal pathogens will not affect their detection by nucleic acid–based techniques, since only fungal cells containing DNA are needed. Using appropriate DNA probes, fungi which are generally not amenable for rapid identification can be detected and identified. Soil-borne fungal pathogens such as *Phytophthora, Gaeumannomyces, Pythium,* and *Leptosphaeria* that cause rela-

tively nonspecific symptoms, such as generalized rotting and death of plants, may be detected by using specific DNA probes. Adoption of PCR-based assays allows enhancement of sensitivity and specificity of detection and quantification of fungal pathogens in plant tissues and assessment of relatedness of pathogens.

9.6.1 Restriction Fragment Length Polymorphism

Application of restriction fragment length polymorphism (RFLP) analyses for fungal plant disease diagnosis has been found to be useful. The RFLP technique is based on the natural variations in the genomes of different groups or strains of organisms. Loss or gain of restriction endonuclease recognition sites or other events such as deletions or insertion in the DNA sequences may result in variations (polymorphisms) in fragment sizes. The DNA of the test organism is digested with restriction enzymes, and the fragments are separated by electrophoresis in agarose or polyacrylamide gel to detect the differences in the size of the DNA fragments. The number and size of the fragments formed after digestion are determined by the distribution of restriction sites in the DNA. Hence, depending on the combination of each restriction enzyme and target DNA, a specific set of fragments, that can be considered a fingerprint for a given strain is formed. The specific sites of fragments are usually identified by Southern blot analysis (Hamer et al., 1989; Leach et al., 1990), but they can also be directly observed by staining the gels with ethidium bromide for observation under ultraviolet light (Klich and Mullaney, 1987; Jones et al., 1989). The DNA fragments are then transferred to a nitrocellulose or nylon membrane and hybridized with an appropriate probe (Appendix 9[vii]).

By using an appropriate probe, detection and identification of pathogenic fungi may be achieved. Nicholson et al. (1994) developed a pathotype-specific DNA probe for the identification of the R type of *Pseudocercosporella herpotrichoides*. They isolated a 6.7-kb DNA fragment from an R-type isolate of the pathogen which showed specific hybridization to R-type isolates and not to N, C, or S pathotypes or to *P. anguioides*. Infection of rye seedlings by R type was detected by hybridization of this probe to DNA extracted from infected plants.

The RFLP data may be used for assessing the genetic diversity of the pathogen population as well as for determining the extent of relatedness of the pathogen groups on the basis of the numerical analysis of the data (Lynch, 1988; Nei and Li, 1979). Restriction fragment length polymorphisms were used to estimate the genetic divergence and relationship among isolates of *Fusarium oxysporum* f. sp. *gladioli* (Me et al., 1994). The usefulness of RFLP analysis has been increasingly recognized in fungal taxonomy (Garber and Yoder, 1984; Anderson et al., 1987, Coddington et al., 1987; Foster et al., 1987; Kistler et

al., 1987; Manicom et al., 1987; Klich et al., 1993). Genomic DNA RFLPs combined with random probes can be used for differentiating species, formae speciales, races, and isolates of *Fusarium* (Coddington et al., 1987; Manicom et al., 1987; Kim et al., 1993). Ko et al. (1993) reported that RFLPs in nuclear DNA were correlated to some extent with the prevailing races of *Magnaporthe grisea* in Korea. However, the relationship between RFLPs in nuclear DNA and virulence of *M. grisea* was inconclusive.

Both total DNA (Manicom et al., 1987; Coddington et al., 1987) and mitochondrial (mt) DNA (Anderson et al., 1987; Foster et al., 1987) have been subjected to RFLP analyses for taxonomic studies. But in many cases RFLP analyses have been performed with mitochondrial DNA (mt DNA) rather than with genomic DNA, since the mt DNA is much smaller. When cut with a single restriction enzyme, mt DNA produces about 10–20 fragments, forming distinct patterns on electrophoresis in agarose gels. The RFLP analysis of ribosomal DNA (rDNA) and mt DNA of isolates of *Cylindrocarpon heteronema*, which causes European canker in apples, by using rDNA from *Saccharomyces carlsbergensis* and mt DNA of *C. heteronema*, revealed intraspecific heterogenicity. Four rDNA and six mt DNA restriction pattern categories were noted among the isolates tested (Brown et al., 1994) (Fig. 9.6).

Okoli et al. (1994) examined the relationship of two host-adapted pathotypes of *Verticillium dahliae* by RFLP analysis. They found that isolates obtained from and adapted to peppermint formed a subgroup (M) distinct from the non-host-adapted subspecific group A of *V. dahliae*. Similarly isolates of *V. dahliae* from cruciferous hosts formed another group (D). By using two specific probes, the isolates from cruciferous plants could be distinguished on the basis of the variation in polymorphisms. Ueng and Chen (1994) reported that isolates of *Phaeosphaeria nodorum* exhibited a significantly lower degree of genetic variation than the isolates of *P. avenaria*, on the basis of RFLP analysis after the digestion of genomic DNAs by Eco RI. Several RFLP alleles useful for differentiation of *P. nodorum* from *P. avenaria* could be identified. The species-specific probes may be used as natural markers in epidemiological studies.

Frei and Wenzel (1993) reported that among 21 clones of *Pseudocercosporella herpotrichoides*, 13 clones exhibited restriction fragment length polymorphisms among isolates, and by combining with specific probes, the pathogen could be detected in infected plant material. Moreover, polymorphic pathogen-specific probes allowed varieties to be differentiated directly in infected plants without isolation of the pathogen. Carlier et al. (1994) observed distinct differences in RFLP patterns and hybridization intensities that suggest appreciable interspecific genetic divergence in *Mycosphaerella musicola* and *M. fijiensis*, which cause banana leaf spot diseases, indicating the possibility of early detection and identification of these pathogens by RFLP analyses.

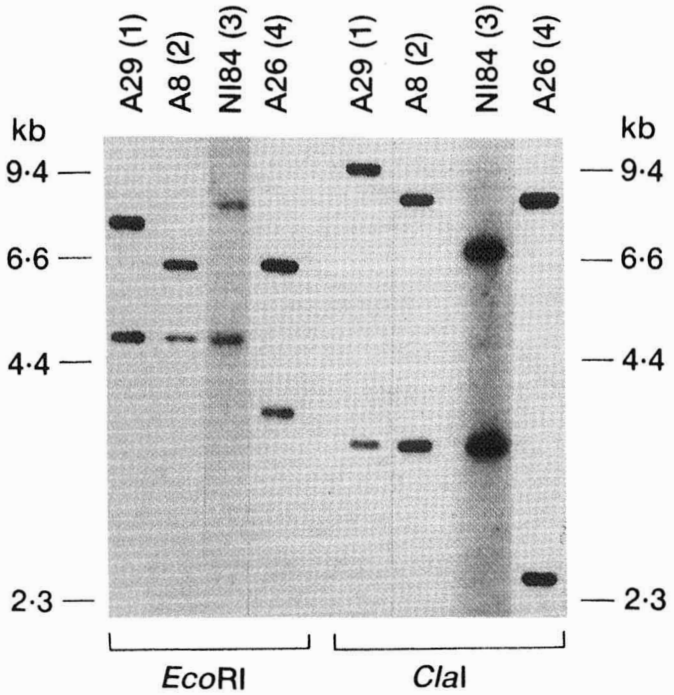

Figure 9.6 RFLP patterns of DNA from four isolates of *Cylindrocarpon heteronema* digested with Eco RI and Cla I and hybridized with the yeast rDNA probe pMY 60; ClaI restriction patterns of all isolates show a third faint band of approximately 1.05 kb (Blackwell Science Ltd., and British Society for Plant Patholgy, U. K.). (Courtesy of Brown et al., 1994.)

Muliple-copy DNA probes prepared from chromosomal DNA have several advantages. Employing highly repetitive DNA sequences enhances both the sensitivity of the assay, as the signal is present in multiple copies, and its reliability, as a result of the lack of influence of variation in one copy in the genome on the total signal observed in a hybridization-based assay. Moreover, repetitive DNA has a very high probability of being species-specific. Repetitive DNA fragments of 12 species of *Phytophthora* tested appeared as continuous discrete bands over a faint smear in agarose gels when stained with ethidium bromide. Similar digestion patterns were observed for the isolates belonging to the same species; different species of *Phytophthora* exhibited different patterns. Very similar *P. cryptogea* and *P. drechsleri* could be differentiated by the repetitive DNA profiles. The heterogeneous status of *P. megasperma* and complete homogenicity of 12 isolates of *P. parasitica* could be established by examining the DNA profiles. As this method is relatively simple, it may be useful

for investigation of taxonomic problems and identification of different species of *Phytophthora* (Panabieres et al., 1989) (Appendix 9[vii]). Cloned DNA probes prepared from chromosomal DNA of *Phytophthora parasitica* hybridized to *P. parasitica* DNA only, but not to DNA of other *Phytophthora* spp. and *Pythium* spp. DNA from all isolates of *P. parasitica*, including *P. parasitica* var. *nicotianae*, hybridized strongly with the probes, indicating their species-specific nature (Goodwin et al., 1989, 1990). Development of such species-specific DNA probes for the detection of *P. citrophthora* (Goodwin et al., 1990), *Gaeumannomyces graminis* (Henson et al. 1993), *Phoma tracheiphila* (Rollo et al., 1987), and *Leptosphaeria korrae* (Tisserat et al., 1991) has been reported. One of the cloned DNA sequences had as many as 50 to 100 copies of the *L. korrae* genome and can be employed for the detection of the pathogen in the root tissues of the turfgrass (Tisserat et al., 1991) (Fig. 9.7) (Appendix 9[viii]).

As mitochondrial DNA is smaller than chromosomal DNA, restriction maps can be developed to determine conserved and nonconserved regions.

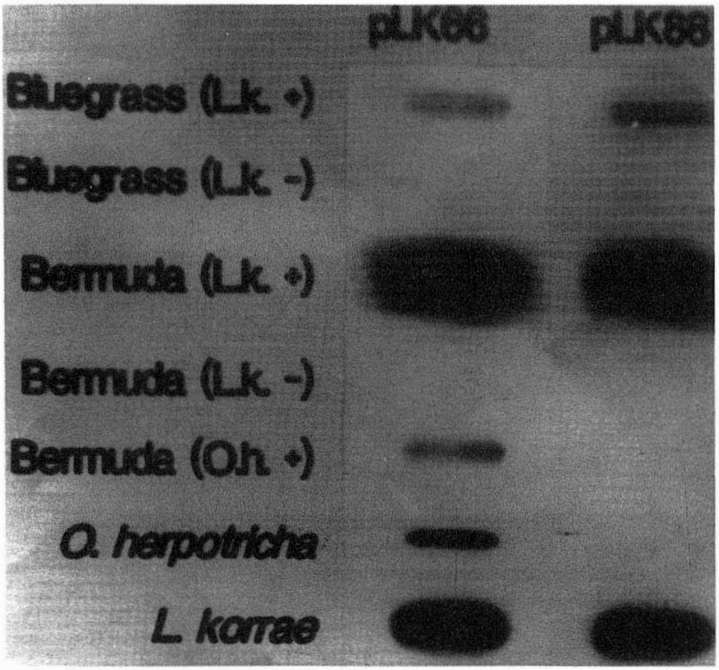

Figure 9.7 Slot hybridization of two probes (pLK 66 and pLK 88) to fungal and plant DNA extracted from Kentucky bluegrass and bermuda grass root samples: LK+, roots colonized by *L. korrae*; LK–, noninfected roots (American Phytopathological Society, Minnesota, USA). (Courtesy of Tisserat et al., 1991.)

Cloned DNA probes generated from mt DNA of *Gaeumannomyces graminis* (Henson et al., 1993) and *Peronosclerospora sorghi* (Yao et al., 1991) were highly specific and did not hybridize with DNA of other fungi. However, in the case of *Pythium* sp., some probes hybridized to a subset of isolates sharing the same mitochondrial restriction map, whereas many probes hybridized to the DNA of more than one *Pythium* sp. (Martin, 1991).

The probe specific for a dispersed repeated DNA sequence (called MGR) was employed to construct genotype-specific Eco RI restriction fragment length profiles (MGR-DNA fingerprints) from field isolates of rice blast pathogen (*Magnaporthe grisea*) in the United States of America. The MGR-DNA fingerprints could be used as the basis for distinguishing major pathotypes of *M. grisea*, identifying the pathotypes accurately, and defining the organization of clonal lineages within and among pathotype groups (Levy et al., 1991). The genetic relationships among isolates of *Pyricularia grisea* from rice and other hosts were analysed by RFLP analysis by using the repetitive probe MGR 586. Rice blast isolates representing four distinct races differentiated by inoculation on Korean differential rice varieties showed multiple bands hybridizing to the probe MGR 586. The study indicated that *P. grisea* populations from nonrice hosts, such as *Digitaria sanguinalis, Eleusine indica, Lolium boucheanum, L. multiflorum,* and *Festuca elatior*, could be sources of inoculum for the rice crop (Han et al., 1995). Ueng et al. (1995) found a correlation between molecular and biological characters of the biotypes of *Stagonospora nodorum* from barley and wheat. The genetic similarity, as determined by RFLP analysis, was very high (70.82) within each of the two biotypes, whereas the similarity was very low (0.12) between the two biotypes (Appendix 9[ix]).

The genetic similarity in 39 isolates of *Fusarium oxysporum* encompassing 5 formae speciales, that cause vascular wilts in cucurbits was studied. The total DNA was digested with three restriction enzymes (Pst I, Hind III, and Eco RI), Southern-blotted, and hybridized with a mt DNA polyprobe from *F. o. niveum*. Within each forma speciale unique RFLPs were present. *Fusarium oxysporum* niveum was found to have least divergence; *F. o. cucumerianum* showed maximum divergence (Kim et al., 1993). A new DNA fingerprinting probe, (Cat)5, was employed to detect genetic variation in two host specialized groups (on pine and fir) of *Heterobasidium annosum* in North America. Several fingerprint bands were specific to these groups, and several bands unique to isolates of either of the host specialized varieties (var. *wageneri* and var. *ponderoseum*) of *Leptographium wageneri* were also distinguished. More polymorphisms could be detected by using (CAT)5 probe than by using 21 isoenzyme markers (DeScenzo and Harrington, 1994).

The genetic fingerprinting and RAPD technique have been used to characterize pathotypes of fungal pathogens. The pathotypes of *Fusarium oxysporum* f. sp. *ciceris* could be differentiated by amplification of DNA by RAPD into

yellowing- or wilt-inducing pathotypes, and identification of pathotypes was confirmed by inoculating chickpea differentials (Kelly et al. 1994). Genetic variation in *Magnaporthe poae* and *Erysiphe graminis* isolates was determined by using RAPD markers by Huff et al. (1994) and Mc Dermott et al. (1994). Kolmer et al. (1995) reported that there was a correlation of 0.58 between virulence and molecular similarity in 64 single uredinal isolates of *Puccinia recondita* f. sp. *tritici*. Virulence and molecular polymorphisms were determined by using near-isogenic wheat differential lines and RAPD employing 10 arbitrary decamer primers.

9.6.2 Polymerase Chain Reaction

Enhancement of the sensitivity of an assay to a high level becomes necessary for the detection of pathogens which cause vascular wilts and root rots. Polymerase chain reaction–(PCR)–based assays allow detection of such pathogens as may be present in low populations in infected tissues or soil (Table 9.3). The presence of *Phytophthora parasitica* in infected tomato roots and soil (Goodwin et al. 1990), *Leptosphaeria korrae* in turfgrass (Tisserat et al., 1991), *Phytophthora citrophthora* in citrus roots (Goodwin et al., 1990), *Gaeumannomyces graminis* in wheat (Schesser et al., 1991; Ward and Gray, 1992; Henson et al.,

Table 9.3 Target Sequences of Fungal Plant Pathogens Amplified by PCR

Pathogen	Target sequence	Reference
1. *Colletotrichum gloeosporioides*	ITS I and conserved rDNA	Mills et al., 1992
2. *Gaeumannomyces graminis*	Mitochondrial (mt) DNA rDNA of mt DNA	Elliot et al., 1993 Ward and Gray, 1992
3. *Leptosphaeria maculans*	ITS I and conserved region of nuclear rDNA	Xue et al., 1992
4. *Mycosphaerella musicola* and *M. fijiensis*	ITS I and conserved region of rDNA	Johanson and Jeger, 1993
5. *Phoma tracheiphilla*	DNA from infected plant	Rollo et al., 1990
6. *Phytophthora* sp.	ITS of rDNA	Lee et al., 1993
7. *Verticillium albo-atrum* and	ITS 1 and 2 of nuclear rDNA;	Nazar et al., 1991; Hu et al., 1993;
V. dahliae	DNA from spores; ribosomal DNA	Moukhamedov et al., 1994.
8. *Septoria tritici Stagonospora nodorum*	ITS of nuclear DNA	Beck and Ligon, 1995

1993; Elliott et al., 1993), *Phomopsis tracheiphila* in citrus (Rollo et al., 1990), *Verticillium* spp. in potato (Moukhamedov et al., 1994), and *Monosporascus* spp. in muskmelon (Lovic et al., 1995) has been detected by employing PCR-based assays.

Polymerase chain reaction was used to amplify a ribosomal DNA fragment from *G. graminis*. This fragment, after labeling, was used as a probe which hybridized to Eco RI digests of target DNA. Consistent differences in the band pattern among three varieties of *G. graminis (G. tritici, avenae,* and *graminis*) were observed, indicating that such probes have considerable potential for use in the identification of these pathogens (Ward and Gray, 1992). Elliott et al. (1993) used a 188 bp DNA fragment derived from boiled mycelium of *G. graminis* for amplification by PCR as a probe for detection and identification of the pathogen from different grass hosts. Amplification of the specific DNA and presence of lobed hyphopodia in culture may be used for the identification of this pathogen. Using PCR assay, on the basis of sequence differences in their ribosomal RNA genes, *Verticillium dahliae*, *V. albo-atrum,* and *V. tricorpus* can be detected reliably, and a diagnostic set is now availble for the investigation and monitoring of the *Verticillium*–potato pathosystem (Moukhamedov et al., 1994). Henson et al. (1993) showed that by using nested primers to amplify a fragment of mt DNA, it is possible to detect *G. graminis* in development and to diagnose resistance in pathogens to fungicides. The biomass of *Verticillium albo-atrum* or *V. dahliae* could be determined by adopting PCR assay (Hu et al., 1993). The assay was used to study the colonization of lucerne by *V. albo-atrum* and sunflower by *V. dahliae* comparatively. The study accurately showed the substantial differences between the two pathogens, and the results could be obtained more rapidly and accurately than by conventional cytological or maceration and plating techniques (Hu et al., 1993). Many fungal pathogens have been reported to have developed resistance/tolerance to methyl benzimidazole carbamate (MBC), as a result of point mutation at amino acid 198 in the β-tubulin subunit, causing a change from glutamic acid to alanine. The resistant and sensitive strains of *Botrytis cinerea* could be successfully diagnosed by a PCR-based assay (Martin et al., 1992).

Sequences of specific regions, such as the internal transcribed spacers (ITS), of ribosomal DNA may be amplified by PCR with universal primers and used to differentiate *Pythium* spp. that are difficult to identify on the basis of morphological characters. The restriction fragment probes from ITS 1 showed a high degree of species specificity to *P. ultimum* when tested by dot-blot hybridization against 24 other *Pythium* spp. There was no difference among 13 isolates of *P. ultimum* var. *ultimum* and var. *sporangiferum* from eight countries and two isolates of *Pythium* group G recently classified as *P. ultimum* (Levesque et al., 1994). Probes generated from the ITS region of ribosomal DNA of *Colletotrichum gloeosporioides (Glomerella cingulata*) amplified by

PCR were employed to study 39 different isolates. These isolates were divided into 12 groups linked to host plant species and geographical origin (Mills et al., 1992). By using species-specific PCR primers for ITS regions, *Cylindrocarpon heteronema, Stagonospora nodorum, Septoria tritici,* and *Monosporasus* spp. were detected by Brown et al. (1993), Beck and Ligon (1995), and Lovic et al. (1995).

9.6.3 Random Amplified Polymorphic Deoxyribonucleic acid Technique

The random amplified polymorphic DNA (RAPD) method is a PCR technique which uses arbitrary primers and can be employed to distinguish races, strains, and pathogenic or nonpathogenic isolates of fungi. The primers used in this method are very short (10 or fewer bases) pieces of DNA from a desired source. It is highly probable that these primers find some complements in target DNA, producing a mixture of DNA fragments of various sizes. When the products from such a reaction are run on an electrophoresis gel, distinct banding patterns are produced, and somes of these patterns may prove to be specific to certain species or varieties or strains. The patterns themselves may be useful for detection and diagnosis of some pathogenic fungi, but some of the bands, in certain cases, may be cut out of a gel and sequenced to produce specific primers for more precise PCR analysis or probes for dot hybridization and other detection protocols.

The DNA sequence with 232 bp obtained from random amplified polymorphic DNA of *Peronospora tabacina* had homology to *P. tabacina* DNA only, and this sequence was amplified by PCR by using required oligonucleotides. Using this DNA fragment, *P. tabacina* was detected in local lesions, systemic vascular infections, and other infected parts of tobacco plants. Prediction of a disease epidemic may be possible by the use of spore traps, followed by amplification of the specific DNA fragment. This procedure may be valuable to regulatory agencies and in epidemiological and ecological studies (Wiglesworth et al., 1994). Johanson et al. (1994) reported that the RAPD technique could be used to differentiate *Mycosphaerella fijiensis* and *M. musicola*, which cause Sigatoka disease of banana, *M. mussae* and *M. minima*, the two other species commonly found on banana. The DNA from these *Mycosphaerella* species produced distinct RAPD banding patterns with all PCR primers tested.

Dobrowolski and O'Brien (1993) reported that fragments obtained from the products of RAPD-PCR amplification of *Phytophthora cinnamomi* DNA were tested for specific hybridization to *P. cinnamomi* DNA. The DNA fragments that hybridized specifically to *P. cinnamomi* were cloned and could be

used for detecting the fungal pathogen. Nicholson et al. (1993) showed that by using specific primers that produced distinct profiles of three races, the races of *Bipolaris maydis* could be differentiated. The pathogenic and nonpathogenic isolates of *Fusarium oxysporum* f. sp. *dianthi* from carnation were distinguished clearly by the RAPD method. Isolates of *Crinipellis perniciosa*, which causes witches'-broom disease of cacao, and members of Sterculiaceae, Solanaceae, and Bixaceae were analyzed by RAPD, which revealed distinct RAPD banding patterns. Banding patterns were found to be similar among basidiocarps on the same broom in *Theobrama cacao*. However, differences among monospore cultures from the same basidiocarp could be detected (Andebrhan and Furtek, 1994). This procedure was found to be simple, rapid, and reproducible when compared to other methods of identification (Manulis et al., 1994). For the identification of strains of *Trichoderma,* increasingly used as biocontrol agents, use of serological methods or isoenzyme patterns has not offered reliable results. By using RAPD with 10 arbitrary oligonucleotide primers, the strains of *Trichoderma* could be consistently distinguished, especially the isolate T-39 (the strain of *T. harzianum* used commercially as a biocontrol agent against *Botrytis cinerea*). The procedure developed by Zimand et al. (1994) requires only a small amount of DNA and less time and does not involve the use of radioisotopes (Appendix 9[x]).

9.7 DETECTION OF BACTERIAL PATHOGENS

9.7.1 Nucleic Acid Hybridization Technique

Detection and identification of plant bacterial pathogens may be possible by following conventional isolation and by employing physiological and pathogenicity tests which are time-consuming. But they yield doubtful results. Development of diagnostic DNA probes specific for the pathogen(s) concerned has helped in detecting, differentiating, and quantifying the population of bacteria very rapidly, especially the pathogens known to be transmitted through seeds and other planting materials. Specific DNA probes are available for detecting *Erwinia carotovora* subsp. *atroseptica* (Ward and DeBoer, 1984), *Pseudomonas syringae* pv. *phaseolicola* in beans (Schaad et al., 1989; Prossen et al., 1991), *Xanthomonas campestris* pv. *phaseoli* in beans (Gilbertson et al., 1989), *Clavibacter michiganense* subsp. *michiganense* (Thompson et al., 1989) and *P. syringae tomato* (Cuppels et al., 1990) in tomato, *Erwinia carotovora* (Ward and De Boer, 1990) and *C. michiganense* subsp. *sepedonicum* (Verreault et al., 1988) in potato tubers, *X. oryzae* pv. *oryzae* and *X. oryzicola* in rice (Cottyn et al., 1994), and *Pseudomonas glumae* in rice seeds infected by bacterial grain rot (Tsushima et al., 1994) (Appendix 9[xi]).

The diagnostic DNA probes may show specificity at the genus, species, pathovar, or race level. A probe that can be used for taxonomic comparisons is prepared by identifying a DNA fragment that is present only in the bacterial species to be identified, but not in other closely related species. The DNA sequences of 16S ribosomal RNA of different bacterial species have been compared for selecting probes specific at genus level. De Parasis and Roth (1990) developed a DNA probe for comparison of partial sequences of 16S rRNA from 52 strains of bacteria including *X. oryzae* pv. *oryzae*. The rRNA molecule is present in large numbers (>10,000 copies/cell) in actively growing bacterial cells and has diverse sequence regions that can be correlated with phylogenetic relatedness. The sensitivity of the probe derived from unique 16S rRNA sequences is greatly increased, because of the large number of copies present in a bacterial cell. A DNA sequence based on the 16S rRNA which hybridized only with plant pathogenic pathovars of *X. oryzae* pv. *oryzae* has been identified. By screening cloned DNA fragments at random, species- and subspecies-specific probes for *Erwinia carotovora* (Ward and De Boer, 1990) and *C. michiganense* subsp. *sepedonicum* (Verreault et al., 1988) and *C. m. michiganense* (Thompson et al., 1989) have been developed. Tsushima et al. (1994) prepared a probe specific for *Pseudomonas glumae*, causative agent of bacterial grain rot of rice for the detection of the pathogen in rice seeds by using two restriction enzymes, Eco RI and Kpn 1. The resultant fragment (PG2Ia) hybridized to all strains of *P. glumae*, but not to *X campestris* pv. *oryzae, X. campestris* pv. *campestris*, other species of *Pseudomonas, Agrobacterium, Erwinia,* and gram-positive *Clavibacter, Arthrobacter*, and *Carobacterium* tested. A probe for plasmid DNA present in *Xanthomonas campestris* pv. *citri* was employed to distinguish the pathotypes of this bacterial pathogen (Pruvost et al., 1992).

Dreier et al. (1995) reported that by employing Southern hybridization with DNA probes derived from plasmid-borne genes *cel* A (encoding an endocellulose) and *pat*-1 (involved in pathogenicity), *Clavibacter michiganensis* subsp. *michiganensis*, causative agent of tomato wilt and canker, could be detected. The *cel* A probe differentiated the subspecies of *C. michiganensis,* while *pat*-1 could distinguish virulent and avirulent strains of *C. michiganensis* subsp. *michiganensis.*

Ryba-White et al. (1995) compared the strains of *Xanthomonas oryzae* pv. *oryzae* (Xoo) from Africa, North America, and Asia by RFLP analysis using three repetitive DNA sequences cloned into Bluescript as probes. Total genomic DNA from each strain was digested with Eco RI, separated by electrophoresis, and blotted to membranes on which hybridization with ^{32}P-labeled probes was carried out. They found that the DNA banding patterns of African and Asian Xoo strains were substantially different, representing distinct geographi-

cally isolated populations. The RFLP patterns for strains from North America had least similarity with any other group of strains. This study suggests that RFLP analysis, though useful for comparing genomes within a pathovar, cannot be used for reclassifying the strains on the basis of RFLP analysis only.

Pathovars are identified by their pathogenic potential, differentiated by a specific set of differential cultivars. Development of a specific probe for the DNA fragment related to the production of toxic metabolite involved in pathogenesis is possible. By using the probe for the DNA related to phaseolotoxin, pathovars of *P. s. phaseolicola* could be identified (Schaad et al., 1989; Prossen et al., 1991). Likewise probe for DNA involved in coronatine toxin production has been developed to detect *P. s. tomato* (Cuppels et al., 1990). The DNA probes for T-DNA in Ti-plasmid can be used for the detection of *Agrobacterium tumefaciens* (Burr et al., 1990). Probes can also be developed to monitor the distribution of genes encoding for copper resistance in *X. c. vesicatoria* (Garde and Bender, 1991) and streptomycin resistance in *P. papulans* (Norelli et al., 1991). Development of probes specific for races has not been very successful, except in the case of a race-specific DNA probe for *P. solanacearum* race 3 (Cook and Sequira, 1991).

To perform colony hybridization, the bacterial suspension is prepared by macerating the infected tissue in liquid and incubated for diffusion of the bacteria into the liquid. The bacterial suspension is then spread onto the culture medium, permitting its growth, and covered with a nylon or nitrocellulose membrane. The filter membrane is removed after sufficient growth of the bacteria. Soil extracts or seed-soak washes may also be tested for the presence of the bacteria. By employing an appropriate DNA probe the bacteria can be detected (Cuppels et al., 1990; Ward and De Boer, 1990).

The sensitivity of dot-blot hybridization for detection of bacterial pathogens may vary, depending on the probe and bacterial species, and the minimum number of cells required for detection, which varies from 200 colony forming unit (cfu) to 10^6 cfu. As the number of bacterial cells in seed-soak washes is likely to be less, it is necessary to grow the bacteria first on a semiselective medium for 96 hr and to concentrate bacteria before spotting on the nitrocellulose membrane as in the case of *P. c. phaseolicola* (Schaad et al., 1989).

9.7.2 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) analysis employing specific restriction enzyme has been used to detect and differentiate bacterial pathogens and their strains. Hartung and Civerolo (1989) used the genomic DNA prepared from 21 strains of *Xanthomonas campestris* pv. *citri*, 14 strains of *X. campestris* isolated from Florida citrus nurseries, and 10 strains of five other pathovars of

X. campestris for RFLP analysis. A significant separation between the strains of *X. campestris* and *X. c.* pv. *citri* was observed, indicating that *X. campestris* that was prevalent in citrus nurseries was not a form or strain of *X. c.* pv. *citri.*

A simplified method for RFLP analysis of *X. oryzae* pv. *oryzae* was developed by Raymundo and Nelson (as cited by Leach and White, 1991). Digestion of bacterial DNA with the restriction enzyme Pst 1 resulted in only a few high-molecular-weight DNA fragments which formed distinct patterns for different strains on electrophoresis. The RFLP patterns could be recognized without Southern blot analysis. Most of the RFLP patterns revealed by digestion with Pst 1 were found to be specific for a group of strains within a single race. The strains of race 2, commonly prevalent in the Philippines, could be distinguished by RFLP analysis. The strains of *X. oryzae* pv. *oryzae* found in the United States are considered to be not closely related to the Asian strains because of the differences in RFLP patterns, and hence it is suggested that the bacterium that causes rice leaf blight may be a distinct pathovar of *X. oryzae* (Leach and White, 1991).

9.7.3 Polymerase Chain Reaction

When the target bacterium is contaminated with saprophytic bacteria, it will be difficult to detect by dot-blot hyrbidization. Employing a PCR-based assay will be useful under such condition in addition to enhancing the sensitivity of the detection. For the detection of *P. s. phaseolicola*, a PCR-based assay using the primers from DNA sequences of the phaseolotoxin gene was employed. The assay was not affected by the presence of a high population of nontarget bacteria, and it was highly sensitive in detecting the target bacteria at 1–5 cfu/ml of seed-soak wash (Prossen et al., 1991). *Xanthomonas campestris* pv. *phaseoli* was detected by using primers from plasmid DNA in PCR assay. As little as 10–100 fg of *X. c. phaseoli* DNA (1–10 CFU) was sufficient for detection of this bacterial pathogen (Audy et al., 1994). The PCR assay could detect 34 *Agrobacterium tumefaciens* strains of *Vitis* spp. reliably, and in most cases the PCR results confirmed the results of pathogenicity tests and DNA-slot blot hybridization (Dong et al., 1992).

The DNA probes generated from the fragment obtained after digestion of genomic DNA of *Xylella fastidiosa* (Grape Pierce's disease bacterium) and amplification by PCR were used to detect the bacterium in grape petiole and citrus stem tissues. Detection of the bacterial pathogen by PCR was 100 times more sensitive than by ELISA; the limits of detection were 1×10^2 cfu/ml for PCR and 2×10^4 cfu/ml for ELISA. The two pathotypes infecting grape and citrus could be differentiated by PCR (Minsavage et al., 1994). Maki-Valkama and Karjalainen (1994) reported that *Erwinia carotovora* subsp. *atroseptica* and *carotovora* could be differentiated by the RAPD-PCR technique.

By using PCR based on primers derived from the *pat-1* region of the plasmid (involved in pathogenicity), virulent strains of *Clavibacter michiganensis* subsp. *michiganensis* could be detected in homogenates of infected tomato plants and contaminated seeds (Dreier et al., 1995).

Li et al. (1995) reported that a pair of PCR primers (Sp1f and Sp5r) specifically amplified a 215 bp fragment of genomic DNA of *Clavibacter michiganensis* subsp. *sepedonicus*, causal agent of bacterial ring rot disease of potato, but did not amplify DNA from phenotypically and serologically related bacteria isolated from potato stem or tubers. Comparison of sensitivity of detection of PCR, ELISA, and immunofluorescence (IF) using monoclonal antibodies (MABs) shows that detection of the bacterial pathogen by PCR was more sensitive than that by the other two assay methods. Tubers from ring rot–infected plants which had negative results in ELISA and IF tests, gave positive results in PCR (Table 9.4). De Boer and Ward (1995) showed that by using primers capable of specifically amplifying the fragment of genomic DNA of *Erwinia carotovora* subsp. *atroseptica*, the pathogen could be reliably detected in potato stem and tuber tissues. The PCR method was more sensitive than ELISA employing MABs (Table 9.5). Since infected seed potatoes are the primary sources of infection, detection of low levels of bacterial pathogens in tubers is essentially required for elimination of infected tubers. Hence PCR, with higher sensitivity of detection, can play an important role in disease management programs.

Schaad et al. (1995) developed the BIO-PCR technique, which combines biological and enzymatic amplification of PCR targets. Bean seeds are soaked overnight, and the aqueous extract is plated onto a general agar medium and incubated for 45–48 hr. The plates are then washed with water to collect the bacterial cells, and aliquots of the washings are subjected to two consecutive cycles of PCR without prior DNA extraction using nested pairs of primers required to amplify the *tox* (phaseolotoxin) gene region of *Pseudomonas syringae* pv. *phaseolicola*. The BIO-PCR has several advantages over other PCR techniques. There is no need for DNA extraction from a test organism before amplification, and false-positive results due to the presence of dead bacterial cells and false-negative results due to the presence of PCR inhibitor in seed can be eliminated.

SUMMARY

Nucleic acid–based techniques are used to determine the nucleotide sequence similarities between related species, races, strains, or biotypes of pathogens. Closely related organisms show greater nucleotide sequence similarity. A highly specific nucleotide sequence present in a strain or isolate of a pathogen may be

Table 9.4 Comparative Sensitivity of Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by ELISA, IF, and PCR Assays

Sample No.	Symptom rating[a]	ELISA (OD at 405 nm)	IF[c]	PCR[d]
1	1	0.059 (-)[b]	0.7 (-)	++
2	2	0.054 (-)	1.2 (-)	+
3	3	0.040 (-)	~40	+
4	1	0.047 (-)	~40	+
5	1	0.244	> 50	+++
6	3	0.287	> 50	+++
7	2	0.253	> 50	+++
8	2	0.098 (-)	> 50	+++
9	1	0.082 (-)	8.0	+++
10	2	0.172	-40	+++
11	2	0.261	> 50	+++
12	2	0.275	> 50	+++
13	1	0.103	7.0	+
14	2	0.052 (-)	8.0	+
15	1	0.040 (-)	9.0	+
16	4	0.272	> 50	+++
17	3	0.097 (-)	7.0	++
18	3	0.198	> 50	+++
19	1	0.080 (-)	6.0	++
20	3	0.318	> 50	+++
21	1	0.093 (-)	1.8 (-)	+
22	1	0.066 (-)	1.0 (-)	+
23	1	0.063 (-)	0.6 (-)	+
24	1	0.045 (-)	1.0 (-)	+

[a]Symptom rating: 1—no symptom; 2—slight symptom, 3—well developed symptom; 4—rot.
[b]Tests negative.
[c]Average number of fluorescing cells/microscopic field.
[d]Intensity of bands in ethidium bromide stained agarose gel; + weak; ++ moderate and +++ strong band.
Source: Li et al. (1995).

used to detect and differentiate that particular strain or isolate present in infected plant material and rapidly establish its identity. Interspecific and intraspecific variations can be assessed by using appropriate probes. Chromosomal and extrachromosomal nucleic acid sequences have been used for the preparation of probes. Radioactive and nonradioactive materials have been employed for labeling the probes. Nucleic acid hybridization methods are particularly useful for the detection of viroids. Dot-blot hybridization, restriction fragment length

Table 9.5 Comparison of ELISA and PCR for Detection of *Erwinia cartovora* subsp. *atroseptica* in Potato Tissues

Potato tissue	No. of samples tested	ELISA	No. of samples positive by PCR
Stem			
Symptomatic	25	+	25
Asymptomatic	8	+	6
Seed tubers	52	-	11
	6	+	2
	14	-	2
Progeny tubers			
Symptomatic	5	+	5
Asymptomatic	35	+	32
	25	-	3

Source: De Boer and Ward (1995).

polymorphism, polymerase chain reaction, and random amplified polymorphic DNA are the techniques that are being employed for the detection, differentiation, and quantification of plant pathogens in seeds, seed materials, soil, and water. The usefulness of these techniques in seed certification programs and epidemiological studies intended to monitor the distribution and spread of the pathogens is discussed.

APPENDIX 9(i): METHOD OF RAPID PREPARATION OF INFECTED PLANT TISSUE EXTRACTS FOR PCR AMPLIFICATION OF VIRUS, VIROID, AND MLO NUCLEIC ACID (LEVY ET AL., 1994)

A. Sample preparation (per Gene Releaser [GR] manufacturer recommendations)
   i. Cut out leaf disks (about 30 mg) by using an inverted pipette tip (larger end) and transfer them to 1.5 ml microfuge tubes.
   ii. For testing of anthers (for the presence of viroid) place 10–20 anthers into a microfuge tube.
   iii. For testing of the phloem tissue for the presence of MLOs/viruses, excise 2–4.5 mm long leaf midrib sections and place them into microfuge tubes.
   iv. Grind the tissues with a disposable pestle in 100 µl/sample of ice-cold TE buffer containing 10 m*M* Tris-HCl; 1 m*M* EDTA, pH 8.0, and 0.4 mg of 120 grit carborundum/µl of buffer.

v. For MLOs, alternatively, grind the tissues in MLO grinding buffer consisting of 95 m*M* K_2HPO_4, 30 m*M* KH_2PO_4, 10% sucrose, 0.15% bovine serum albumin fraction V, 2% polyvinylpyrolidone (PVP-10), and 0.53% ascorbic acid, pH 7.6, with carborundum or liquid N; then grind in MLO buffer without carborundum.

vi. Centrifuge at 12,000 rpm at 4°C for 1–2 min for virus samples; centrifuge for 10–45 sec or gravity-settle for MLO samples, and transfer the supernatant to sterile 1.5 ml microfuge tubes and place them on ice.

vii. Dispense 1 µl (for viruses and viroids) and 2 µl (for MLOs) aliquot of each sample in thin-walled PCR tubes containing 20 µl (for MLOs) and 23 µl (for virus and viroid samples of freshly resuspended gene releaser GR).

viii. Gently vortex GR–tissue extract mixtures at low speed for 30 sec and place them on ice till all samples are prepared.

ix. Place the samples in a microwave safe rack, overlay with 50 µl of mineral oil, close the lid, and microwave at the high-power setting for the required period (heating time multiplied by the oven power rating [watts] to be equal to 4500 watt-minutes).

B. Reverse-transcription–polymerase chain reaction (RT-PCR) amplification

i. Take out 10–20 µl aliquot of GR matrix containing the sample immediately after microwaving; add to a primer annealing reaction mixture containing 6 µl of 5 X reverse transcriptase buffer (consisting of 250 m*M* Tris-HCl, pH 8.3; 375 m*M* KCl; and 15 m*M* $MgCl_2$); 3 µl of 0.1 *M* dithiothreitol [DTT), 1 µl complementary primer, and sterile water to yield a final volume of 30 µl.

ii. Vortex the sample–primer mixture briefly and denature by heating at 100°C for 5 min; chilling on ice for 2 min; and annealing the primers at room temperature for 30–45 min.

iii. Add the annealed reaction mixture to 20 µl of a cDNA reaction mixture consisting of 4 µl 5 X reverse transcriptase buffer, 2 µl 0.1 *M* DTT, 1 µl RNasin (40 units), 5 µl of 0.3 *M* 2-mercaptoethanol, 2.5 µl 10 m*M* dNTPs (2.5 m*M* each dGTP, dATP, dTTP, dCTP), and either 1 or 2 µl of Maloney murine leukemia virus (MMLV) reverse transcriptase (200 U/µl) for virus and viroid, respectively.

iv. Vortex the reaction mixture and incubate at 42°C for 1–1 1/2 hr.

v. Carry out amplification in thin-walled PCR tubes containing the reaction mixture consisting of 5 µl of 10 X PCR buffer (10 m*M* Tris-HCl, pH 8.3; 50 m*M* KCl; and 0.001% gelatin), 3 µl of 25 m*M* $MgCl_2$ (1.5 m*M* final concentration), 1 µl of 10 m*M* dNTPs, 1 µl each of 6 µ*M* complementary and homologous DNA primer, 2.5 units of DNA *Taq* polymerase, and sterile water to produce a vol-

ume of 45 μl. Overlay the mixture with 75 μl of mineral oil and place at 85°C in a DNA thermocycler for 5 min.

vi. Add 5 μl of GR-cDNA mixture and amplify through the following steps: denaturation at 94°C for 30 sec, primer annealing at 62°C for 30 sec, and extension at 72°C for 45 sec for 30 cycles with a final extension at 72°C for 7 min.

C. Analysis of PCR products by electrophoresis

i. Electrophorese PCR products in 5% native polyacrylamide gels of 150 V for 1.5 hr in IX TBE (containing 89 m*M* Tris, 89 m*M* borate, and 2.5 m*M* Na_2EDTA, pH 8.3) and stain the gels with silver nitrate in the case of viruses and viroids; electrophorese in 1.2% agarose gels and stain with ethidium bromide and observe under ultraviolet (UV) illumination.

APPENDIX 9(ii): ONE TUBE PCR AMPLIFICATION PROCEDURE (WETZEL ET AL., 1991)

i. Grind the infected leaf tissue in a plastic bag containing gauze along with sterile water added at the rate of 1:4 (w/v) in a rolling grinder; centrifuge the sap for 10 min in a microfuge at full speed; dilute the supernatant with sterile water (10-fold).

ii. Disrupt the virus particles in 10 μl aliquots of diluted extract by treatment with 1% Triton X-100 for 10 min at 65°C.

iii. Incubate the target nucleic acid with methyl mercury hydroxide (10 m*M* final concentration) for denaturation at room temperature for 10 min; neutralize with 20 *mM* 2-mercapto-ethanol for 10 min at room temperature.

iv. Incubate the denatured target nucleic acid with reverse transcription mixture containing 50 m*M* Tris-HCl, pH 8.3; 50 m*M* KCl; 7.5 m*M* $MgCl_2$; 0.1 V Inhibit ACE (5′3′ Inc); 20 μ*M* dNTPs; 0.1 μ*M* primer; and 0.5 U avian myeloblastoses virus reverse transcriptase in the tube to yield a final volume of 20 μl for 45 min at 42°C.

v. Add 80 μl PCR buffer (10 m*M* Tris-HCl, pH 8.3; 50 *mM* KCl; 1.5 m*M* $MgCl_2$; 0.1% gelatin) containing 1 μ*M* of both primers, 200 μm dNTPs, and 2 V of *Taq* DNA polymerase; overlay with 100 μl of mineral oil; amplify for 40 cycles of template denaturation at 92°C (1 min), primer annealing at 62°C (2 min), and DNA synthesis at 72°C (2 min).

vi. Analyze reactions by electrophoresis of the reaction mixture; observe bands by either ethidium bromide or silver staining.

APPENDIX 9(iii): PCR-MICROPLATE HYBRIDIZATION METHOD (HATAYA ET AL., 1994)

A. Preparation of digoxigenin- (DIG)-labeled cDNA probe for the CP coding region of PVY-RNA
   i. Use the cDNA clone pUCYTCP1-19 as a template DNA; carry out PCR in 50 µl reaction mixture containing 10 m*M* Tris-HCl (pH 8.9); 80 m*M* KCl; 1.5 m*M* $MgCl_2$; 0.5 mg/ml BSA; 0.1% sodium cholate; 0.1% Triton X-100; 0.1 m*M* each dGTP, dATP, dCTP; 0.065 mM dTTP; 0.035 m*M* DIG-11-dUTP; 50 pmol each of PCR primers; 20 ng of template DNA, and 1U Tth DNA polymerase.
   ii. Carry out PCR amplification as follows: 30 cycles of 30 sec denaturation at 94°C (5 min for the first cycle), 1 min annealing at 56°C, and 2 min primer extension at 72°C (10 min for the last cycle); place a drop of light mineral oil to prevent evaporation before the commencement of amplification; extract the reaction product with TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) saturated phenol; chloroform (1:1) mixture to remove light mineral oil and BSA; mix the water phase with 1/4 volume of 10 *M* ammonium acetate and 2.5 vol of ethanol and keep at –80°C for 30 min.
   iii. Purify DIG-labeled probe by an Ultra free C3-TTk (Millipore Ltd.) spun column for removing unreacted dNTP, DIG-II-dUTP, and primers; dissolve the DIG-labeled probe in the upper filter cup on 50 µl of TE.

B. Nucleic acid extraction from potato samples
   i. Extract the nucleic acids from leaves (about 1 g) (symptomatic and asymptomatic) by using a metal rod pestle; transfer about 200 µl of sap to a microcentrifuge tube (1.5 ml); immediately add the extraction buffer containing 100 m*M* Tris-HCl (pH 9.0), 2 m*M* EDTA, 1 m*M* sodium diethyl dithiocarbamate (DIECA), 1% sodium dodecylsulfate (SDS), and 0.1 mg/ml bentonite; centrifuge.
   ii. Treat the aqueous phase with TE saturated phenol; chloroform (1:1) mixture; centrifuge.
   iii. Precipitate the nucelic acids in aqueous phase with 0.1 volume of 3 *M* sodium acetate, pH 5.2; and 0.6 volume of isopropyl alcohol; extract the precipitated nucleic acids with TE saturated phenol; chloroform (1:1) mixture; precipitate the total nucleic acids again with 0.1 volume of 3 *M* sodium acetate (pH 5.2) and 2.5 volume of ethanol.
   iv. Dissolve the nucleic acids in 50 µl of distilled water; mix with 50 µl of 4 *M* LiCl; keep on ice overnight.
   v. Dissolve the precipitated RNAs in 400 µl of distilled water; use samples of 4 µl of RNA for RT-PCR.

C. Amplification of cDNA to viral RNA sequences by RT-PCR
   i. Incubate the reverse transcription (RT) reaction mixture (20 μl) containing 0.5 μg of extracted viral RNA, 0.5 μg of oligo $(dT)_{12-18}$ primer, 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 10 m*M* DTT, 3 m*M* $MgCl_2$, 0.5 m*M* each dNTP and 100 UM-MLV RTase, at 37°C for 60 min.
   ii. Add one half of the reaction mixture (10 μl) to 40 μl of a PCR premixture containing 75 m*M* Tris-HCl, pH 8.9; 81.25 m*M* KCl; 1.125 m*M* $MgCl_2$; 0.375 mg/ml bovine serum albumin (BSA); 0.075% sodium cholate; 0.075% Triton X-100; 50 pmol each of plus and minus sense primer; 187.5 μm each of dNTP and IU of Tth DNA polymerase.
   iii. Place a drop of light mineral oil on the PCR reaction mixture to prevent evaporation; proceed through 30 cycles of 30 sec denaturation at 94°C (5 min for the first cycle), 1 min annealing at 56°C, and 2 min primer extension at 72°C (10 min for the last cycle) by using a Temp Control System PC-700 program.
   iv. Extract the reaction product with TE saturated phenol/chloroform (1:1) mixture to remove light mineral oil and BSA; mix the water phase with 1/4 volume of 10 m*M* ammonium acetate and 2.5 volume of ethanol; keep at -80°C for 30 min; centrifuge.
   v. Dry the precipitate in a vacuum and dissolve in 50 μl of TE.

D. Microplate hybridization
   i. Denature the amplified cDNA fragments at 100°C for 5 min and quickly chill in ice water; dilute 5-fold serially with different dilutions of standard saline citrate (SSC) (1xSSC:0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0) containing 10 *mM* EDTA.
   ii. Dispense 100 μl of diluted DNA into wells of a 96 well polystyrene microplate; incubate at 37°C for 2 hr and wash three times with phosphate-buffered saline (PBS) solution containing 137 m*M* NaCl, 8.1 *mM* Na_2HPO_4, 1.47 m*M* KH_2PO_4, 2.7 m*M* KCl, pH 7.4, containing 0.05% Tween 20 (PBS-T).
   iii. Transfer to each well 100 μl of hybridization solution containing the heat-denatured DIG-labeled probe (1 μl/ml: 1000–fold dilution); 50% formamide; 5X SSC; 10 m*M* EDTA, pH 7.0; 0.1% Tween 20; and 100 μg/ml yeast tRNA; seal the plates with adhesive tape; incubate at 42°C for 12 hr; wash three times with PBS-T.
   iv. Dispense 100 μl of alkaline phosphatase–conjugated anti-DIG antibody diluted 5000-fold with PBS-T; incubate at 37°C for 1 hr; wash three times with PBS-T.
   v. Dispense 200 μl of *P*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) as substrate; incubate at room temperature for

1–2 hr; determine absorbance values at 415 nm by using a microplate reader.

APPENDIX 9(iv): COMBINATION OF IMMUNOCAPTURE AND PCR AMPLIFICATION IN MICROTITER PLATE FOR DETECTION OF VIRUSES (NOLASCO ET AL., 1993)

A. Immunocapture of viruses from plant tissues
   i. Grind infected plant tissues (1:10 w/v) in 500 m*M* Tris-HCl, pH 8.2, containing 2% PVP-40, 1% PEG 6000, 140 m*M* NaCl, 0.05% Tween 20, and 3 m*M* NaN_3; centrifuge at 5000 g for 5 min.
   ii. Dispense 50 μl of the supernatant to each well of the microtiter plate, already coated with antibody specific to the virus to be detected by the method of Clark and Adams (1977); incubate overnight at 4°C.
   iii. Wash the wells three times by flooding with PBS-Tween, taking care to prevent cross-contamination between wells (see Appendix 8 [i]).

B. RT-PCR amplification
   i. Perform an RT reaction in the microtiter plate by adding to each well 20 μl of the reverse transcription mixture containing 50 m*M* Tris-HCl, pH 8.3; 75 m*M* KCl; 3 m*M* $MgCl_2$; 1 m*M* each dNTP; 25 units of ribonuclease inhibitor; 1 μ*M* downstream primer; 200 units of M-MLV reverse transcriptase (BRL); incubate at 37°C for 1 hr.
   ii. Carry out amplification of cDNA in the same wells (using a Techno PHC-3 thermocycler with a microtiter plate adaptor) by adding 80 μl of amplification mixture consisting of 60 m*M* Tris-HCl, pH 9.0; 15 m*M* KCl; 2.1 m*M* $MgCl_2$; 20 m*M* $(NH_4)_2SO_4$; 0.2 m*M* each dNTP; 0.2 μ*M* each primer; 0.005% BSA; overlay with a drop of mineral oil.
   iii. Heat the mixture at 94°C for 2 min; cool to 72°C and add 1.6 units of thermostable DNA polymerase.
   iv. Proceed through 30–35 cycles, each cycle consisting of an annealing step of 1 min at 52°C, an elongation step of 1 min at 72°C, a denaturation step of 30 sec at 93°C, and an elongation step of 5 min for the final cycle.

C. Detection of amplified DNA products by Southern hybridization
   i. Transfer the DNAs to nylon membranes (Hybond N^+, Amersham Inc.) using the alkaline transfer procedure; soak the membranes in sodium citrate (SSC) (20 × SSC = 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0); hybridize to the probes labeled with digoxigenin.

APPENDIX 9(v): DETECTION OF MLO IN INFECTED PLANT WITH DNA PROBES (LEE AND DAVIS, 1988)

i. Excise 0.3 g of leaf midrib or young shoot and grind in liquid nitrogen; transfer to a microcentrifuge tube containing 0.4 ml of extraction buffer (0.1 *M* Tris, pH 8.0; 0.5 *M* EDTA; 0.5 *M* NaCl; 0.5% 2-mercaptoethanol; 0.5% SDS), crush the sample with a minipestle; centrifuge for 10 min at 2000 rpm.
ii. Transfer the supernatant to another clean microcentrifuge tube; centrifuge the sediment again at 8000 rpm for 10 min; combine the supernatant with the one obtained earlier.
iii. Heat the supernatant at 65°C for 5 min; centrifuge at 14,000 rpm for 5 min; transfer the supernatant to another clean microcentrifuge tube.
iv. Extract with 200 µl phenol and 200 µl chloroform isoamyl alcohol (24:1); centrifuge at 14,000 rpm for 5 min; retain the aqueous phase.
v. Denature DNA and transfer to nitrocellulose membrane in a dot-blot apparatus; bake the membrane for 2 hr at 80°C.
vi. Hybridize with DNA probe as for Southern hybridizations.

APPENDIX 9(vi): DETECTION OF MLOS IN INOCULATIVE INSECTS WITH DNA PROBES (KIRKPATRICK ET AL., 1987)

i. Crush the leafhoppers (frozen at –20°C) on a moistened nitrocellulose membrane; place the membrane with crushed leafhoppers on filter paper moistened with 0.3 *M* NaOH; incubate for 3 min.
ii. Transfer the nitrocellulose membrane to a second filter paper soaked in 0.3 *M* NaOH; incubate again for 3 min.
iii. Transfer the membrane sequentially to a pair of filter papers soaked with 1 *M* Tris-HCl, pH 8.0; incubate for 3 min, then to pairs of filter papers soaked with 0.5 *M* Tris-HCl, pH 8.0, and 15 *M* NaCl; remove the insect debris.
iv. Dry the nitrocellulose membrane; bake at 80°C for 2 hr.
v. Hybridize with a DNA probe as for Southern hybridization.

APPENDIX 9(vii): IDENTIFICATION OF FUNGAL PATHOGENS BY REPETITIVE DNA POLYMORPHISM (PANABIERES ET AL., 1989)

A. Preparation of fungal DNA
   i. Grow the fungal pathogen in an appropriate medium; harvest the

cultures by filtration on filter paper under a vacuum; rinse the mycelia in 250 ml of distilled water; store by freezing.

ii. Grind the frozen mycelium (250 mg) in liquid nitrogen; suspend the fungal powder in 0.5 ml of NIB buffer containing 100 m*M* NaCl; 30 m*M* Tris-HCl, pH 8.0; 10 m*M* EDTA; 10 m*M* β-mercaptoethanol; 0.5% (v/v) NP-40; centrifuge for 1 min at 12,000 g.

iii. Resuspend the pellet in NIB buffer; repeat the procedure in (ii) above; resuspend the pellet in 0.8 ml of homogenization buffer consisting of 0.1 *M* NaCl, 0.2 *M* sucrose, and 10 *mM* EDTA; add 0.2 ml of lysis buffer containing 0.25 *M* EDTA; 0.5 *M* Tris, pH 9.2; and 2.5% sodium dodecyl sulfate; incubate at 55°C for 30 min.

iv. Extract twice with 1 volume of phenol-chloroform-isoamyl alcohol (50:48:2); then twice with 1 volume of ether.

v. Add 1 volume of ethanol; centrifuge for 1 min in a microcentrifuge at room temperature; collect the DNA as a pellet.

vi. Wash the pellet with 70% ethanol; centrifuge again; resuspend in 50 μl of TE (10 m*M* Tris, pH 8.0; 1 m*M* EDTA); store at -20°C.

B. Digestion of DNA and electrophoresis analysis

i. Digest 5 μg of total DNA overnight with 20 units of restriction enzyme (per manufacturer's recommendations).

ii. Separate DNA fragments on 1% agarose gels at 5 v/cm in 90 m*M* Tris borate buffer, pH 8.3.

iii. Stain the gels with ethidium bromide; view under UV light.

APPENDIX 9(viii): DETECTION OF FUNGAL PATHOGENS WITH DNA PROBES (TISSERAT ET AL., 1991)

i. Place 200–400 mg of infected plant tissue in a 1.5 ml microfuge tube; freeze by adding liquid nitrogen; grind with a smooth-lipped steel rod.

ii. Suspend the ground samples in 600 μl 2 X CTAB buffer (2 X CTAB = 1.4 *M* NaCl, 2% hexadecyltriethylammonium bromide, 1% 2-mercaptoethanol, 10 m*M* Tris-HCl, pH 8.0); extract twice with chloroform.

iii. Precipitate by adding 0.8 volume of isopropanol; resuspend the pelleted DNA in 40 μl TE buffer (TE = 10 m*M* Tris, pH 7.6; 1 m*M* EDTA).

iv. Denature DNA at 95°C for 4 min; transfer 20 μl to a nylon membrane in a slot-blot apparatus; bake at 80°C for 2 hr.

v. Hybridize with DNA as for Southern hybridizations.

APPENDIX 9(ix): IDENTIFICATION OF FUNGAL PATHOGENS BY RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLP) ANALYSIS (UENG ET AL., 1992, 1995)

A. Preparation of fungal genomic DNA
   i. Cultivate the fungal pathogen in an appropriate medium for 7–10 days at room temperature (25°C–28°C) with constant shaking (150 rpm); harvest the mycelia by filtration and centrifugation.
   ii. Resuspend the mycelial pellet in 150 ml of 0.1 *M* EDTA, pH 8.0; stir for 30 min; centrifuge; collect the pellet.
   iii. Grind the mycelium in liquid nitrogen in a mortar with pestle; add 100 ml of lysis buffer containing 50 m*M* Tris-HCl, pH 8.0; 50 m*M* EDTA; pH 8.0; 3% sodium dodecyl sulfate (SDS); 1% 2-mercaptoethanol; heat in a water bath at 65°C for 60 min.
   iv. Add 100 ml of phenol–chloroform mixture (1:1, v/v); stir for 30 min; centrifuge at 16,500 relative centrifugal force (RCF) for 10 min.
   v. Retain the upper phase and reextract with phenol/chloroform again; precipitate the DNA by adding 0.1 volume of 3.0 *M* sodium acetate, pH 7.0, and 2.5 volume of absolute alcohol.
   vi. Transfer the DNA by using a sterile pipette to a centrifuge tube; wash once with 70% ethanol; drain to dry for a brief period.
   vii. Add 15 ml of 1X TE buffer containing 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 to the pellet; shake gently, heat intermittently at 68°C; add 1X TE buffer, if required, to dissolve the pellet.
   viii. Add 8.6 g of cesium chloride (CsCl) and 0.3 ml of ethidium bromide (5 mg/ml) at the rate of 7.5 ml of DNA solution; centrifuge at 157, 516 RCF for 15 hr in a vertical rotor.
   ix. Collect the DNA band (distinct from the brownish polysaccharide band); extract with CsCl-saturated 1-butanol.
   x. Precipitate the genomic DNA with 3 volumes of water and 4 volumes of isopropanol; wash with 70% ethanol; vaccum dry; resuspend the DNA in 1X TE buffer.

B. DNA hybridization
   i. Cleave the genomic DNA with *Eco* RI (15 units) at 37°C overnight; fractionate DNA fragments by 1% agarose gel electrophoresis at a constant voltage of 25 V, after pretreatment of gel with 0.25 *N* HCl for 15 min, 1.5 *M* NaCl/0.5 *N* NaOH for 25 min, and 1.5 *M* NaCl/0.5 *M* Tris-HCl, pH 7.4, for 25 min, successively.
   ii. Transfer DNA fragments to membranes (Nytran) (pore size 0.45 μ*M*) using X 10 SSC buffer consisting of 1.5 *M* NaCl, 0.15 *M* sodium citrate, pH 7.0.

iii. Bake the blots in a vacuum at 80°C for 2 hr; use the appropriate probe for hybridization.

APPENDIX 9(x): RANDOM AMPLIFIED POLYMORPHIC DNA PROCEDURE FOR THE DIFFERENTIATION OF STRAINS OF FUNGI (ZIMAND ET AL., 1994)

A. Preparation of genomic DNA
   i. Grow the test fungus in appropriate medium for 5–7 days; harvest the mycelium; freeze-dry overnight.
   ii. Grind the mycelium (50 mg) in an Eppendorf tube; suspend in 500 µl of extraction buffer containing 200 m*M* Tris-HCl, pH 8.5; 250 m*M* NaCl; 2.5 m*M* EDTA; 0.5% sodium dodecyl sulfate (SDS).
   iii. Mix the slurry in 350 µl of phenol (melted at 45°C and equilibrated with 1 volume of extraction buffer); then add 150 µl chloroform; mix well; centrifuge at 13,000 g for 1 hr.
   iv. Transfer the upper aqueous phase to a tube containing 25 µl RNase A solution (20 mg/ml in 10 m*M* Tris-HCl, pH 7.5; 15 m*M* NaCl); boil for 10 min; incubate for 10 min at 37°C.
   v. Add 0.7 *M* NaCl and 0.1 volume of cetyltrimethyl ethyl ammonium bromide (CTAB) to remove the polysaccharides; extract with chloroform/isoamyl alcohol (12:1); remove the upper phase; repeat CTAB extraction till no interface is seen.
   vi. Transfer the clean upper phase to an Eppendorf tube; mix with 0.54 volume of isopropanol; DNA precipitates as a lump; remove as much of the supernatant as possible.
   vii. Centrifuge for 5 sec; remove the supernatant with a pipette; rinse the pellet with 70% ethanol; dry under a vacuum; resuspend in 100 µl of 10 m*M* Tris-HCl, pH 8.0, and EDTA.

B. PCR amplification
   i. Perform amplification in 25 µl containing 1–1.5 units of *Taq* DNA polymerase; 0.5 µl each of dCTP, dGTP, dATP, and dTTP (1.25 m*M*); in 10 m*M* Tris-HCl, pH 9.0; 50 m*M* KCl; and 0.1% Triton X-100; 1.9 µl of $MgCl_2$ (25 m*M*); 0.5 µl of (10 µ*M*) primer and 25–50 ng of DNA; overlay the reaction mixture with mineral oil in a Thermo Cycler through one cycle of denaturation at 94°C for 7 min; perform low stringency annealing of the primer at 35°C for 1 min and extension at 72°C for 1 min.
   ii. Run additional 40 cycles with following steps: 90°C for 1 min, 35°C for 1 min, and 72°C for 2 min, final cycle at 72°C for 5 min.

iii. Electrophorese the PCR products in 1.2% agarose gels; stain with ethidium bromide for detection; use size markers obtained by digesting lambda DNA with *Pst* 1.

APPENDIX 9(xi): PCR AMPLIFICATION OF BACTERIAL DNA FOR IDENTIFICATION OF BACTERIAL PATHOGENS (COTTYN ET AL., 1994)

A. Preparation of bacterial DNA
   i. Grow the bacteria in nutrient broth to the early log phase; harvest the cells by centrifugation at 12,000 rpm for 10 min; resuspend the pellet in 2 ml of Tris-HCl, pH 8.3, and 1.0 m*M* EDTA (1X TE).
   ii. Add 250 µl of 10% sodium dodecyl sulfate (SDS) and 50 µl of 10 mg/ml of proteinase K; incubate at 37°C for 1 hr with gentle shaking.
   iii. Add 0.45 ml of 5 *M* NaCl and mix thoroughly; add 0.4 ml of hexadecyl trimethyl ammonium bromide (CTAB) solution (10% CTAB in 0.7 *M* NaCl); incubate at 65°C for 20 min.
   iv. Add an equal volume of chloroform:isoamyl alcohol (24:1); shake the mixture gently for 30 min; centrifuge at 15,000 rpm for 30 min.
   v. Transfer the upper aqueous phase by using a bent Pasteur pipette to a fresh tube containing an equal volume of cold isopropanol; gently shake till completion of DNA precipitation.
   vi. Take out the DNA with a Pasteur pipette; wash in 70% ethanol; dissolve in 1X TE; store at -20°C.

B. PCR amplification and analysis of PCR products
   i. Prepare the reaction mixture consisting of 20 ng of DNA template in a 50 µl reaction volume containing 10 m*M* Tris-HCl, pH 8.3; 50 m*M* KCl, 200 *M* each of dNTP; 0.4 *M* each of primer; and 1.5 units of *Taq* polymerase.
   ii. Carry out amplification on a DNA Thermocycler (Perkin Elmer Cetus) with an initial denaturation step at 94°C for 2 min followed by 40 cycles of the denaturation step at 94°C for 1.5 min, a primer annealing step at 62°C for 2 min, and an extension step at 72°C for 2 min with an additional extension step of 72°C for 5 min as the last cycle.
   iii. Perform gel electrophoresis on 2% agarose in Tris-borate EDTA at 2 V/cm; stain with ethidium bromide.

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Detection of Double-Stranded RNAs

Infected plants may have double-stranded-(ds)-RNAs when a) the infecting viral genome is ds-RNA, as in phytoreoviruses and cryptoviruses, or b) ds-RNA is produced as a replicating form during the process of replication of single-stranded (ss)-RNA viruses. In the case of certain viruses, such as velvet tobacco mottle virus, ds-RNA may accumulate during replication. This technique of detecting the ds-RNA is useful for the early and rapid recognition of virus infection. The presence of ds-RNA can be detected by either polyacrylamide gel electrophoresis (PAGE) or antiserum reaction with ds-RNA. The quantity of ds-RNA obtained may vary with host–virus combinations (Valverde et al., 1986). The ds-RNA may be isolated and labeled or cloned as cDNA for developing nucleic acid probes.

Labeled ds-RNA or cDNA probes may be used to detect RNA viruses (Bar-Joseph et al., 1983; Jordan, 1986). It is possible to detect infection by viruses which reach only very low concentrations or are difficult to purify (Chu et al., 1989) and before symptom expression (Lejour and Kummert, 1986). Groundnut rosette virus (GRV) infection was detected by the presence of the ds-RNA even when the assistor virus was absent. As the antiserum against GRV is not available, detection of ds-RNA will be helpful for diagnosing GRV infection (Breyel et al., 1988). Latent infection by plant viruses can also be recognized by detecting the ds-RNA of the viruses. Habili (1993) reported that widespread latent infection of citrus with tristeza virus in Australia could be detected by ds-RNA analysis. Saldarelli et al. (1994) employed two ^{32}P-labeled cDNA clones specific for ds-RNA sequences as probes for detecting grapevine leaf roll–associated closterovirus III (GLRa V III) in grapevine extracts from leaves, petioles, or cortical tissues infected by the virus. The probes were virus-specific and did not hybridize with total RNA from healthy controls or from vines infected by other viruses.

The ds-RNA profile may help in the classification of an unknown virus or virus strain. On the basis of the characteristics of double stranded forms of subgenomic mRNAs, strains of the viruses may be identified (Valverde et al., 1986). By determining ds-RNA profiles of 26 cucumber mosaic cucumovirus isolates, 7 distinct ds-RNA profile types can be identified. The differences between the profiles are stable and hence may be used as the basis of classifying CMV isolates. They reflect a greater range of biological characteristics than other methods currently available (Pares et al., 1992). Additionally, ds-RNA analysis may help to detect the presence of infection complexes such as satellite RNA, multiple infections, and cryptic virus (Lejour and Kummert, 1986).

The antiserum prepared against polyinosinic–polycytidylic acid (In–Cn) may be used to detect the ds-RNA of plant viruses by indirect enzyme-linked immunosorbent assay (ELISA-I). The ds-RNA from cucumber mosaic cucumovirus (CMV) and plum pox potyvirus (PPV) from infected plants were obtained by heating the leaf extracts at 80°C for 2 min and maintaining a pH of 6. The ds-RNA of PPV from *Nicotiana benthamiana* plants could be detected readily at 50 days after inculation (Aramburu and Moreno, 1994).

There are, however, certain limitations that prevent the procedure from being adopted widely. The presence of non-pathogenesis-related ds-RNA in healthy controls, lower level of sensitivity than bioassay or electron microscopy, and laborious nucleic acid purification methods prevent the wider use of ds-RNAs as an aid for virus detection and classification (Chu et al., 1989).

SUMMARY

Plants infected by plant viruses containing RNA as a genome may have ds-RNA, because some viruses have ds-RNA as their genome, or a ds-RNA may be produced as a replicating form during the process of viral nucleic acid synthesis. Detection of this ds-RNA by ds-RNA or cDNA probes is possible. The ds-RNA profile is useful for the classification of unknown viruses or virus strains.

11
Diagnosis and Monitoring of Plant Diseases

In the past, diagnosis of different diseases affecting crop plants was considered a form of art, and disease was disgnosed by an intuitive judgment as to its nature after examination of the visible symptoms of the disease concerned. Later, using determinations based on biochemical and physiological studies of the mechanisms of infection or the metabolic characteristics of pathogens, the diseases were diagnosed (McIntyre and Sands, 1977). It is now well recognized that the techniques for pathogen detection and diagnosis of diseases caused by them are critical factors to be considered for developing strategies for effective crop management and regulatory programs. The techniques are also required for determining the cause, epidemiological characteristics, and distribution of diseases in a geographical location.

During the past two decades remarkable advances in the fields of molecular biology and immunology have been responsible for significant improvements in the accuracy, rapidity, and sensitivity of diagnostic techniques. The diagnostic methods also enhance the effectiveness of quarantine and control measures, resulting in saving of millions of dollars (Leach and White, 1992).

Crop disease diagnosis is required essentially to recognize the primary disease-causing factor(s). The general procedure followed involves examination of symptoms, microscopic examination of diseased tissue, isolation and purification of the pathogen, and inoculation on the appropriate host to induce the disease for comparison of the symptoms with those observed on the plant submitted for diagnosis. Pathogens may then be grouped taxonomically on the basis of their morphological, physiological, or biochemical characteristics. If the pathogen occurs in the form of physiological races, a set of differential varieties of plant species has to be inoculated to identify the pathogen race or biotype. But this procedure is frequently found to be tedious and impractical when early diagnosis is desired.

The diagnosis of the disease without the requirement of isolation of the pathogen and subsequent adoption of Koch's postulates became the imperative need. The molecular diagnosis of plant diseases based on the immunological properties and genomic characteristics of plant pathogens is being increasingly preferred by diagnosticians, since the pathogens can be reliably identified very rapidly and suitable management decisions can be made at the appropriate time. Immunochemical assays employing polyclonal antibodies were the first molecular techniques tested for the detection of pathogens. Polyclonal antibodies have been found to be very effective for detection, identification, and taxonomic classification of viruses by employing immunosorbent electron microscopy or immunosorbent assays (Chapter 7). The use of polyclonal antibodies for bacterial or fungal pathogens is somewhat limited because of lack of specificity at the species, pathovar, or race level. However, the development of hybridoma technology leading to production of monoconal antibodies which react specifically with different epitopes helped to overcome some of the problems associated with polyclonal antibodies. Further improvements could be obtained by using nucleic acid–based techniques which can resolve the differences in the nucleic acid sequences which are not involved in the immunological properties of the pathogen. The immunoassays and nucleic acid–based methods provide the level of sensitivity and specificity required by diagnosticians.

The precision, sensitivity, and rapidity of the diagnosis may determine the quality and utility of the diagnostic tool employed. Molecular probes can appreciably reduce the time required for assay and increase the sensitivity of assay, allowing detection of the pathogen before symptom expression. Detection of viruses in presymptomatic plants or in single-vector insects can be achieved by ELISA (Hibino et al., 1988). As the viruses have much simpler structure than bacterial and fungal pathogens, immunological assays are more useful in detection of viral infections. Use of monoclonal antibodies, however, has enhanced the usefulness of immunological assays for the detection of bacterial and fungal pathogens.

11.1 PLANT DISEASE DIAGNOSTIC CENTERS

The need for the establishment of disease diagnostic centers (DDCs) was realized because an organized, systematic, and professional effort is required to assist in the rapid detection of pathogens and accurate identification of plant disease problems to implement suitable solutions. It is also rightly acknowledged that establishment of DDCs will ultimately reduce plant disease losses, both quantitative and qualitative since there can be no effective disease management system without proper diagnosis. Most of the departments of plant pathology

in the agriculltural or traditional universities at the state or province level or advanced research centers at the national level perform crop disease diagnostic services. In some countries, DDCs form part of the state's department of agriculture or are private or commercial services.

The responsibilities and motivation of DDCs may vary, depending on the nature of the agency/institution offering such a service. An extension-university clinic may offer disease diagnosis as a service, usually along with various aspects of the education programs of the cooperative extension service. The state departments of agriculture in the United States have diagnostic laboratories primarily to perform regulatory functions with the aim of identifying pathogens so as to limit or prevent their interstate or intrastate dissemination. Commercial or private laboratories also are engaged in disease diagnosis, mainly on a customized basis, as observed in many North American states (Barnes, 1994).

The capacity to handle diseased specimens may vary with DDCs, depending on the facilities available. They may receive several hundreds of plant disease samples in a year, and the number may go up progressively in future. The increase of over 900% in the number of specimens submitted to the Texas Plant Disease Diagnostic Laboratory over the past 11 years clearly indicates both the need for and acceptance of diagnostic service by the clientale group. Yet, in developing countries adequate facilities and expertise may not be available, and such a deficiency has to be overcome, wherever necessary, for the efficient and useful functioning of DDCs.

Adoption of modern methods will lead to dramatic changes in the sensitivity and reliability of disease diagnosis, increasing the credibility of the agency offering diagnostic service. The enzyme-linked immunosorbent assay (ELISA) and nucleic acid hybridization–based diagnostic procedures have been widely adopted, and these techniques have provided the modern clinics with a continued conduit of state-of-the-art diagnostic techniques. Many clinics in North American states routinely employ highly technical diagnostic procedures, which have attained the level of sophistication that prevails in medical diagnostic facilities, which require careful maintenance of equipment and instruments and professional expertise. Modern clinics also possess powerful computers for maintenance and retrieval of information. Traditional diagnostic methods involving isolating pathogens and culturing them on differential or selective media, inducing fungal sporulation by moist chamber incubation, performing examinations under the light microscope, proving pathogenicity to satisfy Koch's postulates, and inoculating onto differential diagnostic hosts or cultivars for race, strain, or biotype identification, etc., have to be carried out under certain conditions for the interpretation of some disease problems.

The disease diagnostic centers have also been involved in activities associated with disease diagnosis, including disease surveys on a regional or national

basis, host–pathogen indexing, and phytosanitary certificate services. These activities also demand accurate and rapid detection of plant pathogens for diagnosing the various crop diseases without loss of time in taking required plant protection measures. With the realization of the importance and need for effective functioning of DDCs, it was decided to bring out the *Plant Diagnostics Quarterly* (PDQ), which is published by and for diagnosticians and those interested in disease diagnostics.

11.2 PLANT QUARANTINES

After the liberalization of regulations for import of plants and plant materials that followed General Agreement on Tariffs and Trade (GATT) ratification by participating countries, there is a potential risk of introducing several destructive pathogens and pests, especially viruses, viroids, and mycoplasma-like organisms. Implementation of adequate quarantine safeguards has become imperative to prevent the introduction of new pathogens. Establishment of a postentry quarantine facility along with expertise in propagation of plants by tissue culture techniques will lead to large-scale production of pathogen-free plants within short periods.

Various methods are used for the detection of viruses, viroids, and MLOs in vegetatively propagated planting materials. The grow-out test, indicator-inoculation test, indexing method, histopathological test, electron microscopy, and serological tests are employed for the early detection of infection and elimination of infected materials or plants. Effective functioning of postentry quarantines will greatly help to prevent the introduction and subsequent spread of plant pathogens.

SUMMARY

The history of development of plant disease diagnosis and the need for disease monitoring to prevent introduction/incidence and spread of various crop diseases are described. Establishment of disease diagnostic centers (DDCs) will help to detect and differentiate plant pathogens, and these centers can help farmers undertake effective disease management programs.

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