AN INTRODUCTION TO THE DIAGNOSIS OF PLANT DISEASE

Chelston W. D. Brathwaite

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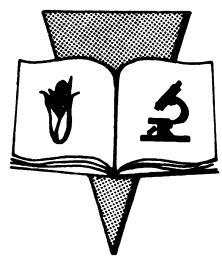

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CONTENT

PREFACE

Plant diseases are undoubtedly one of the major factors which limit the productivity of crops throughout the tropics. In order to control these diseases and so limit their impact on food production, correct diagnosis is important. This publication was prepared to assist students who are being introduced to plant pathology for the first time, to acquire the rudimentary principles and techniques which are required for plant disease diagnosis.

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1

THE DIAGNOSIS OF PLANT DISEASE

Diagnosis of a plant disease is dependent mainly on observations of the symptoms of the disease and the presence of a pathogenic agent in or on diseased tissues.

There are three main symptom types that are characteristic of plant disease.

a. Necrosis or death of infected tissue – e.g. leaf spot of Banana caused by *Mycosphaerella musicola.*

b. Hyperplasia and hypertophy – increase in cell number and size respectively, resulting in galls, tumours or witches' broom, e.g. witches' broom disease of cocoa caused by *Crinipellis perniciosus.*

c. Hypoplasia and hypotrophy – reduced growth and stunting, characteristic of plants attacked by plant parasitic nematodes.

The major agents producing these symptom types are either fungi, bacteria, viruses, mycoplasma, nematodes, insects or so-called physiological disorders or non-parasitic disease agents. The latter include adverse climatic conditions, lack of a particular nutrient, phytotoxic atmospheric pollutants, e.g. sulphur dioxide, fluorine, ozone, etc.

The determination of the causal agent of a disease is usually the first step in attempts to control the disease. Where the disease is common and experience in diagnosis is available, there is no need to isolate the pathogen; however, in some cases the information is not available and isolation may be necessary.

It is sometimes felt that diagnosis is a simple matter of looking at the plant and immediately the disease is known and complete recommendations can be given for control. But this is not like the medical doctor/patient situation. Plants cannot talk, and consequently, the approach to diagnosis must be cautious. A tomato

plant which wilts in the field on a sunny day may be affected by one or more of the following:

a. Bacterial wilt caused by *Pseudomonas solanacearum.*
b. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici.*
c. Nematode attack – e.g. *Meloidogyne* spp.
d. Insect damage to roots.
e. Lack of water.
f. Toxic conditions in the soil.
g. Poor root development owing to poor soil physical conditions.

Consequently a careful evaluation of the total situation is necessary before a correct diagnosis is made.

Steps in diagnosing a disease

a. Examine the plant, carefully noting the following:

   1) parts of the plant attacked – young vs. old leaves, etc.

   2) the kinds of symptoms produced; for example, if there are leaf spots, are they angular? Coalescing? Surrounded by a halo? Do they have a distinct margin?

   3) evidence of the pathogen; sometimes, conidiophores or conidia of the pathogen are obvious. They can be scraped from lesions and examined under the microscope.

   4) field conditions and cultural practices.

b. The total disease sindrome, is compared with the description of the diseases in a recognized text.

c. If there is no text or description of the desease, it will be necessary either to obtain the services of a competent plant pathologist or attempt to isolate the pathogen.

Precautions in diagnosis

a. Never overlook the possibility of several diseases occurring on the same plant or part of the plant.

b. There are several cases of insect damage that can be mistaken for disease caused by fungi, bacteria or viruses.

c. The cultural conditions under which a crop is grown must be carefully considered.

COLLECTING SAMPLES

Very often, it is not possible to diagnose the disease, and samples must be sent to competent authorities. The following hints in sampling should be useful:

a. Collect good quality specimens.

b. Collect all parts of the plant which show evidence of disease.

c. Wrap specimens in moist paper and enclose in a plastic bag.

d. Where it is necessary to preserve specimens for long-term storage, place plant parts in Formalin-acetic acid-alcohol (FAA). This is made up of 50% ethanol (100 ml), Formaldehyde (10 ml) and acetic acid (10 ml).

e. Photographs of the diseased specimen may also be helpful in diagnosis.

2

THE MICROSCOPE

Because most organisms that cause plant disease are too small to be seen with the naked eye, a microscope is an important tool in Plant Pathology. From the standpoint of construction and operational details, there are many different kinds of microscopes; however, the principles underlying their operation are the same. The microscope basically consists of two systems:

a. an optical system (for magnification).
b. an illumination system (for rendering the specimen properly visible).

The optical system

Magnification in the compound microscope is obtained by a series of two lens systems. The lens nearest the specimen is called the **objective**, and that nearest the eye is called the **ocular** or **eyepiece**. The total mignification is the product of the ocular and objective magnifications. The objective lens is made of a combination of convex and concave lenses. The lenses are usually made of different kinds of glass to correct for various chromatic and spherical aberrations. Microscopes are usually fitted with three objectives: (1) the low power objective (16 mm, magnification x 10), (2) the high-dry objective (4 mm, x 40) and (3) the oil immersion objective (1.8 mm, x 95). The millimetre reading designates the focal length of the objective. The shorter the focal length, the shorter is the distance between the specimen and the objective. Some microscopes also carry an extra low power objective (x 4). The objectives are held and rotated into place by a revolving **nosepiece**. Two adjustment wheels focus the lens system on the specimen. The **coarse adjustment** moves the body tube (or the stage on some models) over a greater vertical distance and brings the specimen into approximate focus, while the **fine adjustment** moves the body tube less for precise focusing.

Some microscopes are fitted with a condenser, a lens system which gathers the light and focuses it on the specimen. The condenser is usually equipped with an iris diaphragm which regulates the amount of light entering the condenser. There may be provision for the use of filters.

Magnification of the light microscope

The smallest diameter that can be seen with the standard light microscope is about 0.2 μ (1 μ = .001 mm). Bacteria, fungi and nematodes are all larger than 0.2 μ, but viruses and mycoplasma are smaller than 0.2 μ and can only be "seen" with the electron microscope.

The illumination system

The illumination system may consist of a simple mirror or a built-in light source.

CALIBRATION OF THE MICROSCOPE

For measuring a microscopic object or specimen, a calibrated microscope is necessary. In order to calibrate the microscope, an ocular micrometer and a stage micrometer are needed.

Procedure:

a. Unscrew the upper lens eyepiece and place the ocular micrometer (engraved side up) on the metal shelf (i.e. the ocular diaphragm) on the inside of the eyepiece.

b. Place the stage micrometer (right side up) on the stage of the microscope under an objective. Locate the scale on the stage micrometer under the objective.

c. Superimpose the scale on the ocular micrometer onto the scale of the stage micrometer, such that the graduations or divisions on one scale are parallel with those on the other.

d. At one end of the scales, select 2 lines that coincide. Then look for another line on the ocular micrometer that coincides with one on the stage micrometer.

e. Determine the number of divisions on the ocular micrometer and those on the stage micrometer between the lines that coincide.

f. If the scale on the stage micrometer is 1 division = .01 mm, and 45 ocular divisions equal 35 objective (or stage) divisions, then 1 ocular division equals $\frac{35}{45}$ x .01 = 0.0077 mm or 7.7 μ.

g. The values obtained are valid only so long as the same ocular micrometer in the same eyepiece is used on the same microscope and at the same tube length.

h. The procedure must be repeated for each objective or when any of the factors indicated in (g) above is changed.

USE OF THE MICROSCOPE

a. Place the slide on the stage, specimen side up, and centre the section to be examined as accurately as possible over the hole in the centre of the stage.

b. Adjust the mirror so that the maximum amount of light reaches the specimen.

c. With the low-power objective in position, lower the body-tube until the objective is about 0.5 cm from the slide.

d. Look through the eyepiece and slowly raise the objective until the specimen is in approximate focus. **N.B.** Never focus downward while looking through the eyepiece; that may be the end of your specimen.

e. After examining the specimen at low-power, shift to high-dry objective by rotating the nose-piece until the objective clicks into place.

f. Look through the eyepiece and slowly raise the tube, using the coarse adjustment, until the specimen comes into focus. Bring the image into accurate focus with the fine adjustment.

g. Care must be taken in using the oil immersion lens. Raise the body tube and rotate the nose-piece until the oil immersion objective clicks into position. Now place a drop of immersion oil on the portion of the slide directly under the objective. Watching the objective from the side, carefully lower it into the oil. Do not allow the objective to touch the slide. Look through ocular and slowly focus upward, using the fine adjustment. The image should come into view.

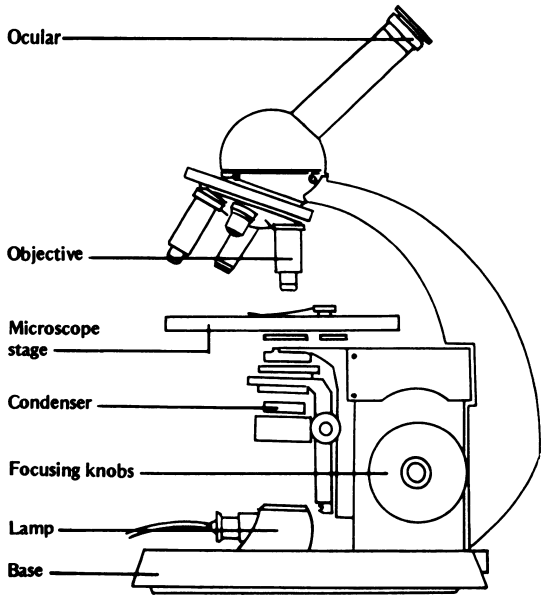

Fig. 1. Diagrammatic representation of a typical compound light microscope.

Care of the microscope

a. Never touch microscope lenses with the fingers.
b. Clean the lenses by gently wiping with lens tissue only, or with lens tissue dipped in xylene or diethylether.
c. Keep the stage of the microscope clean and dry.
d. Do not tilt the microscope when working with oil immersion lenses. The oil may drip onto the stage condenser and harden, thus destroying the condenser.
e. Never force the microscope – all components should work freely.
f. Never allow the objective to touch the slide or coverslip.
g. Never lower the body-tube with the coarse adjustment while looking through the microscope.
h. Never exchange lenses of different microscopes.
i. Store microscope in microscope box when not in use.
j. Before using any microscope, always take a few minutes to read the instruction manual. The few minutes spent at this time may make the difference between frustration and success.

3

THE AUTOCLAVE

The autoclave is a sterilizing device which uses moist heat in a pressurized chamber to destroy microorganisms. It is an essential tool used in Plant Pathology in the preparation of media, sterilization of equipment and in the drying of sterilized materials. The pressure cooker is a miniature autoclave. Autoclaves are of two types: 1) those that depend on live steam; 2) those in which steam is generated within the autoclave.

The autoclave is effective in killing microorganisms because: 1) the material to be sterilized is first moistened; 2) the steam is at a temperature of about 121°C; 3) the pressure is about 15-20 psi.

The moist heat is believed to cause irreversible coagulation of proteins in the living cells.

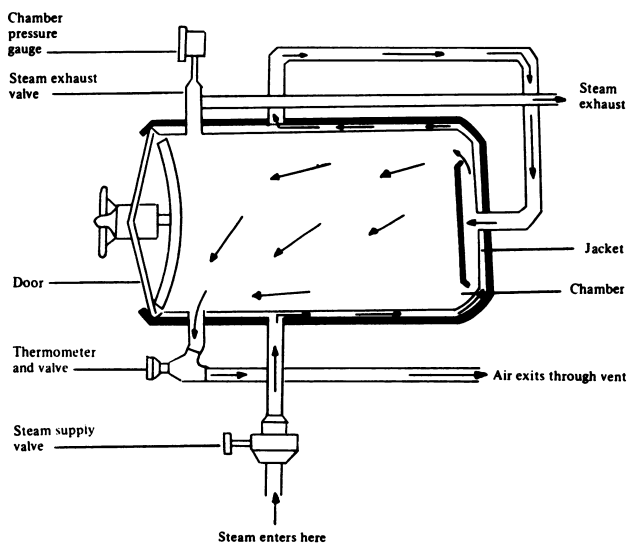

Fig. 2. Diagrammatic representation of a typical steam autoclave.

THE OPERATION OF AN AUTOCLAVE IN WHICH STEAM IS GENERATED IN THE UNIT

a. Check to ensure that sufficient water is present in the autoclave.

b. Load the autoclave and close lid securely.

c. Open the steam valve and switch on electricity supply.

d. Let steam issue from the valve for about 5 minutes, then close the valve.

e. Allow the autoclave to operate for the required time.

f. Turn off electric supply.

g. Let pressure drop to zero, then open steam valve.

h. Wait 5 minutes, then open lid.

4

THE PREPARATION OF MEDIA FOR FUNGAL AND BACTERIAL GROWTH

CONSTITUENTS OF A MEDIUM

A variety of solid and liquid media is used for the growth of fungal and bacterial pathogens. A suitable medium for growth of these pathogens should include available sources of carbon, nitrogen, inorganic salts and, depending on the organism, even vitamins or other growth substances. These may be supplied in the form of natural products such as sterilized plant parts (leaves, shoots, roots, seeds etc.), infusions or extracts of these materials, or chemically defined synthetic mixtures of known compounds.

The plant pathogenic nematodes and viruses and certain fungi such as some rust fungi, powdery fungi, and some of the downy mildew fungi, have not yet been grown successfully on a nutrient medium. These pathogens are called obligate parasites.

Liquid media usually have the same composition as their solid counterparts, except that agar is included in solid media. Agar is a complex polysaccharide which is usually obtained from a sea alga. Agar dissolves in water at 100°C and solidifies at 40°C to form a relatively clear gel. Agar is usually added to media at 1.5 to 2 percent, i.e. 15-20 gm per litre of medium.

Most of the media used routinely in plant disease investigations are easily prepared. In general, the procedure is as follows:

Add the ingredients, in the amounts indicated in the formula or recipe, to a beaker or Erlenmeyer flask that will hold 1 – 1 1/2 or 2 times the final volume of medium. Measure the required volume of water. Begin adding water to the ingredients, a little at a time, to prevent the formation of lumps, stirring constantly. When the ingredients are thoroughly moistened, the rest of the water can be added. Aluminium foil may be used to cap the flask or beaker. Heat the flask and its contents to dissolve the ingredients. When agar is a component of the medium, the mixture must be heated to the boiling point, to dissolve the agar. When antibiotic and other heat labile sub-

stances are being used in media, they must be sterilized by filtration and added to the cooled, autoclaved medium.

pH OF THE MEDIUM

An important factor to be considered in media preparation is the pH of the medium. Fungi usually grow better under acid conditions (about pH 5.5-5.8), while the growth of bacteria is usually favored by media approximately neutral in reaction (about pH 6.8-7.0). The reaction of the medium can be determined readily by means of pH indicator papers, indicator dyes, or a pH meter. The reaction of the medium can by adjusted to the desired level by adding IN NaOH or HCl to the hot solution. The pH of a medium can also be controlled by making up the medium in a buffer rather than water.

DISPENSING THE MEDIUM

While it is still hot (in the case of an agar medium), the mixture is despensed into appropriate containers for sterilization. If the medium is to be used for propagation of a fungal or a bacterial pathogen, it is usually dispensed into test tubes. Six-inch tubes with a 3/4" diameter are the most common. If the medium is to be stored or used in petri plates, it can be dispensed into 300-ml Erlenmyer flasks or 8-oz screw-top medicine bottles. Dispensing the medium can be facilitated with the use of a 500-1000 ml capacity funnel, fitted with a short piece of rubber tubing that ends in three inches of glass tubing. The funnel is supported by a ring stand, and delivery of the medium to the tubes or bottles is controlled by a pinch-cock attached to the rubber tubing. About 12 ml of medium is dispensed into each tube. The flasks should not contain more than 250 ml of medium; they can then be plugged with cotton or capped with aluminum foil. If medicine bottles are used, they should not be filled beyond the shoulder of the bottle; the caps should be in position during sterilization, but fitted loosely to allow for expansion of the medium and air within the bottle during sterilization. The caps are tightened prior to removing the bottles from the sterilizer.

STERILIZATION OF THE MEDIUM

A medium must be sterilized the same day it is prepared in order to prevent microbial growth. Sterilization of the medium should be done in a steam autoclave or in a pressure cooker. Tubes should be

arranged loosely in wire baskets or other containers, and flasks or medicine bottles should be located in the sterilizer so as to permit free movement of flowing steam around them. Sterilization is completed in 20 minutes at 15 lbs pressure and a temperature of 121°C. After the required time has elapsed, the pressure should be allowed to fall gradually to zero before the medium is removed from the sterilizer. If the pressure is released too quickly, the medium may boil over from the containers, wetting the cotton plugs and causing the medium to be lost from the containers. Jarring or swirling the tubes or flasks while they are being removed from the sterilizer may also cause the medium to boil over and result in painful burns to the investigator. It is important to handle them with great care during this operation until they have cooled.

PREPARING THE MEDIUM FOR SLANT CULTURES AND PLATE CULTURES

Tubes containing an agar medium are arranged in a slanted position to cool so that when the agar has solidified, a sloped surface will extend from about 1/2" to 3"– 3 1/2" from the bottom of the tube.

If the medium is to be added to petri plates, it should be allowed to cool to about 50°C (at this temperature the flask can be held against the face or forearm). The sterile petri plates are stacked in groups of 5 or 6. The melted agar is then added to each dish in turn, beginning with the lowest dish by raising its lid just enough to pour the agar from the flask into the dish. Add enough agar to provide a medium depth of about 5 mm, or about 15-20 ml for standard petri plates.

SOME COMMONLY USED MEDIA

a. Potato dextrose agar – PDA

Ingredients:	Distilled or tap water	1000 ml
	Sliced potatoes	200 g
	Agar	17 g
	Dextrose	20 g

Procedure: Cook potatoes in 500 ml of water for 20-30 minutes over a low fire. Strain the potato broth through 2 or

3 layers of cheesecloth to remove potato debris. Melt the agar in 500 ml of water in an autoclave or pressure cooker and add 20 g of dextrose to the melted agar. Combine the hot potato broth and melted agar solutions and bring to a volume of 1000 ml. Mix thoroughly and dispense the medium into test tubes or flasks plugged with cotton and sterilized.

b. **Nutrient agar (NA)**

Ingredients:	Distilled or tap water	1000 ml
	Beef extract	3 g
	Peptone	5 g
	Agar	15 g

Procedure: Heat the beef extract, peptone, and agar in water until they dissolve. Dispense the medium into tubes, flasks or bottles which are plugged with cotton or suitably capped (e.g. with aluminium foil) and sterilized. The final reaction of the medium will be about pH 6.8. Nutrient broth is prepared in the same manner, except that agar is omitted from the preparation.

c. **Water agar (WA)**

Ingredients:	Distilled water	1000 ml
	Agar	15–20 g

Procedure: Dissolve agar in the water by heating. If desired, adjust the pH with colorimetric pH indicators or a pH meter and the addition of 1N HCl or NaOH. Dispense the medium into tubes, flasks or bottles, plugged with cotton or capped and sterilized.

d. **Lima bean agar**

Ingredients:	Distilled water	1000 ml
	Lima beans	200 g
	Agar	15 g

Procedure: Blend frozen lima beans and water in a kitchen blender until the mixture is smooth. Add agar to the mixture, then heat to dissolve the agar. Dispense the medium into tubes etc., and autoclave.

e. **Oatmeal agar**

Ingredients:	Distilled water	1000 ml
	Oatmeal	100 g
	Agar	15 g

Procedure: Stir Oatmeal into water at about 70°C, and maintain at 60°C for about 1 hour. Restore the volume, add agar, and heat to dissolve. Dispense the medium into tubes etc., and sterilize.

SELECTIVE MEDIA

The addition of certain chemicals to a basal medium can repress the growth of certain microorganisms while permitting the growth of the organism required. This repressive selection is usually used for isolating plant pathogens from soil or from infected tissue. The addition of antibiotics to the medium removes many bacteria, as required for the isolation of fungal pathogens. Generally, the presence of 100 ppm of streptomycin will inhibit growth of most bacteria. Altering the pH can also be effectively used to eliminate bacteria during isolation. Because bacteria grow best at pH 6.8 to 7.0 and most fungi at 5.0 to 5.8, a medium with a low pH will thus inhibit bacterial growth, while favoring fungal growth. One or two drops of a 25% solution of lactic acid are usually added to 10 ml of melted agar to inhibit bacterial growth when isolating fungi.

CLASSIFICATION OF MEDIA

Media for microbial growth may be described as **synthetic, semisynthetic** or **natural.** Synthetic media are those whose chemical composition is known precisely. Semisynthetic media are those in which a few of the ingredients are known precisely, while natural media are derived from complex plant or animal products and their precise composition is unknown, e.g. oatmeal agar. The composition of oatmeal agar will depend on the composition of the oat used in the medium which, in turn, will depend on the variety of oats, the age of the grain and several other factors.

STERILIZATION OF EQUIPMENT

When sterile glassware such as pipettes, petri plates or flasks are required for an experiment, the glassware should be washed in a detergent, thoroughly rinsed with tap water, then with distilled

water, and allowed to dry. Flasks need only to be capped with cotton or aluminium foil. Pipettes should be sorted to size and then wrapped in newspaper or wrapping paper; petri plantes can be placed in lots of six or twelve in paper bags of appropriate size or wrapped in newspaper or aluminium foil. The paper wrapping should be secured with sticky paper tape prior to heating the glassware. Sterilization is accomplished by heating the glassware in an oven at 180°C or 356°F for a mimimum of 2 hours. To prevent damage, containers or equipment containing rubber or plastic parts should be sterilized in an autoclave for 20 minutes.

5

DETECTION OF FUNGAL PATHOGENS IN INFECTED PLANT TISSUES

In some cases, the presence of a fungal pathogen in infected tissues is revealed by the presence of the reproductive or vegetative structures of the fungus on the surface of these tissues, e.g. spores of powdery mildew on cucumber leaves. In other cases, however, some magnification of the specimen is necessary. This may be done either with a simple hand lens (15-20 x magnification) or with a microscope (either stereoscopic or compound).

Many fungi produce their reproductive units called spores or conidia on stalk-like structures that emerge from the plant tissues. The spores are variously shaped, sized and colored. Examples include: the large, dark, cigar-shaped spores of *Helminthosporium* that cause lesions on cereal and grass leaves; the small hyaline oval or round spores of *Botrytis* that are produced in grape-like clusters on flowers during wet periods; the hyaline, barrel-shaped spores of the mildew fungus *Erysiphe* that are produced in chains on the leaves of cereals, lettuce and certain other plants. Numerous fungi produce their spores in tiny flask-shaped structures called pycnidia. When moist conditions prevail, the spores ooze out in a mucilaginous matrix and appear as glistening droplets surmounting the tiny pycnidium.

Sometimes the fungal pathogen is not apparent when diseased tissue is examined microscopically. In such instances, if the plant material is washed in a detergent solution, rinsed well in water and then placed in a closed container (petri dish, test tube, jar, etc.) containing moist filter paper or paper towels, the pathogen usually will grow out of the diseased tissues and produce its reproductive structures on the surface.

Mycelium in plant tissues is readily differentiated by using the differential stain, cotton blue, in lactophenol. Lactophenol solution is prepared from 20 grams of phenol (warmed until melted), 20 ml lactic acid, 30 ml glycerine and 20 ml distilled water. To one-half of the lactophenol solution is added 2.5 ml of a 1.0% cotton blue (aniline blue) solution in distilled water. Thinly sliced sections or

bits of diseased tissue are placed on a slide, and a few drops of the stain are added. The slide is warmed in a flame until the solution fumes. The plant material is then rinsed in the clear lactophenol solution, transferred to 1-2 drops of lactophenol solution on a new microscope slide, covered with a coverslip and observed with the microscope. The fungal mycelium is stained blue while the plant tissue remains unstained. **Care should be taken not to allow the microscope objectives to contact the lactophenol solution, thus causing damage to the objectives. Caution should be exercised in handling the phenol since it may cause severe burns.**

ISOLATING A FUNGAL PATHOGEN

Bacteria and fungi are ubiquitously present as contaminants on plant tissue. Since the goal is to isolate the pathogenic fungus from the diseased tissues and to grow it as a pure culture, it is important to exclude bacteria and fungi that are present as contaminants on the surface of the working area, on equipment to be used, and on the surface of the diseased tissue.

The surface of the working area may simply be wiped with a cloth moistened with water, or with a disinfestant such as mercuric chloride (1:1000) or sodium hypochlorite solution.

Prior to using a scalpel, dissecting needle, forceps etc., the instrument should be dipped in 70% ethanol and then passed through a flame to eliminate contaminants from that portion of the instrument that will be used in handling the diseased tissue. The instrument should be air cooled for a minute before use.

Contaminants on the surface of woody plant parts such as twigs and roots are easily destroyed by dipping the plant part in 70% ethanol, passing the material through a flame and allowing the alcohol to burn off. This treatment is usually too drastic for succulent tissues, such as leaves, shoots, flower parts or seedling tissues. For these, household bleach is used, often in combination with a wetting agent if the surface of the plant tissue is difficult to wet.

The active ingredient in preparations of household bleach is sodium hypochlorite at a concentration of about 5.25% by weight. The bleach solution is diluted with water (10:90) to give a 10% (volume/volume) solution of disinfestant. It is sometimes necessary to ensure complete surface disinfestation by rinsing plant materials that are difficult to wet in 70% ethanol or in a solution of a wetting agent (Agral, 1-2 drops/1000 cc of water) or a 2% solution of Decon 90 prior to immersing them in the disinfestant. If the diseased area is small, as in leaf-spots or stem lesions, the diseased tissue and a small amount of adjacent tissue is cut out, rinsed in wetting agent and immersed in the disinfestant. If the affected area involves a large portion of the leaf, shoot, etc., as in some blights,

a portion of plant tissue, which preferably includes both diseased tissue and healthy tissue adjacent to the margin of the diseased area, is removed, rinsed in a wetting agent and immersed in the disinfestant. About 1 cm^2 of plant tissue can be handled conveniently in this manner and provides enough tissue to prevent the disinfestant from penetrating the tissues and killing the pathogen. The time that the tissue is left in the disinfestant varies according to the type of tissue involved and may range from 1-2 minutes for thin, very succulent tissue to 10-15 minutes for hard seeds.

Additional disinfestants are:

a. 95% alcohol – dip for 3 seconds.
b. 50% hydrogen peroxide – dip for 15 seconds to 5 minutes.
c. 0.4% Formalin – dip for 15 seconds to 5 minutes.

PLATING THE INFECTED TISSUE

The disinfested tissue may be blotted dry between paper towels and then cut into small pieces 1-2 mm^2 in size which are transferred to the surface of an agar medium in petri plates. The lid of the petri plate should be raised only enough to allow the bits of tissue to be placed readily on the agar. Four or five pieces of tissue should be spaced approximately equidistant on the plate. The bark of woody tissue is removed using sterile forceps, and small chips or bits of tissue are transferred to the medium in petri plates, as described for more succulent tissue.

The plates are usually kept at a temperature of 25-30°C for a few days to allow the pathogen to grow out of the tissue and develop on the agar medium. When the type of fungal growth from the tissues is consistent for several pieces of tissue, it can be assumed that the pathogen has been isolated.

ESTABLISHING PURE CULTURES

Some of the fungal growth from each of several colonies should be transferred to individual tubes of the same agar medium to provide pure cultures of the pathogen. This is most readily done by means of a transfer needle. The needle is heated in a flame until it glows red, and then cooled by touching it to a portion of the agar medium that is free of microbial growth. A tiny portion of the medium and fungal growth is then removed from the fungal colony and transferred to the midpoint of the sloped surface of

the medium in a tube. The pathogen usually can be maintained in pure culture for long periods by storing it at about 5°C and subsequently transferring it to tubes of fresh medium in a similar manner at intervals of 2-3 months.

DETERMINING THE CHARACTERISTICS OF THE ISOLATED FUNGUS

Characteristics of the growth of a fungal colony growing on an agar medium in a petri dish can be observed through the microscope by placing the uncovered petri dish on the stage of the microscope and gently placing a cover slip over the advancing margin of the fungal colony. Characteristics of mycelium and spores can be observed by using a dissecting needle or transfer needle to transfer bits of the fungal growth from a culture to a small drop of water on a microscope slide, placing a cover slip gently on the drop and viewing the material through the microscope.

The slide culture technique is especially useful for determining the mode of sporulation of a fungal pathogen. A petri dish is fitted with two pieces of 9 cm diameter filter paper, a bent glass rod, a microscope slide and a 22 x 30 mm cover slip. The petri dish and contents are sterilized at 15 lbs. pressure and a temperature of 121°C for 15-20 minutes. Sterile water agar or PDA (2-3% agar) in petri plates is cut with a sterile scalpel into squares smaller than the cover slip. A block of agar is then transferred to the sterile slide in the petri plate, and the cover slip is placed on it with sterile forceps. Bits of mycelium and/or spores are transferred from a culture of the fungus to the mid-point of each of the four sides of the agar block. The filter paper in the dish is moistened with an aqueous solution of 5% glycerin to maintain the humidity of the dish. The plates are kept at 25-30°C for a few days to allow the fungus to grow and sporulate. The slide cultures are removed to the microscope stage for observation. For many fungi it is possible to determine the characteristics of the mycelium, the manner in which the spores are produced and characteristics of the spores.

Fungal structures may be more clearly apparent if they are stained with 1% solution of acid fuchsin or cotton blue in lactophenol. One or two drops of the stain are added to a microscope slide, and the fungal mycelium is transferred to the drop, which is then covered with a cover slip. If the fungal material is mounted in water, stain can be added to the mount by placing a drop of stain on the slide at the edge of the cover slip and then drawing the drop of stain under the cover slip by bringing a piece of filter paper into contact with the water at the opposite edge of the cover slip.

OBSERVING THE DEVELOPMENT OF A FUNGUS ON LEAF SURFACES

The germination of fungal spores on the leaf surface, and their penetration into leaf tissues, can be followed readily by what is called the whole-mount technique. At intervals (e.g. 6, 18, 32 and 48 hours) after a plant is inoculated with spores of a fungal pathogen, leaves are collected and placed in Carnoy's solution (2:1 mixture of glacial acetic acid and absolute ethyl alcohol) for 24 hours, then transferred to lactophenol solution for 24 hours, stained in 0.1% acid fuchsin in lactophenol solution for 8-10 hours, rinsed in lactophenol solution and finally mounted in lactophenol solution.

6

DETECTION OF BACTERIAL PATHOGENS IN INFECTED TISSUES

The presence of plant pathogenic bacteria in infected plant tissue can be demonstrated, and their size and shape determined, by a technique that involves negative or background staining. A drop of 2% solution of Congo red stain in distilled water is placed on a clean microscope slide. A sterile scalpel is used to remove a small piece (1-2 sq. mm) of diseased tissue from the margin of healthy and diseased tissue, which is placed in the drop of Congo red solution and crushed with the scalpel. After 1-2 minutes the bits of tissue are scraped from the slide, and the remaining stain solution is spread in a thin film over the slide. The stain is allowed to dry without heating and then flooded with 2-3 drops of acid alcohol (3 drops concentrated HCl in 30 ml 95% ethanol). The acid alcohol turns the stain blue. The alcohol is allowed to evaporate. A drop of immersion oil is added directly to the stained portion of the slide, and the slide is examined directly with the oil immersion lens of the microscope. If an oil immersion lens is not available, a drop of mineral oil covered with a cover slip can be used with the high power lens. The bacteria appear as unstained (white) short, rod-shaped cells against a blue background. If the stain is not spread thinly enough over the slide, the slide will appear black or very dark blue, and the bacteria will not be visible. If too much plant tissue is used to prepare the slide, the stain may be precipitated and fail to give the desired result.

ISOLATION OF BACTERIAL PATHOGENS

A simple procedure for isolating bacterial pathogens in pure culture involves preparation of a dilution series.

a. Arrange 6 sterile petri plates in a row and label them 1 through 6.

b. Place 4 drops of sterile water separately and equidistantly in the first petri plate.

c. Place a single drop of water in each of the remaining petri plates.

d. Use a sterile scalpel to remove a small piece of diseased tissue from the affected plant and place the tissue in one of the drops on the first petri plate.

e. Use the scalpel to crush the diseased tissue in the drop of water.

f. After 5-10 minutes (to allow the bacteria to separate from the plant tissue), use a transfer loop that has been flamed and cooled to transfer a loopful of water and bacteria from the first to the second drop in the same plate. The second drop is mixed well with the transfer loop, and **without flaming the loop**, a loopful is transferred from the second drop to the third drop in the plate. This operation is repeated successively from drop to drop and plate to plate and results in a progressive dilution of the number of bacteria in the original suspension.

g. Add cooled, melted nutrient agar to each plate and swirl the plate to distribute the bacteria uniformly throughout the agar.

h. Store the plates at room temperature (25-30°C).

i. In plates prepared at the higher dilutions, the bacteria will grow as distinct colonies; these presumably develop from single bacterial cells.

j. When bacterial colonies appear in the plates, transfer cells from a single bacterial colony to a slanted tube of nutrient agar or nutrient broth.

k. After 24 to 48 hours of growth, add sterilized distilled water to the nutrient agar slant and wash the cells into suspension with a flamed inoculating needle.

l. Streak the suspension from the nutrient agar slant or the nutrient broth onto 2-3 agar plates, and observe the colonies after incubating the plates at room temperature for 24-48 hours. If all the colonies on the plates are identical in appearance and cultural and morphological characteristics, a pure culture has been obtained. Pathogenicity and other tests are then necessary to confirm the identity of the isolated bacterium.

OBSERVATION OF LIVING BACTERIA

Living bacteria are somewhat difficult to observe because the cells are small and contrast only slightly with their environment. However, they can be observed, and their motility can be studied, by using a hanging drop mount.

The procedure is as follows:

a. Apply a smear of Vaseline around the depression of a depression slide.

b. Using the inoculating loop, aseptically transfer one small drop of the culture to the centre of a clean square cover slip.

c. Invert the depression slide and centre it over the drop of culture.

d. Press down on the edge of the cover slip so that the Vaseline makes a firm seal.

e. Quickly and carefully turn the slide over so that the drop is suspended in the depression of the slide. The drop must not touch the bottom of the depression.

f. Examine the bacteria in the drop by first focusing on the edge of the drop with low power objective. Then, switch to high dry and re-focus on the drop. Bacteria should now be visible moving in the drop.

THE GRAM STAIN FOR BACTERIA

The Gram Stain is a differential staining technique which divides bacteria into two groups. The first of these groups is stained purple by the Gram Stain and is called Gram Positive, while the second group is stained pink and called Gram Negative. The technique is very useful in plant pathology because almost all genera of phytopathogenic bacteria except *Corynebacterium* and *Nocardia* are Gram Negative.

Four solutions are used in the Gram Stain:

a. Crystal violet
b. Gram's iodine
c. 95% alcohol
d. Safranin

The method of preparing these solutions is described at the end of the procedure.

Procedure:

a. Place a loopful of the bacterial culture on a clean glass slide.
b. Spread the drop to form a thin film.
c. Allow the film to dry in air.
d. Pass the slide rapidly through a Bunsen flame about three times (film side up). This fixes the bacteria to the slide.
e. Stain with crystal violet for 30 seconds, either by adding drops of the stain to the smear, or by dipping the slide into the stain.
f. Wash with tap water until the stain ceases to flow from the slide.
g. Cover the film with Gram's iodine and allow to act for 30 seconds.
h. Rinse in tap water.
i. Rinse with 95% alcohol for 10 to 20 seconds (i.e. until drippings from slide are colorless).
j. Rinse with tap water.
k. Stain with safranin for 10 seconds.
l. Rinse with tap water and blot dry.
m. Examine under the oil immersion lens of the microscope.
n. Determine the Gram reaction by noting the color of the cell wall.

Reagents for Gram Stain

Crystal Violet. One volume of a saturated alcoholic solution of crystal violet in four volumes of 1% aqueous ammonium oxalate. Allow ammonium oxalate solution to stand overnight or heat gently until in solution. Then mix with crystal violet solution.

Gram's iodine. Iodine crystals 1.0 gm, distilled H_2O 300 ml, potassium iodide 2.0 gm. Mix all the ingredients together.

Safranin. 100 ml of a saturated alcohol solution of safranin-O in one litre of distilled water.

DETERMINATION OF THE BACTERIAL POPULATION OF INFECTED TISSUES

The bacterial population of infected plant tissue can be determined by modifying the dilution plate-count technique. The tissue is measured (either by weight or by area), then surface disinfested

and ground in a mortar or blender with a small measured quantity of sterile distilled water. One millilitre of the suspension is transferred to 9 ml of sterile distilled water, and the resulting suspension is thoroughly mixed. One ml of this suspension is serially transferred to 9.0 ml of sterile distilled water to obtain a 10^9 dilution of the original suspension. Remove 1 ml of suspension from each dilution and place into a sterile petri plate. Add 15 ml agar medium to each plate and incubate the petri plates at room temperature. After 48 to 72 hours at room temperature, count the number of bacterial colonies on each plate. Determine the number of viable cells in the original sample by multiplying the number of colonies on the plate that have between 30 and 300 colonies, by the dilution factor. The results are given as the number of bacteria per gram or cm^2 of tissue.

7

KOCH'S POSTULATES

In determining the causal agent of a particular disease, it is not enough to have isolated a fungus or a bacterium from the infected plant. To demonstrate that an organism is the cause of a disease, the following procedure must be followed:

a. Associate the organism with all cases of the disease.

b. Isolate the organism and grow it in pure culture.

c. Inoculate susceptible plants with the isolated organism and obtain identical symptoms to those previously observed.

d. Re-isolate the organism and compare it with the original isolate. They should be identical.

These four steps have been called Koch's Postulates or Koch's Rules of Proof of Pathogenicity. They are named after Robert Koch, a pioneer in bacteriology, and when they are satisfied, the causal agent of the disease has been established.

8

INOCULATION TECHNIQUES

METHODS OF INOCULATING PLANTS WITH PHYTOPATHOGENIC BACTERIA

There are several techniques available for inoculating plants with phytopathogenic bacteria. The inoculation technique varies with the kind of disease.

Disease Type	Method of Inoculation
a. Soft rot, e.g. soft rot of carrots caused by *Erwinia carotovora.*	A bacterial suspension is dropped onto the cut surface of the tissue.
b. Wilt diseases, e.g. bacterial wilt of tomato caused by *Pseudomonas solanacearum.*	1) The roots of the plants can be cut back to 1-2 cm and then placed in a bacterial suspension for about 2 hrs. 2) The stem may be pricked with a needle dipped in bacteria suspension. Pricking is usually done in the area between the cotyledon and the first leaf.
c. Leaf spot diseases, e.g. Angular leaf spot of cucumber caused by *Pseudomonas lachrymans.*	1) A bacterial suspension may be sprayed onto the surface of the plant. 2) The surface of the leaf may be "painted" with a bacterial suspension. 3) A bacterial suspension may be injected into the tissue with a hypodermic needle.

After inoculation with bacteria, plants are usually placed in a moist chamber for 48 hours. This increases the chances of infection taking place, since plants are generally more susceptible to bacterial infection under conditions of high humidity.

METHODS OF INOCULATING PLANTS WITH PHYTOPATHOGENIC FUNGI

The method of inoculating plants with phytopathogenic fungi will vary depending on the nature of the disease and, more especially, on the process of infection. The inoculation techniques can be grouped into three main categories:

a. **Leaf inoculation.** When the pathogen enters the plant through the leaves, leaf inoculation is used. A spore suspension or dry spores of the fungus may be rubbed, sprayed or brushed onto the surface of the leaves. Spore germination will take place, and infection follows. Moist conditions are usually maintained after inoculation.

b. **Soil infestation.** Where the pathogen is soil-borne, spores, mycelia or sclerotia of the pathogen are mixed with sterilized soil, and healthy plants or seeds are then planted into the infested soil.

c. **Seed infestation.** Seeds of the test plant may be mixed with spores of the fungus, and the seeds are planted into sterilized soil. Seed infestation is commonly used with smut diseases of cereals.

9

THE DIAGNOSIS OF A NEMATODE PROBLEM

Plant parasitic nematodes are small, worm-like animals which live primarily in soil and feed on plant roots. As a result of nematode feeding, the following symptoms may be induced:

COMMON SYMPTOMS OF NEMATODE INFECTION

a. Root galls.
b. Lesions on root.
c. Root rot.
d. Excessive branching.
e. Wilting.
f. Absence of side rootlets (Stubby root).
g. Poor stands in field.
h. Stunting, yellowing and distortion of above-ground parts of plant.

Because these symptoms may be caused by other agents, e.g. insects, fungi, etc. and because plant parasitic nematodes are not visible to the naked eye, the diagnosis of a nematode problem in the field is often difficult. Collection of root and soil samples, extraction of the nematodes, and identification of the nematodes is the only way of establishing beyond a doubt that a particular disease is due to plant parasitic nematodes.

COLLECTING SAMPLES FOR DIAGNOSIS

A soil and a root sample taken from 3 to 5 of the affected plants, and a similar sample from healthy plants, are usually adequate for a diagnosis. If the plants are small, such as carrots, lettuce, etc., the best sample is the complete root system with the

adhering soil (rhizosphere soil). If the plants are large, such as bananas or papaws, samples are taken in the region of the feeder roots, i.e. 1 to 3 feet from the base of the plant, at a depth of 6 to 8 inches. Usually 6 to 8 sub-samples are taken around the plant using a garden fork and spade, and they can be combined to make a composite soil sample. A root sample may be obtained at the same time by cutting away some of the fine feeder roots of the plant. About 1 pint of soil (500 cc) and a handful of roots (100 gm) is an adequate sample. Samples should be placed into labelled plastic bags and kept in a cool container until processed. Nematodes are very susceptible to heat and drying, and samples should be processed as soon as possible. However, samples may be kept for about one week in a refrigerator at 5°C when immediate processing is impossible.

EXTRACTING NEMATODES FROM PLANT ROOTS

There are several methods for extracting nematodes from plant roots. Only the modified Baermann funnel technique will be described here.

Extracting nematodes from the plant roots, using the modified Baermann funnel technique:

a. Wash roots to remove soil.

b. Cut the roots into pieces measuring 1 to 2 cm in length, using a sharp scalpel or razor blade.

c. Place the cut roots, together with 100 cc of sterile distilled water, into a blender, and blend at medium speed for 10-20 seconds.

d. Pour the resulting suspension into a modified Baermann funnel* and let stand for 24 hours.

e. The nematode will leave the upper pan of the apparatus and collect in the water contained in the lower pan. The suspension is poured into a petri dish and examined under the microscope.

(*) The modified Baermann funnel (or pie-pan technique) consists of two ordinary 8-inch pie pans placed one inside the other. The base is removed from the upper pan and replaced by wire mesh with holes about 1/8 inch in diameter. A facial napkin is placed over the mesh, and the root sample is poured over this napkin. The lower pan contains about 100 cc of distilled water. Nematodes will move through the filter, leave the upper pan and collect in the clear water in the lower pan.

STAINING NEMATODES IN PLANT ROOTS

The presence of nematodes in plant roots is a good technique for diagnosing nematode problems. Nematodes in plant tissue may be stained as follows:

a. Wash the soil from the roots.

b. Immerse the roots in a hot lactophenol acid-fuchsin solution* for 1 to 3 minutes, depending on the tissue. The more succulent the tissue, the shorter the time.

c. Remove the roots from the stain and wash them with cold tap water.

d. Place the roots in clear lactophenol. The roots will lose the stain, but the nematode retains the stain and appears pink under the microscope.

EXTRACTING NEMATODES FROM SOIL

There are several methods for extrating nematodes from soil; however, the modified Baermann funnel technique described above is one of the simplest techniques. The soil sample is thoroughly mixed and an aliquot (about 50 cc) is placed on the filter of the upper pan, which is then placed in the lower pan. The nematodes can be collected from the lower pan after 24 hours.

A technique that may be used when pie pans are unavailable is:

a. Place about 100 cc of infested soil in a 4" diameter plastic planter.

b. Secure a piece of muslin or cloth, which has been lined with a double layer of facial tissue, over the top of the pot.

c. Invert the pot into a bowl containing tap water so that the muslin or cloth is a few centimetres from the base of the

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(*) Lactophenol acid-fuchsin solution:

Phenol	20 gm
Lactic acid	20 ml
Glycerine	30 ml
Water	20 ml

Add 5 cc of a solution of 1 gm of acid-fuchsin and 100 cc of water. Cotton blue (aniline blue) may also be used.

bowl. The pot may also be inverted into water in a funnel, the base of which is closed with a piece of rubber tubing and a pinch clamp. Nematodes will emerge from the soil and enter the water of the bowl or funnel. The suspension is collected and examined under the microscope.

KILLING AND FIXING A NEMATODE SAMPLE

The nematode suspensions obtained from the pie pans can be poured into a beaker.

Allow the beaker to remain at room temperature for one (1) hour. **Carefully** pour off the top of the suspension, leaving the nematodes suspended in about 5 cc of water. Add 5 cc of boiling water to the beaker and mix quickly. The temperature of the suspension is raised to about 70°C, and the nematodes are killed. Alternatively, place the beaker in boiling water bath for 2 minutes, stirring constantly. If the nematodes are subjected to higher temperatures, they become worthless for identification. Add 14 cc of commercial (38%) formaldehyde to 86 cc of water. Mix thoroughly and add 10 cc of this mixture to the 10 cc of nematode suspension. Alternatively, 10 cc of a mixture of 14 cc of formalin, 4 cc of triethanolamine and 82 cc of water (TAF) may be used. Place the fixed nematodes in a screw cap phial. The nematodes can then be submitted to a nematologist for identification.

IDENTIFYING PLANT PARASITIC NEMATODES

The identification of plant parasitic nematodes is a difficult task and can be satisfactorily carried out only by a trained nematologist. However, without identifying the specimens, it is usually possible to distinguish between a plant parasitic nematode and a saprophytic nematode. This is because all plant parasites possess a protrusible stylet at the anterior end of the body; saprophytic nematodes do not usually have this stylet.

DIAGNOSING A ROOT-KNOT NEMATODE PROBLEM

In the tropics, root-knot nematodes, *Meloidogyne* sp. are very common. They attack a large number of host plants and their population density in soil is usually high. The root-knot nematode causes galling of roots, and the presence of these galls is a good indication of a root-knot nematode problem. In diagnosing the problem, the following should be noted:

a. Galls induced by root-knot nematodes are sometimes confused with nodules on leguminous plants. Nodules usually occur on the side of the root and are attached by an isthmus or neck; galls are formed along the axis of the root.

b. Galls may also be induced on roots by insects and nematodes not belonging to the root-knot group.

c. The shape and size of galls induced by root-knot nematodes vary with crop, variety and environmental conditions.

d. The presence of the root-knot nematode in the galls may be demonstrated by staining the tissue or by teasing out the tissue under the microscope where the pear-shaped, white, mature females will be observed.

10

VIRUSES AND PLANT VIRUS DISEASES

Viruses are submicroscopic entities which cause diseases in plants and animals. The viruses cannot be cultured on media as fungi or bacteria can, but their presence can be detected when their symptoms are transferred from one plant to the next, or through electron microscopy.

Several techniques are available for transferring a virus from one plant to another. All of them require that one or more virus particles enter an injured but living plant cell. Mechanical inoculation is often used, as with the tobacco mosaic virus (TMV) in tobacco. An abrasive, Carborundum, dusted on the leaf of a healthy plant will injure the leaf hairs and epidermal cells when gentle pressure is applied with a gauze pad dipped in juice from leaves of a plant with tobacco mosaic. The virus multiplies in the cells into which virus particles are introduced, and the newly synthesized virus moves to adjoining cells. This movement may be restricted in some plant varieties, and the resulting injury to the leaves takes the form of small necrotic lesions visible to the naked eye.

Insects such as aphids, may also transfer virus particles from diseased plants to healthy plants. Many viruses may be transferred from one plant to another by grafting, provided a suitable union is formed between the translocation elements of both plants. Dodder, a parasitic seed plant, may also be an effective agent of transfer of virus, transporting the virus from a diseased plant to a healthy one through its own phloem vascular system. Insects, mites, nematodes and at least one fungus are also able to transmit some viruses from one plant to another.

Individual particles of some viruses may be seen with electron microscopy. Special preparations are made of the virus and exposed to a beam of electrons in an electron microscope. Differential passage of electrons through the virus preparation creates an image that may be magnified and viewed directly on a screen, or

reproduced on a photographic plate for processing. With this tool, much information has been obtained on particle size, shape and structure of common viruses.

It is also possible to purify and concentrate some viruses. When this is done, the final preparation is somewhat comparable to a pure culture of a fungus or bacterium. The purified material may then be examined critically for purity and homogeneity, and studies can be made of the structure, composition and properties of the particles found in these preparations. Extensive analytical studies are then needed to determine whether or not a particular kind of particle is the virus. These studies usually involve subjecting the preparation to several procedures that separate particles with different chemical and physical properties, and inoculating healthy plants with each fraction obtained. If infectivity is always associated with a particular kind of particle, and if the two cannot be separated, the evidence indicates that the virus has been "isolated" and a "pure culture" obtained.

11

MYCOPLASMA AS AGENTS OF PLANT DISEASE

In recent years, it has become clear that many plant diseases that were once thought to be caused by plant viruses are caused by small bacteria-like agents called mycoplasma. Mycoplasma are nothing new; in the 19th Century, Louis Pasteur described the first mycoplasma – called *Mycoplasma mycoides* – as the causal agent of pleuropneumonia in cattle. Since that time, mycoplasma have been described as the cause of disease in humans, cattle, birds and rodents. In 1967 however, Doi and Ishiie in Japan conducted a study of a disease called Mulberry dwarf disease and reported that agents resembling mycoplasma were present in the sieve tubes and occasionally in the phloem parenchyma. No similar bodies were found in healthy plants. The disease was cured by the application of tetracycline antibiotics to the plants; this led to the conclusion that the disease was caused by mycoplasma.

Since the work of Doi *et al,* about 20 other plant diseases have been shown to be caused by mycoplasma.

The characteristics of mycoplasma

a. Mycoplasma are the smallest living organisms known which can be grown in a cell-free medium. They usually vary between 125 and 250 mμ in diameter and can therefore be observed only with electron microscopes.

b. They lack cell walls and tend to be pleomorphic, varying in shape usually as a result of small changes in the osmotic concentration of the medium.

c. They are gram negative.

d. They reproduce by producing long filaments which break up, giving rise to elementary bodies.

e. They are very similar to bacterial protoplasts or L-forms of bacteria, i.e. bacteria from which cell walls have been removed or which have been grown in media to prevent cell wall formation.

Characteristics of diseases caused by mycoplasma

a. They are almost always transmitted by leaf hoppers.

b. The mycoplasma-like bodies are present in the phloem.

c. They can be controlled by tetracycline antibiotics such as aureomycin.

d. The symptoms of the diseased plant are invariably yellowing, stunting, rosetting or witches' broom effects.

Diseases known to be caused by mycoplasma now include rice yellow dwarf, sugarcane white leaf, tomato big bud and sweet potato little leaf.

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