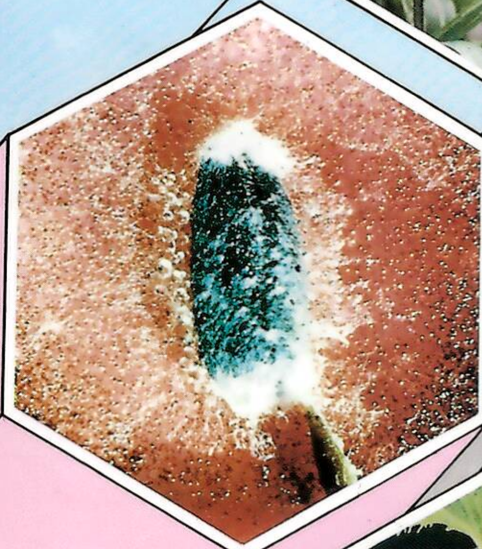
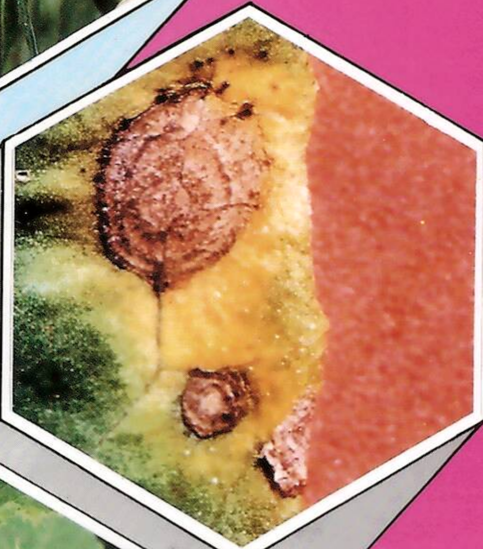
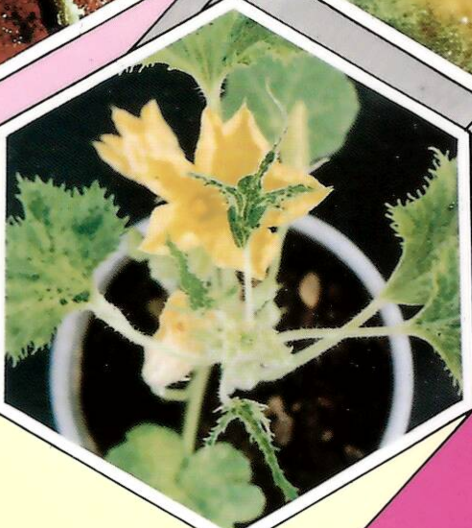
PHYTOPATHOLOGICAL DIAGNOSTIC TECHNIQUES







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PHYTOPATHOLOGICAL DIAGNOSTIC TECHNIQUES

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1992

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PREFACE

A training course on plant disease diagnosis was organized jointly by the Crop Diseases Research Institute and the Training Centre, NARC, from August 15 to 31, 1988. The trainees of the course included research workers, subject matter specialists from different provincial and federal institutions, internship trainee students from various agricultural universities of the country, and students of Barani Agricultural College, Rawalpindi. Twenty professionals belonging to various research and educational institutions of Pakistan took part in the training process. The contributions of these professionals were presented in the form of a manual for the guidance of the trainees.

The manual was very much appreciated not only by the trainees but also by other professionals involved with plant disease diagnosis in the country and a number of demands poured in for it. Since the manual was in mimeographed form and a limited number of copies were available, soon none was left for meeting the demand. It was decided to bring out the manual in printed form. The manual has been revised and covers plant pathological techniques and methods used in the plant disease diagnosis process including methods of sterile techniques, seed health testing, identification of plant pathogenic bacteria, viruses and special techniques for rust and pythiaceous fungi.

It is hoped that this manual will be a useful source of information on phytopathological techniques to plant pathologists working in various research and diagnostic labs and students both in public and private sector.

We thank all the professionals who contributed to this manual. We are grateful to Malik Mushtaq Ahmad Director, Publications, PARC, for critical reading and editing of the manuscript and making arrangements for its publication. To Dr. B.C. Wright COP, MART Project, PARC, we owe special thanks for patronising the publication of the manual.

IFTIKHAR AHMAD,
M. ASLAM
and ANJUM MUNIR

INTRODUCTION

Plant diseases caused by fungi, bacteria, viruses and nematodes take a heavy toll of our crops and the total losses, if calculated at modest rates of 20-25% run into Rs.3600 million per year apart from substantial destruction of crops in years of epidemics (as it happened in Pakistan with wheat during 1978 through rusts and with chickpea in 1980 through blight). Thus plant pathology, which constitutes a big sector of plant protection, is one of the major components in obtaining higher yields and better quality of field crops, vegetables, fruits and forest trees. It then falls upon the plant pathologist to devise ways and means to combat plant diseases so that the losses caused by them to the crop plants are minimized.

For planning an efficient programme of plant disease control proper and correct identification of the plant disease is essential. Plants, unlike animals, do not possess the faculty of speech. Accurate diagnosis of a plant disease, therefore, depends entirely on the technical knowledge and skill of the plant pathologist. Considering the variety of agents that are capable of inducing plant diseases, correct identification of a disease and its cause calls for a high degree of professional competence on the part of the plant pathologist. In addition to possessing the needed basic knowledge of the subject he has to be able to apply expertly all the scientific methods of analysis that might be required and deduce the results without substantial doubt.

Compared with the enormity of plant disease problems that prevail in the country we realize also the fact that there is a dearth of plant pathologists to tackle these. The plant pathologist, therefore, despite his particular area of specialization has, at times, to work as a generalist. Realizing this the Pakistan Agricultural Research Council does its level best to provide the country with well trained manpower for work in various disciplines of agricultural science. This manual on plant disease diagnosis prepared by the Crop Diseases Research Institute, for young scientists who either are on the verge of, or have just entered that field of plant pathology, is a part of this endeavour. It is expected that it will serve as a valuable teaching aid for research workers, extensionists and students of plant pathology.

CONTENTS

	Page
Preface	i
Introduction	iii
List of Contributors	vii
Plant Disease Diagnosis *Iftikhar Ahmad and Khurshid Burney*	1
Sterile Techniques used in Plant Pathology *Khurshid Burney*	5
Microbiological Techniques *Muzaffar Ahmad Khan*	15
Wheat Rust Methodology *Munawar Hussain*	20
Isolation Techniques and Identification for Pythiaceous fungi *Akhtar Ali Shah, Iftikhar Ahmad and M. Aslam*	29
Seed Health Testing *Shamim Iftikhar*	37
Identification of Bacterial Plant Pathogens *M. Afzal Akhtar*	45
Plant Nematology Laboratory Techniques *M.A. Maqbool and Anjum Munir*	52
In-vitro Culture of Plant Parasitic Nematodes *Manzoor H. Soomro*	58
Histological Methods in Host-parasite Studies *Mahmood-H. Nasir*	65
Detection and Identification Methods for Plant Viruses *Saif Khalid*	68

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PLANT DISEASE DIAGNOSIS

Iftikhar Ahmad and Khurshid Burney

INTRODUCTION

An efficient programme of crop disease management is dependent on proper and correct identification of the disease. There are hundreds of diseases that affect crop plants. Each kind of crop plant can be affected by a number of diseases and a particular pathogen can affect several species of crop plants. Therefore, grouping of diseases is necessary to facilitate identification and subsequent management of any given crop disease. Crop diseases can be classified in a number of ways, e.g., according to plant part affected, according to symptoms produced or according to the type of crop affected. The classification of crop diseases according to the causal pathogens is considered most appropriate. In this system diseases caused due to biotic factors are grouped into (1) diseases caused by fungi; (2) bacteria; (3) mycoplasma; (4) parasitic higher plants; (5) viruses and viroids; (6) nematodes; and (7) protozoa. In addition to these a number of disorders are caused due to abiotic factors, such as nutrient deficiencies, mineral toxicities, high/low temperature, light, etc. Diseases caused by pathogens (biotic factors) are infectious and can spread from plant to plant.

To diagnose a crop disease, one has to first determine whether a pathogen is responsible for causing the disease or it is a physiological disorder caused by an environmental factor or deficiency. It is easy for an experienced person to do so but in most cases a detailed examination of symptoms is required.

Pathogen causing the disease is either present on the surface of the organ showing symptoms or inside the plant. Most of the pathogens are present inside the plant. Presence of a pathogen on the surface of a diseased plant indicates that it may be the probable cause of disease. An experienced person can identify it with naked eye or a magnifying lens. However, in many cases microscopic examination of the specimen is necessary. When the pathogen is not present on the surface of the plant, additional symptoms are studied and effort to isolate the pathogen is made. The ease with which a particular disease can be identified is also dependent on the particular group of a pathogen.

DISEASES DUE TO PARASITIC HIGHER PLANTS

Diagnosis of diseases due to parasitic higher plants is rather simple. Parasitic higher plants like mistletoe, dodar, whitch weed and broom rape are found growing on either stems or roots of the host plant.

NEMATODE DISEASES

Nematodes are worm-like, without segmentation on the body. The small size (300 - 1000 μm) of plant parasitic nematodes make them invisible to the naked eye, but they can easily be seen under the microscope. All plant parasitic nematodes have a hollow stylet with which they puncture the plant cells.

Nematodes may infect roots, or above ground parts of the plants. On the roots symptoms appear as root knot galls, lesions or excessive root branching. The plant becomes stunted, chlorotic as in nutrient deficiency, or wilt. When the above ground part of plant is infected, the leaves are distorted, galls or necrotic lesions are formed. The presence of a plant parasitic nematode on or in a plant, indicates that the nematode is most probably the pathogen.

FUNGAL AND BACTERIAL DISEASES

Many fungal and bacterial diseases can be diagnosed by observation with the naked eye or with the help of microscope. For these, isolation of the pathogen is not required.

Presence of fungal mycelium and spores or bacteria on the affected area of a diseased plant indicates that they are either causing the disease or are saprophytes growing on the tissue that has been killed by a pathogen.

In cases where the fungus or the bacterium is mixed with other organisms, or when it has not yet produced its characteristic fruiting structure or spores or when it seems to be a new pathogen, the organism has to be isolated and grown in pure culture. Spores and fruiting structure can also be obtained by placing the diseased tissue in a wet chamber.

When the fungus has been identified by looking at the diseased tissue under the microscope or by isolation in pure culture, it can be checked in appropriate books of mycology or plant pathology to see whether it has been reported to be pathogenic or not on the plant on which it was found. When symptoms on the plant are similar to those given in the book as caused by the fungus in question then the diagnosis is considered complete.

The most appropriate approach to prove that a bacterium is a causal organism of a particular disease is to isolate it in pure culture and inoculate healthy plants using a single colony. The symptoms produced are compared with symptoms produced by a species of known bacteria.

VIRAL, MYCOPLASMIC AND OTHER DISEASES

A number of diseases are caused by viruses , viroids, mycoplasma, rickettsia like bacteria and protozoa. The diagnosis of diseases due to viruses and related

pathogens is difficult due to two reasons. Firstly due to their very small size and secondly because of non-specific symptoms which in many cases resemble those produced by environmental factors, insect damage or by other pathogens.

To determine that the disease is caused by one of the above pathogens and not an environmental factor, the pathogen is transmitted from a diseased plant to a healthy plant and the symptoms are studied.

Transmission of the pathogen is obtained by:

1 Budding or grafting the diseased part on to the healthy plant.
2. Rubbing the sap from the diseased on to the healthy plant.
3. Allowing potential vector of the pathogen to feed on the diseased plants and then transferring them on the healthy plants.

If symptoms appear resembling those of diseased plant, then the disease is certain to be caused by one of the above pathogens and are not a result of the environment. Although very little is known about mycoplasmas, rickettsia like bacteria and protozoa, it is known that they are probably transmitted by budding or grafting or by certain insect vectors. Transmission of the pathogen through the sap or nematodes indicates that the pathogen is a virus or viroids. Further diagnosis to ascertain one of the above pathogens involve a series of tests. Most common of which are:

1. Inoculation of several host plants with the pathogen and comparing the symptoms produced on these hosts with the symptoms produced on the same host by other pathogens.
2. Electron microscopy of the infected tissue and comparison of the morphology of the pathogen with known pathogens.
3. Application of certain antibiotics: (Mycoplasma are often sensitive to tetracycline. Rickettsia like bacteria are often sensitive to penicillin. No effect of the antibiotic indicates the possibility of virus).
4. Heat treatment of diseased plant parts and recovery of symptoms indicate the presence of mycoplasmas or rickettsia like bacteria. If symptoms do not reappear, it would suggest virus etiology.
5. If the causal organism like certain virus can be isolated and purified, serological tests can be used for diagnosis.

IDENTIFICATION OF A PREVIOUSLY UNKNOWN DISEASE

When a pathogen appears to be the most probable cause of a disease but no previous record of the pathogen causing that disease is found, then Koch's postulates have to be performed to verify the hypothesis that the isolated pathogen is the cause of the disease. In such a situation, following steps must be verified:

1. The pathogen must be found associated with all the diseased plants examined.
2. The pathogen must be isolated and grown in pure culture on nutrient media or on susceptible host plant in case of obligate parasites. The appearance and characters of the pathogen must be noted.
3. The pathogen from pure culture must be inoculated on healthy plants of the same species and it must produce the same disease on the inoculated plant.
4. The pathogen must be isolated in pure culture again and its characteristics must be exactly like those observed in step 2.

In many cases Koch's postulates are easily verifiable, in others it may be difficult due to many factors and may require improvement of techniques of isolation, culture and inoculation.

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STERILE TECHNIQUES IN PLANT PATHOLOGY

Khurshid Burney

INTRODUCTION

In nature, pathogens exist in mixed populations with other pathogens and microorganisms. Therefore, study and examination of a particular pathogen requires two kinds of operations: isolation, the separation of pathogen in question from the mixed population that exists in nature; and cultivation, the culture of pathogen in artificial environment in pure form. This requires an understanding of the principles of sterile techniques. These principles are applied in the sterilization of equipment and media, and in the isolation and transfer of cultures.

Following are the five principles of sterile techniques:

1. *Ubiquity of Microorganisms*: An understanding of the fact that microorganisms are present everywhere, therefore, isolation of the desired one is essential from the others.

2. *Creation of Sterility*: To achieve isolation, destruction or removal of microorganisms undesired ones is necessary.

3. *Maintenance of Sterility*: Continued isolation from unsterile objects and contaminants is essential to maintain purity.

4. *Prevention of Contaminants*: Contaminants are microorganisms where they are unwanted. Care is taken to prevent pure cultures from being contaminated.

5. *Confinement of Microorganisms*: Microorganisms causing disease should be prevented from getting out so as to limit their spread.

Application of the first three principles of sterile techniques results in the creation of sterility and application of the last two principles results in the isolation and maintenance of cultures of microorganisms to be studied.

Sterilization and isolation can be brought about by a number of methods which will be discussed under the following headings.

STERILIZATION

Various sterilization methods available include use of heat, chemicals and radiation.

Sterilization by Heat

Steam under Pressure (Autoclaving)

Autoclaving is commonly used because it is usually effective, cheap and requires relatively short period of time. Autoclaves equipped with a jacket, shorten time and reduce the amount of moisture left on the material. Temperature and time required for adequate sterilization of different glassware containers is given in Table 2.1.

Table 2.1. Temperature and time required for adequate sterilization of different glassware containers

Container	Minutes exposure at 121-123°C
Flasks and Beakers	
1 l Erlenmeyer	20 - 25
500 ml	17 - 22
200 ml	12 - 15
125 ml	12 - 14
Test Tubes	
18 x 150 mm	12 - 14
38 x 200 mm	15 - 20

The following recommendations should be followed when using an autoclave.

1. Rely on temperature, not pressure gauge.
2. Keep thermostatic trap clear of plugging substances, check trap if temperature is not being reached.
3. Avoid filling containers more than 2/3rd if not sealed to prevent boiling over and plugging trap.
4. Do not exhaust autoclave rapidly if liquids are being sterilized, plugs will be blown and agar may boil over.
5. Do not use overly tight plugs, these may be blown.
6. Do not fill autoclave with materials requiring widely different times for sterilization.
7. Do not pack load too tight.
8. Increase time for materials that are hard to heat or are heavily contaminated.
9. If agar has solidified, allow time for melting.
10. Do not over-wrap materials.
11. Do not leave paper wrapped items on bottom of autoclave.
12. Investigate heat labile properties of substances to be sterilized relating properties to conditions of test such as pH.

13. Do not autoclave cellulose nitrate tubes, they may explode.
14. If volume of liquids is critical, use tightly capped containers to prevent the usual 3% to 5% loss by evaporation, but allow for a longer sterilization time.
15. Acid agar media will not gel properly after autoclaving, either increase amount of agar or acidify after autoclaving.
16. Empty and dry containers should be tightly stoppered and if possible inverted or laid on side.

Care should be taken while autoclaving as it changes the pH and certain substances like enzymes are denatured by heat.

Steam without Pressure (Tyndilization)

Usually steam without pressure is used for melting agar. As steam without pressure requires more time it brings about breakdown of different substances. Its main use is freeing substances of fungal contaminants.

Hot Dry Air

This process is also longer than autoclaving but it is preferred for sterilization of glass ware because of the lack of moisture residue. Petri dishes can be autoclaved without wrapping. Recommended time and temperature for sterilization with dry air is given in Table 2.2.

Table 2.2. Recommended time and temperature for sterilization with dry air

Temperature(°C)	Time in minutes
170	60
160	120
150	150
140	180
121	overnight

Sterilization by Gases

Gases are mostly used in the treatment of heat labile substances, plant tissue and bulky materials or where steam is not available. Their biggest drawback is that they are slow acting. Most commonly used gases are propylene oxide and ethylene oxide. Propylene oxide is easy to handle. One to three ml of propylene oxide is used to sterilize agar filled petri dishes which are placed under a hood at room temperature. The plates can be used after 24 hrs. Similarly, tissue to be sterilized is moistened and put in a jar to which is added 1ml of propylene oxide. The material can be used after 12 hrs. This does not remove bacteria successfully from seeds. Ethylene oxide is used commercially to sterilize petri plates and other equipment. It is used with a carrier-CO_2 or flourinated methane which eliminates inflammability. Ethylene oxide is toxic and should be handled in an aerated room.

Liquid Ethylene Oxide Sterilization

1. Cool carbohydrate solution to be sterilized to 3-5°C using an ice bath.
2. Add 1% by volume of chilled liquid ethylene oxide using a chilled pipette and agitate.
3. Keep solution in the ice bath for 1 hr.
4. Transfer to water bath at 45°C and allow to volatilize under a hood.

Formaldehyde at different rates is used to sterilize soil.

Sterilization by Filtration

Sterilization by filtration is a superior way to get rid of microorganisms from liquids without changing the properties of the material. Although there are many different types of filters, the most commonly used membrane filters are millipore filters. These filters are available with autoclavable filter holders. The filters with the holders are autoclaved at 121°C for 15 minutes. Care should be taken to keep the membranes dry when autoclaving and the autoclave exhausted very slowly. Filters are believed to have a negligible absorption and do not contaminate a filtrate if ordinary solvents like water, dilute acid or alkali is used.

Sterilization by Radiation

Ultra violet lamps can effectively reduce air-borne contaminants but are somewhat hazardous. Ultra violet lights are usually used to get rid of microorganisms from laminar flow tops. Care should be taken not to look into the UV lamps or loiter around it. Noticeable sign should be posted on it. Antiseptics and disinfectant used for miscellaneous purposes are given in Table 2.3.

ISOLATION OF PLANT PATHOGENS

Most plant diseases can be diagnosed by observation with the naked eye or examination under the microscope. At times the pathogens cannot be identified because it is mixed with other contaminants. Therefore, the pathogen is isolated from the diseased tissue for identification and further study.

Preparation for Isolation

The preliminary operations to be performed before attempting to isolate a pathogen from a diseased tissue are important for successful isolation of the organisms and are mentioned as follows:

1. Glassware such as petri dishes, test tubes, pipettes, etc., are sterilized by dry heat at 160°C for 1 hr or by autoclaving. Glassware can also be sterilized by dipping in one of the cleaning solutions mentioned in Table 2.1. All chemically treated glassware should be rinsed thoroughly in sterile water before use.

2. The surface of infected or infested tissue is cleaned with a suitable cleaning solution so as to get rid of the surface contaminants.

Suitable Solutions

a) 5.75% sodium hypochloride (1:9) with water.
b) 95% ethyl alcohol - leaf dip for 3 seconds.
c) 1:100 mercuric chloride for 4 seconds.

Table 2.3. Antiseptic and disinfectant materials and their uses

Chemical	Conc.	Use	Procedure
Ethanol	70-90%	Disinfection of instruments	Dip instrument, shake excess ethanol, flame
Mercuric Chloride	0.1%	Isolation of microorganisms	Plant parts submerged for a few seconds to a minute
Sodium Hypochlorite (make a fresh solution and store in cool dark place)	1%	Isolation of microorganisms	Plant parts submerged for a few seconds to several minutes. (considered superior to $HgCl_2$)
Formaldehyde alcohol	8% in 70% iso-propyl alcohol	Sterilizing instruments with large amount of organic matter	Expose for eight hours to get rid of all organisms
Formaldehyde	1 part and 10 parts water	Elimination of plant pathogens from green house tools	Clean off debris, soak in this solution for several minutes, rinse with water

3. *Preparation of culture media for the growth of the organism to be isolated*: The most commonly used media for the isolation of fungi is potato dextrose agar and nutrient agar for bacteria. These media and other selective media are prepared according to recipes mentioned in a separate chapter. Solutions of culture media are prepared in flasks which are then plugged and placed in an autoclave at 120°C and 15 psi for 20 minutes. Sterilized medium is then allowed to cool to 45°C and then poured into sterilized petri dishes, test tube or appropriate containers (Fig 2.1). Pouring of the media into petri dishes, tubes, etc., is carried out as aseptically as possible either in a separate culture room, laminar flow hood or in a clean room free from drafts and dust. In either case, the work top table should be wiped with a 10%

chlorox solution, hands should be clean and tools such as scalpels, forceps and needles should be dipped in alcohol or rectified spirit and flamed to prevent introduction of contaminating microorganisms.

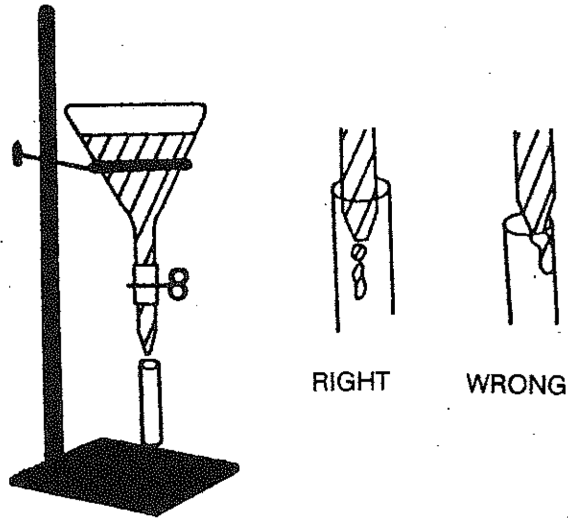

Figure 2.1a. Apparatus and procedure for pouring slants.

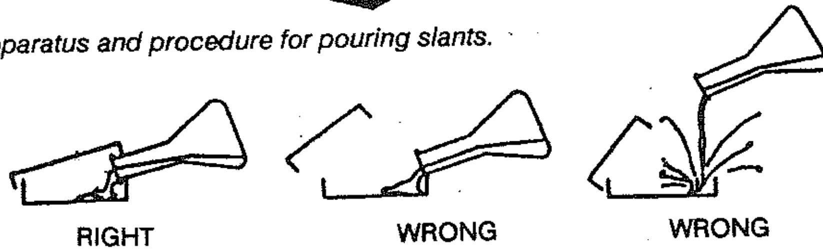

Figure 2.1b. Procedure for pouring plates

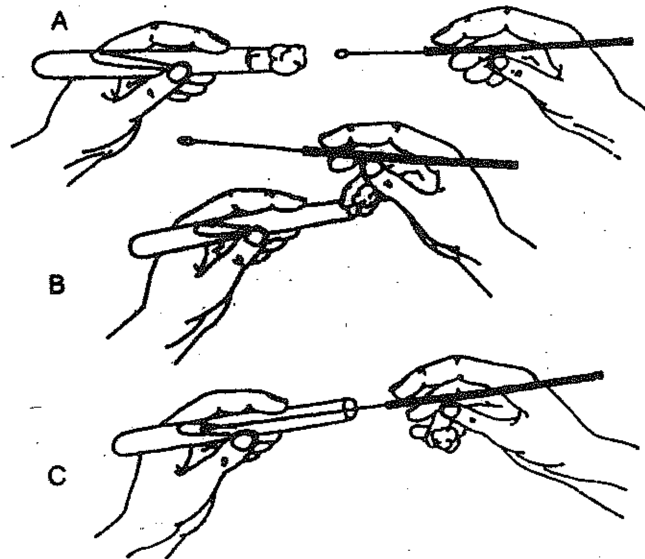

Figure 2.1c. Techniques of handling tubes and transfer loops during transfer of cultures from one tube to another: (A) position of the tube and transfer loop in the hands; (B) removal of the cotton plug; and, (c) insertion of the transfer loop.

Figure 2.1. Techniques for pouring media into slants and petri plates and transfer of cultures.

Among the plant pathogens only all bacteria can be grown on artificial media. Most fungi can be grown on artificial media except the obligate parasites.

ISOLATING THE PATHOGENS

i) *Isolation from Leaves*

In case of fungal leaf spots or blight if spores are present on the surface, a few spores can be shaken on to the media in a petri plate. If the spores germinate the fungus is obtained in the form of mycelial colonies from where they can be sub-cultured on to clean plates.

In case of surface of leaf having other contaminants, pieces of the infected leaf are surface sterilized in 1% chlorox for a few seconds. The pieces are then placed on the medium in petri plates. Plates are then incubated for a few days. Pathogen appears in the form of mycelial colonies, sometimes with contaminants. Pure colonies can be picked up and transferred to sterilized plates of suitable media (Fig 2.2). This work is performed aseptically in a transfer chamber, so that the pathogen in pure culture does not get contaminated.

If on examination under a stereoscopic microscope fruiting structures are present, like pycnidia or perithecia, they can be picked up with a sterile needle surface sterilized in a mild sterilizing solution and placed on a sterilized petri plate having a suitable medium. Mycelial colonies are formed from the spores in the fruiting structures after incubation at suitable temperature.

ii) *Isolation from Stem, Fruits and Roots*

In case of infected stem and fruits the infected portion is surface sterilized with chlorox, then the infected portion is cut and placed on a suitable medium in petri plates. The pathogen will grow from the infected tissue from where it is transferred aseptically to be further studied.

Pathogen from diseased tissue which have been in contact with soil are often with saprophytes. To isolate the pathogen, the roots or any other plant part which has been in contact with soil is first thoroughly washed with running water to remove the soil and decayed plant part in which most of the saprophytes are usually present. After washing, the same procedure is used as described for isolation from other plant parts.

MAINTENANCE AND PRESERVATION OF CULTURES

The pathogens isolated are maintained on suitable media on slants in test tubes. Pure cultures are transferred aseptically from petri plates to the slants. Care should be taken to prevent any contamination while transferring cultures (Fig 2.1).

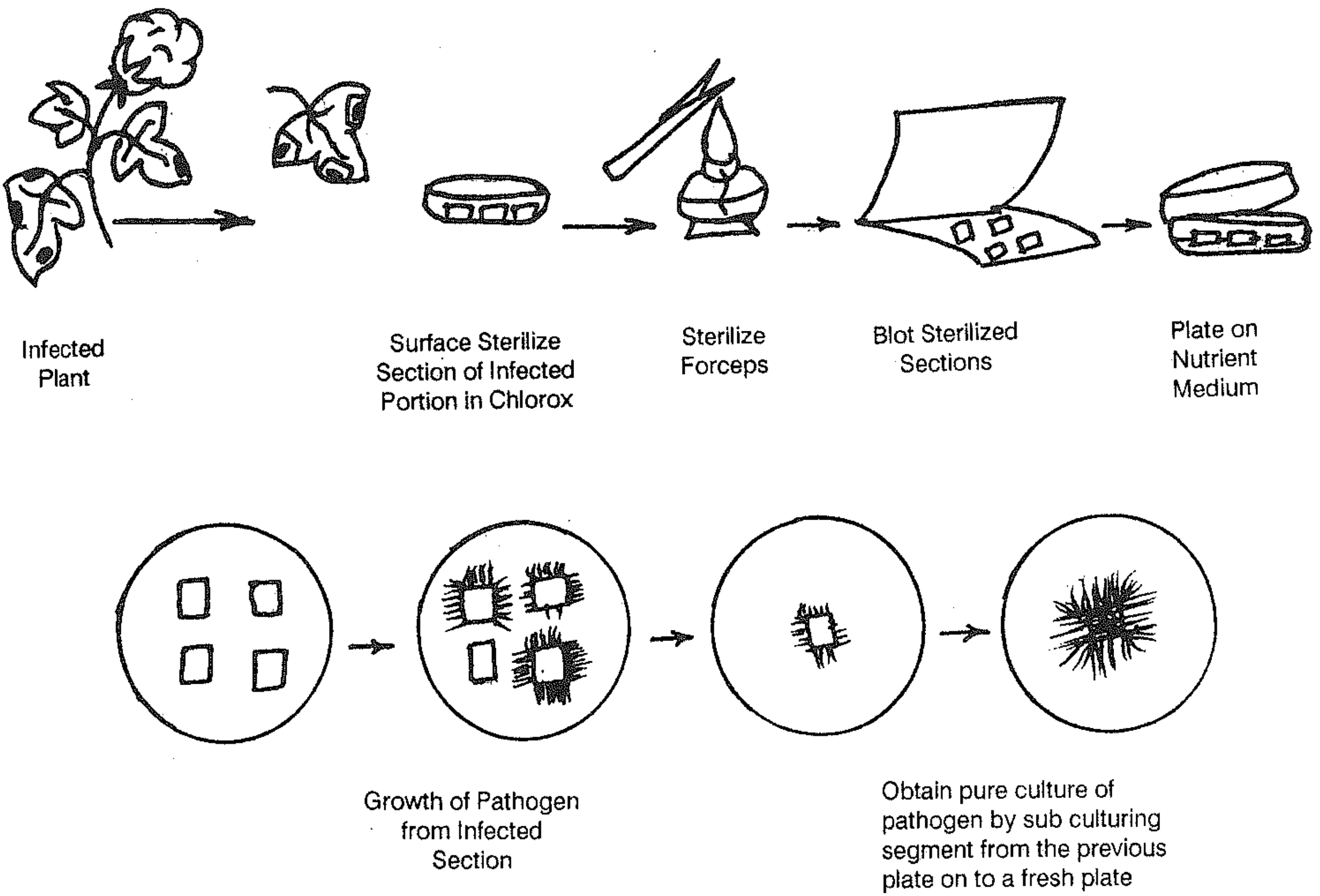

Figure 2.2. Isolation of fungal pathogen from infected plant tissue

Once the pathogen has grown on the slants they are stored at 10°C in refrigerators. For studying the pathogen, the cultures are routinely transferred from slants to petri plates with suitable media.

For long term storage, fungi and bacteria can be preserved by one of the following methods:

1. *Lyophilization*

In this method air is removed from spores or mycelium which have previously been freeze dried with liquid nitrogen. The spores or organisms are suspended in a sterilized colloid such as skim milk, 20% glucose or sucrose solution or beef serum. Lyophil tubes are sealed and cultures stored at 0-4°C.

2. *Oil Overlay*

The organism is allowed to grow vigorously on slants in screw cap tubes. They are then covered with sterilized mineral oil to one cm above the slant and stored at 5-10°C.

3. *Soil*

In this method the tubes with 5g of loam soil are autoclaved for 1 hr at 12°C, cooled and then suspension of the organism added to bring water holding capacity of the soil to 25%. A single tube in this method can be used for continued supply of the organism.

4. *Cryogenic*

In this method cultures are kept in small volumes. Ten to twenty percent glycerol or 10% glucose or sucrose solution is added which aids survival of the organism. The temperature of the organism is slowly brought down by about 1°C per minute down to -20°C. This is brought about by immersing the glass ampules of cultures in liquid nitrogen. These are then kept in deep freezers. When activating the cultures they should be quickly thawed by immersing in a water bath 38-40°C for 1 -2 minutes

IMPORTANT

All through isolation and preservation care should be taken to prevent contamination. Following steps should strictly be followed.

a. Transfer of cultures and pouring of plates should be done in the morning when air is still so that there is less chance of contamination.

b. Transfer of culture should be done aseptically in a place where there is the least turbulence of air and no air drafts.

c. Flame the transfer needle properly.

d. Always flame the neck of the flask while pouring and keep the flask tilted so that no particles fall on the media in the neck of the flask.

e. All bench tops should be swabbed with a sterilizing liquid before use.

f. Transfer chamber should be placed in such a way that there should not be any air draft going in.

g. Transfer chamber should be clear of any obstacle in the way of air coming out of the air filters. Only the plates being poured and necessary equipment should be in it when in use.

h. Always wear an overall and wash hands before and after use.

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MICROBIOLOGICAL TECHNIQUES

Muzaffar Ahmad Khan

INTRODUCTION

In order to study the different aspects of life strategy of an organism, the first thing that one would desire is to obtain the organism in its pure form. To isolate the organisms from their natural habitats in some cases may not be that straight forward, and one might have to perform several types of practices before achieving the desired goal. This write up will mainly deal with microbial isolation, purification, quantification and some other related techniques to study the life strategies of microorganisms.

ISOLATION

A. From Soil System

1. *Soil Dilution Plates*

The most used method is either by incorporating 1 ml of the final dilution in the cooled, melted agar in a petri dish, or by spreading 0.2 - 1.0 ml of the suspension over the surface of a poured set agar plate. Final dilutions of the order 10^{-5} usually give 20 - 50 colonies per plate.

Direct Soil Plating

Crumbs of soil up to the size of a dried pea are placed intact on the surface of a prepared plate (3 or so). After overnight incubation hyphal tip isolation yields fungi representatives of early colonization stages.

Soil Plates

Small soil samples (0.005 - 0.015 g) or suspensions of them, are dispersed across the base of sterile plates and melted cooled agar added.

Immersion Tubes

Plastic centrifuge tubes drilled with 3/4" holes and wrapped with plastic electricians tape are used for burial in soil. After sterilization, a heated needle is used to perforate the tape over the tube perforations.

Screened Immersion Plates

The agar for burial in soil is carried on a glass slide inside a Perspex box with a

lid having 10 spaced holes. After a suitable period of burial, the boxes are removed and any fungal or other type of microbial growth obtained is planted.

Slide Traps

Two standard hanging drop slides are clipped together so that their concavities, lying opposite one another, enclose a small quantity of suitable agar. This technique largely helps to isolate fast growing species.

Hyphal Isolation Method

The method uses the fact that when a soil suspension is made, many of the fungal hyphae remain with heavier soil particles and sediments (and so will be lost from the dilution plating method). The appropriate fraction of the soil is concentrated by sedimentation (different times can be used to separate different fractions) or fractions can be separated by using a series of graded sieves. The concentrated residue is examined microscopically for the presence of fungal hyphae which are gently removed with a sharp pointed forceps or a needle to a agar plate.

Baiting

Suitable baits are numerous, e.g., stems, leaves, surface sterilized roots, seeds, fruit, pollen grains, insect exoskeleton, hair, filter paper, snake skin casts, feathers, etc., and may be chosen with a specific interest.

Partial Pre-sterilization

The soil is treated with heat or chemicals before plating out. Chloropicrin, formalin, carbon disulphide at certain strengths, act selectively killing most, but not all fungal species. This method often yields a high proportion of ascosporic species.

Floating Method

Soil (10 g) mixed with a small amount (5 ml) of mineral oil is shaken up in water. The emulsion which collects on the surface of the water contains 80 - 90% of the spores originally in the soil.

B. Aerial Flora

1. *Exposure of Gel Plates*

Aerial flora/spores can be easily isolated by exposing agar gels of suitable media in air. Germinating spores and hyphal fragments can further be isolated using a stereo microscope.

2. *By Trapping in Aqueous Tween Suspension*

Air spores may also be trapped in Tween 20 or 80 (0.1 - 0.5%) aqueous

suspension. The air is passed through the Tween suspension by either using a vacuum creating device (water jet pump or an air flow pump). The air may be passed for a desired time and then the suspension may be plated as such or in serial dilutions on a suitable agar medium, to obtain growth of microorganism. Further isolation could be made from the agar plates.

C. Aquatic System

1. *From Foam*

The foam accumulated along the stationary objects in an aquatic system is normally very rich in aquatic spores. This foam can be easily collected with the help of a tea spoon and later may be diluted for plating method. Large conspicuous spores are easily picked up by a sewing needle under a stereo microscope.

2. *Millipore Filter*

Spores and other colony forming units of fungi can be trapped on the surface of a millipore filter disks. Then these disks are floated in sterile water gently shaken to remove spores, etc., adhering to the surface of the disk. Later, this suspension can be concentrated or diluted for the plating purpose.

3. *Substrate Incubation*

The substrate of aquatic fungi are collected and brought back to lab in polythene bags. The samples are washed under running tap water for quite some time, and then are given a final wash with sterile distilled water. Later the substrate are incubated while immersed in sterile site water or distilled water for a couple of days to obtain propagule formation. The large conspicuous spore/conidia are easily picked up using a flamed sowing needle under a stereo microscope. For smaller spores plating technique may be used.

Induction of Reproductive Phase

At times certain fungi do not sporulate or form reproductive bodies on artificial culture media, which are normally basis for the identification of fungi. For some fungi, growth on a relatively weaker medium induces sporulation. The dilution can be achieved by immersing slivers of cultures of a fungus in sterile distilled water.

Use of Near Ultra Violet Light

Fungal cultures exposed to N.U.V. for 12-24 hrs in plastic petri plates induces sporulation in certain cases. An ordinary light box having connections for 5 tubes is quite adequate. Even the use of only two tubes gives 80 uw/cm^2 N.U.V. at base.

Purification of Isolates

This is a simple technique to purify a fungal culture heavily contaminated with

bacteria when grown on agar medium. In this situation simply aseptically invert the agar gel in the petri dish that will expose its lower surface to the top. Since fungi can grow into the substratum while bacteria cannot, bacteria free hyphae tips will emerge through the gel that can be then isolated.

Quantification Of Microbial Population

1. *Petroff-Haussen Counting Chamber and Haemocytometer*

Use of Petroff-Haussen counting chamber and haemocytometer are two easy methods to quantify directly spore population or density of colony forming units or a microorganism. The Petroff-Haussen chamber and haemocytometer are precision machined glass plates that have sunken platform at their centre. The surface of this platform is at precision depths (usually 0.02 mn) from the surface of the plate and is etched with a grid system (consisting of squares of various sizes). Because of the precisely machined gap between the grid surface and the overlying glass coverslip, it is possible to relate the number of spores/colony forming units observed in a field bounded by the sides of these squares to the volume of fluid in which they are suspended. Knowing the volume of each square the concentration of spores/c.f.u. ml can be calculated.

2. *Use of Spectrophotometer for Measuring Microbial Population*

Especially in case of bacteria, optical density of their broth cultures can be interpreted against a standard curve to estimate their population ml^{-1}.

Some selective growth media

1. *For isolation of cellulolytic organisms (fungi)*

$(NH_4)_2SO_4$	0.50	g
K_2HPO_4	1.00	g
KCl	0.50	g
$MgSO_4.\ 7H_2O$	0.20	g
$CaCl_2.\ 2H_2O$	0.10	g
Yeast extract	0.50	g
agar	12.00	g
Ball milled cellulose powder	10.00	g
water to	1000.00	ml

2. *Petri modified cellulose agar medium*

$Ca(NO_3)_2.\ 4H_2O$	0.40	g
$MgSO_4.\ 7H_2O$	0.15	g
K_2HPO_4	0.15	g
KCl	0.06	g
Ball milled cellulose powder	10.00	g
agar	12.00	g
water to	1000.00	ml

3. *Malt glucose agar* (*For Fusarium oxysporium Pigmentation in light*)

Malt extract	30.00 g
Glucose	20.00 g
agar	12.00 g
water to	1000.00 ml

4. *Septa and cell wall staining ammonical congo red*
   To stain cell walls and septa use saturated congo red in 10% ammonia.

5. *Ammonical erythrosin*
   Erythrosin 0.1% ammonia is useful to make temporary mounts to examine hyaline walls and septa of species of *Fusarium, Cylindrocladium* and *Gliocladium*.

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WHEAT RUST METHODOLOGY

Munawar Hussain

INTRODUCTION

In Pakistan, wheat is the most important staple food crop. Heavy dependence upon wheat culture has enabled a large number of pathogenic organisms to parasitize this crop. As a result wheat diseases have collectively become one of the major constraints to wheat production. Of these, wheat rusts are by far the most important ones. They are most widespread, regularly occurring and potentially dangerous.

CLASSIFICATION OF WHEAT RUST FUNGI

The three wheat rust diseases result from parasitism of the wheat plant by three different highly specialised fungal pathogens that grow only on living plants. They are called obligate pathogens because of this character. The wheat rust fungi are classified in the order Uredinales, family Pucciniaceae. Thus, three species associated with wheat rust diseases are:

Puccinia striiformis West. Associated with stripe rust; also known as yellow rust or glume rust.

Puccinia recondita Rob. ex Desm. f. sp. *tritici.* associated with leaf rust; also known as brown rust.

Puccinia graminis Pers. f. sp. *tritici.* Eriks & E. Henn. associated with stem rust; also known as red rust or black rust.

Each of these fungi is further divided into physiologic races that are determined on the basis of the differing reactions of certain differential varieties of wheat to different cultures of the rust fungi.

METHOD OF IDENTIFICATION OF PHYSIOLOGIC RACES OF WHEAT RUSTS

Identification of races is based mostly on their behaviour on a set of varieties of wheat known as standard differential varieties. In case of leaf rust, eight varieties have been used to identify more than 200 races of leaf rust. Twelve varieties are used to identify more than 300 races of stem rust. More than 60 races of stripe rust have been identified by using fifteen differential varieties.

The following is the list of wheat varieties used in identification of physiologic races of the rusts:

P.recondita	*P.graminis*	*P.striiformis*
Malakoff	Little Club	Michigan Amber
Carina	Marquis	Ble'rouge d' Ecose
Brevit	Reliance	Strubes Dickkopf
Webster	Kota	Holzapfels Fruh
Loros	Arnautka	Vilmorin 23
Mediterranean	Mindum	Heines Kolben
Hussar	Spelmar	Carstens Dickkopf V
Democrat	Kubanka	Spalding prolific
	Acme	Chinese 166
	Einkorn	Fong Tien
	Vernal	Rouge Prolific Barbu
	Khapti	Petkuss roggen
		Heils Franken
		var. tricoccum
		Webster

SURVEY TECHNIQUES

For the purpose of rust studies the surveys are vital in order to identify new races/virulences as soon as they arise and to keep track of the fluctuations in pathogen populations that occur year to year and place to place. The ultimate aim is to develop suitable disease management strategies. There are two basic types of survey; i) use of mobile units and ii) static units e.g., establishing trap nurseries in different agro-ecological zones of the country.

SAMPLING

To assess the level of rust attack and distribution of races accurately it is necessary to take samples from different locations/cvs by using the procedures, mentioned below:

Random Sampling

Appraising wheat fields at every 10-15 km

Area Sampling

Examining all fields in selected areas.

Stratified Sampling

Sampling five wheat fields for every field of barley if the wheat area is five times that of barley. Plotting of the long distance movement of specific diseases

provides an effective early warning system so that susceptible varieties may be exploited to their maximum without the danger of falling victim to disease.'

COLLECTION AND PRESERVATION OF RUST SAMPLES

a. Rust samples are collected in the uredial stage from all kinds of wheat cvs., collateral hosts, etc., grown in different localities of the country.
b. Care is exercised not to contaminate the hand with spores in order to avoid contamination of successive samples.
c. Rust samples are put in envelops measuring about 10 x 22 cms.
d. Necessary information for each sample such as crop, name of variety, growth stage, locality, date of collection, disease and its intensity and collector, etc., is written on each envelop.
e. Before despatch to rust laboratories it should be dried at room temperature for about 24 hours.
f. On receiving the rust samples at the laboratories they are entered in the register and each sample is given an accession number.
g. The samples are then preserved in cool and fairly dry place in a refrigerator at about 4°C until the time of race identification.

INOCULATION TECHNIQUES

For purpose of race identification, plants of susceptible variety are grown in any suitable container, such as 10 cm clay or plastic pots. It is always better to use disinfected soil and seed to reduce danger of root rots.

Plants should be well spaced in pots, about 12-15 cm pot. They should be grown in good soil, under good light at a suitable temperature and in a rust free room. The seedlings can be inoculated when 5-10 cm tall.

In case of all methods of surface inoculations it is advantageous to rub plants gently between moist fingers in order to remove the wax layer, then automize with water, apply the inoculum and immediately automize again in order to produce initial dew on the plants.

The seedlings can be inoculated by one of the following methods:

a. Applying inoculum by means of spatula.
b. By dusting or gently rubbing the leaves with rusted plants.
c. By using baby cyclone.
d. By using tooth pick covered with cotton moistened with water agar.
e. By using fine hair brush.
f. Automizing with non-phytotoxic mineral oil.

After inoculation, plants are incubated for 24 hr in incubation chambers with almost 100% relative humidity. For leaf and stem rust plants are incubated between

18-22°C and for stripe rust at 9-10°C. After inoculation the pots are placed on the green house benches at 20-25°C and 16-20°C for leaf and stem rusts and stripe rust, respectively.

TAKING OF NOTES (INFECTION TYPES)

Depending on the temperature, full development of uredia usually occurs after 10-14 days of incubation in case of leaf and stem rust and 14-16 days in case of stripe rust. Readings are then taken according to infection types on the differential varieties.

For race identification and assaying the virulence of a particular pathotype, system developed by Stakman and his co-workers in 1919 for stem rust of wheat is used. Later, this system was adopted to most of the cereal rusts except stripe rust. There are three reaction classes viz., resistant, susceptible and mesothetic, which are summarized as under:

Resistant includes			
0	=	Immune	No uredia or other microscopic sign of infection.
0	=	Nearly immune	No uredia but hypersensitive necrotic or chlorotic flecks.
1	=	Very resita	Uredia minute and surrounded by chlorosis or necrosis.
2	=	Moderately resistant	Small to medium uredia often surrounded by chlorosis or necrosis.
Susceptible includes			
3	=	Moderately susceptible	Medium sized uredia associated with chlorosis or rarely necrosis.
4	=	Susceptible	Large uredia without chlorosis or necrosis.
Mesothetic includes			
X	=	Heterogenous	Random distribution of different uredia on single leaf.

USE OF KEY

For leaf rust key devised by Johnston is used to determine the race number. Similarly for stem rust key developed by Stakman, Stewart and Loegering is used. More recently, the genetic basis of physiologic specialization was discovered and physiologic races are now identified on the basis of the combination of genes for

virulence and avirulence that each pathotype has. For this purpose single gene lines are available for studying leaf and stem rust.

In case of stripe rust extensive studies were made in Germany by Gassner and Straib (1932, 1934) and Straib (1935, 1937) introducing a set of 15 cvs of wheat, barley and rye which was widely used.

Johnson et al (1972) developed a binary notation system for race nomenclature. In this system each differential host is allocated a fixed value, known as decanery value. Rust reactions are classified as either resistant, with binary score 'O' or susceptible having binary score '1'. The total decanery values of susceptible cvs is thus added to obtain a race number.

The differential cvs currently used in Europe, Australia and elsewhere consist of world set and European set. The sets along with decanery values and presence of Yr gene is presented in Table 4.1.

Table 4.1. Wheat differential sets currently being used for yellow rust studies

Set	Designated resistant Yr gene	Decanery Value
World set		
Chineses 166	Yr 1	1
Lee	Yr 7	2
Heines Kolben	Yr 6	4
Vilmorin	Yr 3	8
Moro	Yr 10	16
Strubes Dickopf		32
Suwon 92/omer		64
Riebesel 47/51 or Clement	Yr 9	128
Triticum spelta album	Yr 5	256
European set		
Hybrid 46	Yr 4	1
Reichersberg 42	Yr 7+	2
Heines peko	Yr 6+	4
Nord Desprez	Yr 3	8
Compair	Yr 8	16
Carsten V		32
Spalding prolific		64
Heines VII	Yr 2+	128

The infection types are recorded according to the scale proposed by McNeal et al., 1971. The infection types from 0-6 are treated as resistant and 7-9 are considered as susceptible. The scale is given in Table 4.2.

Table 4.2. Scale of infection types used for yellow rust

Description of ITL	Code symbol	Index value
No visible infection	0	0
Necrotic/chlorotic flecks no sporulation	VR	1
Necrotic/chlorotic stripes no sporulation	R	2
Necrotic/chlorotic stripes with traces of sporulation	MR	3
Necerotic/chlorotic stripes with light sporulation	LM	4
Necrotic/chlorotic stripes with intermediate sporulation	M	5
Necrotic/chlorotic stripes with moderate sporulation	HM	6
Necrotic/chlorotic stripes with abundant sporulation	MS	7
Necrotic/chlorotic sporulation area with abundant sporulation	S	8
No necrosis/chlorosis with abundant sporulation	VS	9

INOCULATION TECHNIQUES IN FIELD

Inoculation techniques employed in the field are different to those already mentioned, as the objective is to create epidemic conditions in thousands of plants over a large area. Moreover, there is no control over environmental conditions.

a) Dusting

This method is very simple and most effective way of inoculating large number of plants. The urediospores are mixed with talc. The ratio of spores to talc depends on the quantity of inoculum available, their germination percentage and the size of the area to be inoculated. The spore/talc mixture is dusted by the help of dusters available in the market in different sizes.

Dusting is always recommended to be carried out in late evening preferably just prior to dew formation and when the air is still.

b) Injection

This method of inoculation is most reliable in drier climates. Spore suspension in water to which a few drops of Tween 20 (a mild detergent) are added to break the surface tension, are injected into the leaf sheaths, using a hypodermic syringe. The inoculations are conducted at the late tillering stage of plant growth. Although this method is very time consuming but it always help in good disease establishment. For initiation of disease in the nursery the inoculations are conducted only on border rows and usually 2-3 tillers are inoculated after every 30 cm. By evolution of automatic syringe this method is more popular and convenient.

c) Oil Inoculation

The rust spores are suspended in non-phytotoxic oil and applied on the plants either by knapsack sprayer or a battery operated turbo micro sprayers. Oils with different trade names are sued e.g., Mobilsol 100, pegasol and soltrol 70. About a gram of spores in a litre of oil is enough to inoculate 4000 sq. m. with the help of turbo micro sprayer.

d) Planting Infected Plants

The plants previously infected in a greenhouse are transplanted between border rows. Care is taken to establish these plants. Good results have been obtained using this method as the infected sources produce spores rapidly and continuously.

METHOD TO RECORD RUST DATA IN FIELD

Rust data in the filed is recorded as severity and response. For the sake of uniformity Cobb published a scale in 1892 representing five degrees of rustiness 1, 5, 10, 50 and 100% of the leaf area occupied by rust pustules. Later in 1971, this scale was modified and adopted by USDA. The modified Cobb's scale is now widely used to assess the rustiness world over. There are six degrees of severity viz., 5, 10, 25, 40, 65 and 100 percent. The 100% severity accounts for 37% of the actual leaf area covered. Below 5% severity trace to 2% intervals are used. Peterson et al. (1948) proposed a further modification of the scale in which different sizes of the pustules and their distribution are depicted in four series of diagrams (each series containing twelve actual diagrams) covering a range of pustule sizes and their distribution (Fig. 4.1).

The severity (percentage of rust infection on the plants) and response (type of disease reaction) are recorded according to the scale developed by Loegering in 1959. For rust severity it is common to observe the following intervals:

Trace, 2, 5, 10, 20, 30 ,40, 50, 60, 70, 80, 90 and 100% infection.

The response of a variety refers to the infection type and is classified according to the following letters.

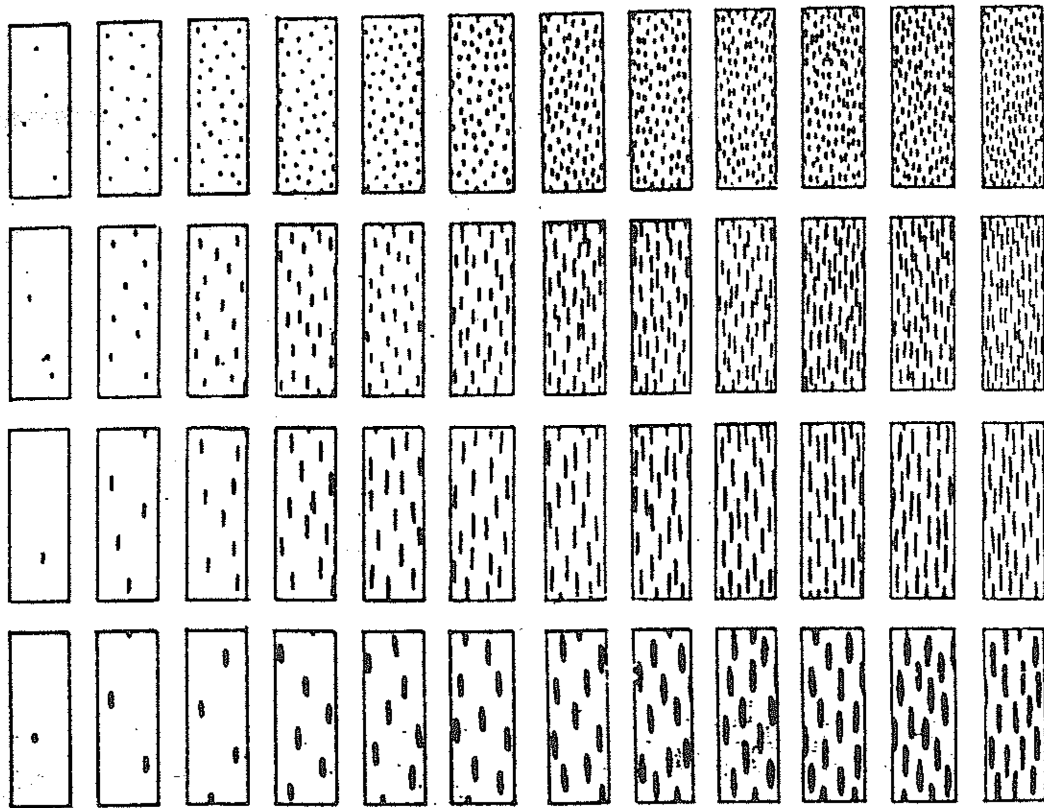
O - No visible infection
R - *Resistant*: necrotic areas with or without small pustules.
MR - *Moderately resistant*: medium sized pustules; no necrosis, but some chlorosis possible.
MS - *Moderately susceptible*: medium sized pustules; no necrosis but some chlorosis possible.
S - *Susceptible*: large pustules; no necrosis or chlorosis.
X - *Intermediate*: pustules of variable size; some necrosis and/or chlorosis.

Severity and response readings are recorded together with severity first. Some examples are given below:

TR - Trace severity of a resistant type infection.
10MR - 10% severity of a moderately resistant type.
50S - 50% severity of a susceptible type infection.

Sometimes variability in reaction within a line under test is observed. This variability may occur in several forms.

1. Clear cut separation of plants into two or even three classes (10R-30MS).
2. A range of reaction without clear separation (10R-10S).
3. A range of reaction on each plant.



A.	0.37	1.85	3.7	7.4	11.1	14.8	18.5	22.2	25.9	29.6	33.3	37.0
B.	1	5	10	20	30	40	50	60	70	80	90	100

Figure 4.1 Diagrams to illustrate degrees of rust severity when the uredia are of different sizes (after Peterson, Camphell and Hannah, 1948) A is the actual percentage of the surface covered by lesions and B is the visual percentage.

The third reaction is most probably due either to a race mixture or to an X reaction of a variety whereas the first two reactions may result from either segregation or seed mixture.

Sometimes difficulty is encountered in recording observations. In such eventualities the following letters are used;

e - *(escape)* sometimes a variety of line will have little or no rust or it matures early.

n - often diseases such as stripe rust, powdery mildew or other leaf spots are so severe that it become difficult to score for leaf rust, then the letter 'n' should be used.

blank - When the data cannot be recorded for any other reasons, the space should be left blank.

Most of the techniques narrated above are as a result of long experience in rust handling by the author.

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ISOLATION TECHNIQUES AND IDENTIFICATION FOR PYTHIACEOUS FUNGI

Akhtar Ali Shah, Iftikhar Ahmad and M. Aslam

INTRODUCTION

Pythiaceous fungi are relatively difficult to isolate from diseased tissues and soils. However, the recent availability of new baiting techniques and selective media has made it easier to isolate this group.

SELECTION, COLLECTION, TRANSPORT, STORAGE AND PRE-ISOLATION TREATMENTS OF SAMPLES

Incorrect selection of samples at the time of collection or the loss of viable propagules during transport or storage due to improper handling of samples may result in negative recovery of *Phytophthora* and related fungi from tissues or soils in spite of proper choice of isolation techniques or careful isolation procedures in the laboratory.

Soils near the root zone or next to other underground plant parts are expected to harbour higher populations. As Pythiaceous fungi succumb easily to dryness and because soil near the ground surface is more subject to high temperature from solar radiation and to drying. Therefore, soil samples should be chosen from at least 3-5 cm below the soil surface.

Proper recognition of symptoms of *Phytophthora* and its related fungi considerably improves the chances of isolation. *Phytophthora* infections on stems and roots result in discoloration and are usually restricted to the barks or cortical regions, with hyphae extending internally to the cambium but not deep into the wood. Dark lesions, in the wood are largely from secondary invaders. *Phytophthora* lesions on fruits, leaves and petioles are almost always brown, purplish brown, or black and are firm in texture. Soft water soaked lesions are probably not those produced by *Phytophthora*.

Exposure of samples to drying or high temperature during transit will result in loss of viability of Pythiaceous fungi. Samples should be collected in plastic bags or similar closed containers and kept in a portable, insulated ice chest or similar containers. Most *Phytophthora* spp. are sensitive to extreme cold as well. Therefore, excessively low temperatures during sample transport should also be avoided. Avoid direct contact of samples with the icy water from melted ice accumulated at the bottom. Use amount of ice in the container which is necessary only to maintain a cool, non-lethal temperature during transit.

Avoid both excessively high and low temperature during pre-isolation storage of samples. The general practice of storing samples in a refrigerator (4-6°C) to reduce growth of secondary bacteria and fungi also reduces the chances of *Phytophthora* recovery because of its sensitivity to extreme cold. A cool incubator at 10-16°C is generally more desirable than a cold refrigerator. The chance of recovery is best if isolation is performed immediately after samples are collected. Lengthy storage at any temperature is not desirable. Under certain circumstances, such as with extremely dry soil samples, a pre-isolation moistening of the sample might enhance *Phytophthora* recovery.

ISOLATION AND QUANTITATION OF PROPAGULES OF PYTHIACEOUS FUNGI FROM SOIL

Several methods have been devised for the direct isolation and quantitation of Pythiaceous fungi from soil. These are:-

1. *Soil Dilution Plate Method*

Two gram soil sample is suspended in 18 ml sterilized distilled water which gives a dilution of 1/10. Using sterilized pipette 2 ml of soil suspension is mixed in 18 ml water and a serial dilution of 1:100, 1:1000 and 1:10000 is prepared. Using a fresh sterile pipette 1 ml aliquot sample is poured in a 9 cm sterilized petri dish and mixed into approximately 10 ml sterile agar medium pH 5.5. The dishes are incubated at 25°C. Fungi growing in dilution plates are counted and identified. The number multiplied by the dilution factor gives total number of propagules per g soil (Waksman, J. Bact., 7:339-341, 1922).

2. *Soil Plate Method*

Small soil sample (0.005-0.01g) dispersed in 1 ml of sterile distilled water in a petri dish and approximately 10 ml melted cool agar (pH 5.5) is poured and mixed. The soil particles are distributed throughout the medium by rotating the dish. The dishes are incubated at 25°C. Fungi growing in soil plates are identified (Warcup, Nature, Lond., 178:1477, 1950).

3. *Capillary Immersion Tube Method*

Glass capillary tubes about 0.5-1.5 mm in diameter are prepared from pieces of glass tubes 8 cm long and 6 mm in diameter. One end of the capillary tube is plugged with cotton wool and the capillary is placed in a beaker filled with melted agar about 5 cm height and autoclaved. When solidified the capillary tubes are cleaned and examined under the microscope for growth of the fungal mycelium. The ends are broken off and transferred on agar to get a fungal colony. (MacWithey, Report of 36th Amm. Conv. of North West Assm. of Horticulturists. Entomologists and plant pathologists, pp. 5-6, 1957).

4. *Floatation Method*

Soil mixed with a small amount of liquid paraffin is shaken up in water. The emulsion which collects on the surface of water contains 80-90% of spores originally in the soil. This is transferred on agar medium and fungi identified (Leedingham & Chimm. Can. J. Bot., 33: 298, 1955).

The soil dilution plate method is generally considered the most accurate method and by far is the most commonly used one for quantitation of soil populations of pythiaceous fungi, particularly *Phytophthora* spp. However, it is effective when inoculum densities in soil samples are relatively high (e.g., greater than 10 propagules per gram (ppg, of soil on a dry-weight basis). It is especially useful in quantitation of artificially infested experimental soils, containing high populations. Naturally infested field soils usually contain low *Phytophthora* populations, often less than 1 ppg and therefore, this method is not suitable in such cases. In natural field soils *Phytophthora* propagule distribution is generally uneven, therefore, soil sample used for making the initial dilution should not be less than 10g for each replicate. In case of uniformly mixed, artificially infested soils 2-3 g may be sufficient.

The above mentioned methods are good and can be used for isolation of Pythiaceous fungi from soil with the use of appropriate selective medium.

DETECTION AND ISOLATION OF *PHYTOPHTHORA* FROM SOIL WITH BAITING TECHNIQUES

Various plant parts (e.g. fruits, seeds, seedlings, cotyledons, leaves and leaf discs) of susceptible host plant can be used as baits, or traps for selective isolation of target *Phytophthora* and *Pythium* spp. from soil.

Various baiting methods can be grouped into three general categories. (1) Inserting soil or (infected tissue) into a wound or hole made on a fleshy fruit (e.g., apple, cacao pods or watermelon). This method requires a relatively large fruit to provide a thick substrate and can involve only a small sample of soil or tissue per replicate. (2) Planting seeds, seedlings, root cuttings, etc., to soil in pots or in the field, followed by heavy watering. This method can sample a larger volume of soil but is generally of longer duration and more time consuming. (3) Floating, partially immersing, or otherwise incubating baits of various types in a water and soil mixture, oversaturated with water or with a high water/soil ratio, in containers of various types. Floating is the most widely used baiting method and is considered effective for isolating most *Phytophthora* spp. Some of the commonly used baiting techniques are given as under:

1. *Apple Fruits*

Place a moist soil sample into a hole made in a firm unblemished apple, cover

with tape or petroleum jelly and incubate at room temperature. *Phytophthora cactorum, p. cinnamomi* or *P. citricola* cause a firm decay within 5-10 days. This technique is modified by making two holes at an angle so that they meet at the apple core. One hole is filled with the moist test soil and the other supplied daily with sterile water and incubated at room temperature. Fruits showing decay are split and isolations made from advancing margins on VYS agar medium

Another modification places soil sample 2 cm thick in a plastic tray and tap water added to 0.5 mm above the soil. Healthy firm apple fruits are placed gently into the sand and incubated for a few days at 15-20°C, when firm brown lesions develop, the fruits are removed from tray, surface sterilized, peeled and tissues from advancing margins are plated on VYS agar. The *P. cactorum* and *P. citricola* are isolated at 20°C and *P. syringae* at 16°C. Unblemished semi-ripe pears as well as firm avocado fruits can be used to isolate *P. cactorum* and *P. cinnamomi.* The optimum incubation is 48-96 hours at 27°C.[Schwinn (1961), Campbell (1940)].

2. *Citrus Fruits and Leaves*

These are used to isolate *Phytophthora* spp. attacking citrus. Place about 100 ml of test soil in a container and flood with water 1-2 cm. Cut citrus leaf pieces 3-5 mm. square or as round discs from mature leaves and float on the surface of water for 3-4 days at 22-28°C. Zoospores gather along the cut edges and form sporangia. Sporangia will not form on sunk leaf tissues or along natural margins. After incubation examine it microscopically under binocular microscope. For preparation of pure culture surface sterilize the leaves and plate on agar medium.

If citrus fruit is used, saturate the test soil with copper free water, place clean unblemished lemon or citrus fruits washed with O.1N HNO_3 and rinsed with distilled water on the soil surface for 5 days at 20°C or until brown lesions appear. When firm brown lesions develop fruits are surface sterilized and tissues from advancing margins are planted on selective media given below. Potato slices about 5 mm thick covered with 0.7 ml of the test soil, wetted and incubated in a humid chamber for about one week at 20°C are also used to detect *P. infestans* (Grimm & Alexander, 1978).

3. *Safflower Seedlings*

Safflower seedlings are used to detect *P. cactorum* in apple orchard soils. Safflower seedlings are grown in steamed soil. To one week old seedlings 15 mm layer of test soil was applied, watered regularly, incubated at 22-28°C for 3-4 weeks. Usually dark brown lesions appear at the crown portion after one week. Seedlings are then thoroughly washed, surface disinfected and plated on media for growth and identification of the pathogen.

4. *Apple Cotyledons*

Apple cotyledons are used to isolate *P. cactorum* from apple orchard soils.

Healthy cotyledons selected, are placed immediately in tap water to avoid desiccation. Four to six cotyledons floated over 30 ml sub sample of test soil, in a sterile deep glass petri dish (100 x 20mm) floated with 60 ml distilled water. Firm brown colonized cotyledons were immersed in fresh water and examined microscopically. For preparation of pure culture, infected cotyledons were plated on VYS agar medium. *Eucalyptus* cotyledons are also used in this manner. (Jeffers & Aldwinckle, 1987).

5. *Alfalfa Seedlings*

Alfalfa seedlings are used for isolation of *Phytophthora megasperma* as well as *Pythium* spp. Germinate surface sterilized alfalfa seeds on moist filter paper under aseptic conditions for 2-3 days at 22-28°C or when the radical is between 6-10 mm long and the ruptured testa can be slipped off. Injure the radical by squeezing the tips with forceps, then float them on water covering 30 ml of test soil to 3-4 mm in a 9 cm culture plate using six seedlings per plate and incubate for 4-6 days at 20°C. Water soaking appears in 2-3 days and sporangia form on the water surface around the bait which can be seen under a dissecting microscope. (Marks & Mitchell, 1970).

Although infection of bait materials generally produces characteristic *Phytophthora* lesions or symptoms, they are not always a reliable indication of *Phytophthora* infection; similar lesions may result from infections by some other pathogens. Sporangium formation on the bait, as determined by microscopic examination, is considered more reliable and widely used.

If sporangia do not form on bait, transfer colonized bait tissues to soil extract, petri solution, or other mineral salts solutions and incubate under light to induce sporulation to confirm presence of *Phytophthora*. Soil bacteria and *Fusarium* spp., have been reported to infect the bait, suppressing sporulation and making it difficult to observe the sporangia of the target *Phytophthora* sp. Therefore, workers prefer the more reliable method of positive *Phytophthora* identification by plating infected bait tissues on a selective medium

DETECTION AND ISOLATION OF *PHYTOPHTHORA* WITH SELECTIVE AGAR MEDIA

Phytophthora was among the first plant pathogenic fungi for which selective isolation media were devised. Since then a great number of good selective media have been reported and widely used for isolation and quantitation of many *Phytophthora* spp. A summarized list of these media is given in Tsao 1983. Some of the representative media and their preparation are given here.

1. VYS-PCNB AGAR FOR *PHYTOPHTHORA* ISOLATION

Distilled water	1000.0	ml
V-8 Juice	40.0	ml

$CaCO_3$	0.60 g
Becto yeast extract	0.20 g
Sucrose	1.0 g
Cholesterol (2ml.N.N.dimethyl formamide sol.)	0.01 g
Benlate (50% Benomyl)	0.02 g
Terrechlor (75% pentachloronitrobenzene)	0.027g
Neomycin sulfate	0.100g
Chloroamphenicol	0.010g
Becto agar	20.0 g

Autoclave the V-8 Juice, $CaCO_3$ and distilled water for 15 minutes at 121°C, filter through whatman # 1 filter paper with a 1 cm pad of celite 45 (John Mansville co.) filter aid; add chloroamphenicol; disperse the cholesterol by shaking , add agar; and autoclave. The V-8 Juice concentrate can be prepared by autoclaving 200 ml juice with 3 g $CaCO_3$ and 200 ml distilled water, and stored unfiltered until needed.

2. SA-PCNB AGAR FOR *PYTHIUM* ISOLATION

Distilled water	1000.00 ml
Sucrose	2.40 g
Aspargine	0.27 g
K_2HPO_4	0.15 g
$K_2 HPO_4$	0.15 g
$MgSO_4. 7H_2O$	0.10 g
$ZnSO_4. 7H_2O$	0.0010 g
$MnCl_2. 4H_2O$	0.0007 g
$FeSO_4. 7H_2O$	0.0044 g
Thiamine HCl.	0.002g
Ascorbic acid	0.010g
Cholesterol(2ml N.N.-dimethyl formamide sol.)	0.010g
Benlate(50% Benomyl)	0.020g
Terrachlor (75% Pentachloronitrobenzene)	0.027g
Neomycin sulfate	0.100g
Chloroamphenicol	0.010g
Becto agar	20.00 g

The ingredients should be added in the order in which listed and all can be added prior to autoclaving. The solution should be shaken thoroughly immediately after addition of cholesterol solution to suspend it. PCNB can be increased to 50 mg/l if difficulty is anticipated from *Rhizopus* or *Alternaria*. If a low calcium agar is used, 80gm/l $CaCl_2.2H_2O$ should be added. Vancomycin at 100 mg/c is as good as Neomycin- chloramphenicol for bacterial control.

ISOLATION OF *PYTHIUM* AND *PHYTOPHTHORA* FROM DISEASED TISSUES

Isolation of Pythium

Pythium spp., are relatively easy to isolate as they grow rapidly on a variety of media commonly used by plant pathologists. Problems may occur when material is badly rotten due to contamination. This can be handled with the washing technique. Rotted tissues are washed in running water or by frequently changing the water followed by submersion of the tissues in water. Pieces of tissues are observed under a microscope. Pieces of tissues with *Pythium* mycelium are removed, blotted and placed on media given above. *Pythium* spp., is reported to grow better than other fungi under submerged conditions, therefore, another method of handling *Pythium* is to invert agar over small pieces of roots. This method is considered very useful for freeing *Pythium* of contaminating bacteria.

Isolation of Phytophthora

In general, techniques used for isolation of *Pythium* are also suitable for *Phytophthora*. The dilute clarified V-8 Juice agar has been found excellent for most *Phytophthora* spp. Select actively progressing lesions, or diseased tissue that has not been desiccated or corked-off from healthy tissues. Diseased tissues in direct contact with soil should be thoroughly washed and the outer layers of the bark stripped off. Selective inhibitors can handle all bacteria or common saprophytic fungi such as *Alternaria, Fusarium, Trichoderma, Phomopsis, Batryosphaeria, Rhizopus,* so surface sterilization is considered unnecessary. Suitable diseased tissue is selected near the advancing margin, or anywhere in the diseased area which has remained wet. *Phytophthora* spp., grow more readily on VYS agar than on the SA agar. Inverting agar over the pieces of diseased tissue is useful for establishing a bubble (moist chamber) over each tissue piece, and facilitates isolation of pure culture. Small pieces of diseased tissues (0.5 x 0.5 x 0.25 cm) are prepared if the agar is to be inverted. This technique has been used successfully in the field during disease survey, because of the inhibitors in the media, contamination by air borne species is of little concern. (Schmithenner, 1973).

For the identification of Pythiaceous fungi following literature is useful.

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SEED HEALTH TESTING

Shamim Iftikhar

INTRODUCTION

Diseases always play a negative role in agriculture. About 16% i.e., 550 million tonnes of total crop production in the world per annum is lost due to plant diseases (James, 1980) and according to Richardson (1929) more than thousand diseases of 500 crop species are seed borne. Coincidently, the most devastating crop diseases are seed borne. Therefore, seed health testing is very essential for reducing losses in crop production. Diseased seeds can sometimes be detected by visual examination of dry seeds. However, most of the time a detailed study involving special techniques is required for confirmation of seed health. These techniques have been of great value in plant quarantine situations, where the most sensitive tests are needed to detect seed borne pathogens.

DETECTION OF SEEDBORNE FUNGI

1. Direct Examination

This method helps in preliminary screening to detect heavily infected seed stocks by observation. Diseased seeds are shrivelled, colour less, small sized and colour difference with yellow shine, or waxy pink staining is usually due to *Fusarium* and brown spots are indicative of infection with *Rhizoctonia* but cultural studies are necessary for confirmation and identification. Sori of rust, pycnidia of *Pleospora* and setae of *Colletotrichum* may be seen on seeds. Sclerotia or ergot of *Typhula* can easily be observed.

Extension of direct examination are:

a. Washing Technique

Soak the seeds to allow spores or nematodes to float off and then detect in washing fluid or by collecting on filter paper. Using this method it is possible to detect *Pyricularia oryzae* in rice, *Cercospora bataticola* in beat, microsclerotia of *Verticillium* in cotton seeds and *Tilletia* spp., in wheat. For this test take out hundred seeds in a 250 ml conical flask containing 25 ml of sterilized water, then shake the seeds on a mechanical shaker for 10 minutes and centrifuge at 3000 rpm for 20 minutes and then examine the sediments under compound microscope for identification of fungal spore (Khanzada and Jamil, 1980). Electron microscopy of washing detects the superficial viruses.

b. Embryo Test

This technique has been described for the detection of loose smut infection in

wheat embryos (Khanzada et al., 1980). The 120 g of seed are soaked for 22 hours in one liter of 5% NaOH to which 0.15 g of trypan blue has been added and kept at 22-24°C. The sample is transferred to eelworm floating vessel (Fenwich can) connected by a tube to a hot water tap. A stream of hot water at 60 - 65°C agitates the vessel where they pass over the top with the flow of water and are run off into as series of graded sieves arranged in descending order of 3.5, 2.0 1.0 mm perforations. The seives are washed through to carry to the bottom and embryos are caught in the first two sieves.

The embryos are washed into a wire basket and dehydrated in methylated spirit or 95% alcohol for two minutes. The embryos are transferred to a filter funnel and a lactophenol and water mixture (3:1) is added, the embryos float and the chaff sinks and can be run off. This process may be repeated until a reasonable clean sample of embryos is obtained.

Finally, the embryos are placed in a 250 ml beaker with 75 ml fresh water free lactophenol and are cleared by boiling for about two minutes. Embryos are best examined, submerged in lactophenol at 12X and 25X magnification using a stereoscopic binocular microscope with substage illumination. Bluish mycellium of *Ustillago tritici* is easily seen in the scutellum of the infected embryos.

2. Incubation Method

i) *Blotter Method*

This method is used for the detection of fungi associated with seeds of all the crops, as suggested by ISTA (1976). Plate 400 seeds of samples at equidistantly (depending on size) over three layered well soaked filter papers in petri dishes of 9 cm diameter under sterilized condition. Incubate the plates at 20 ± 2°C under 12 hours of alternating cycle of ultraviolet light (NVY) and darkness for seven days. The NUV light enhances the sporulation of many fungi. On 8th day examine the plates for presence of fungi under stereobinocular and further examine under compound microscope for confirmation.

ii) *Deep Freezing Method*

After plating the seeds as blotter method, incubate first at 20°C for 24 hours and then again incubate at 20 ± 2°C under 12 hours alternating cycles of NUV and darkness for five days. The deep freezing kills the seed and the subsequent fungal development is often more abundant, but it depends on particularly host-pathogen combination.

iii) *Ragdoll (Rolled Towel) Method*

Place 100 seeds on two well-moistened blotters (48 x 48 cm) and covered with another well moistened blotter of the same size. Roll the blotters in many folds and incubate at 20 ± 2°C for seven days.

3. *Agar Plate Method*

The agar plate or Ulster's method of Muskett and Malone (1941) is to prepare potato dextrose agar media under sterilized conditions.

Potato	200 g
Dextrose	20 g
Agar	15 g
Water	1000 ml

Pour the media in petri dishes and allow to solidify it (addition of small quantity of streptomycin or small antibiotic will limit the growth of bacteria). Plate the seeds on PDA plates at the rate of 10 seeds per dish (depending on size) and incubate at 20 ± 2°C under 12 hours alternating cycle of NUY and darkness for seven days.

In all cases, treat the seeds with 1% sodium hypochloride from 1-10 minutes depending on type of seeds) and other pre-treatment chemicals may be $CaOCl_2$, H_2O_2 and $HgCl_2$. After treatment, transfer the seed to sterilized distilled water and then to blotter paper to drain excess liquid before plating. Different selected media can be used for recognizing the particular pathogen without interference or combination by other organisms.

DETECTION OF SEEDBORNE VIRUSES

Direct Methods

Serological and other Methods

Serological method is mainly used to detect viruses (Hill, 1984) although it is applicable to seedborne bacteria (Gultric et al., 1965) and fungi (Hall, 1971). The precipitation of antibodies with antigen in serum is the basis of serological tests. These include:

1. Tube precipitation test
2. Gel diffusion
3. Latex test
4. ELISA test

Details of serological techniques are given in viral techniques in this manual.

Electron Microscopy (EM)

The EM is one of the most important tool of modern research. It gives a rapid idea of the type of particle involved in infection and the morphological properties of a given virus are mainly useful for assigning the virus to a particular group, but with

serological specific electron microscopy (SSEM) one can easily visualize virus antibody complex direct on the electron microscope grid.

Indirect Method (inoculating the seed extract to indicator plants)

Mechanical inoculation is the application of the virus bearing fluids to the surface of leaves in such a way that the virus can enter the cells of a healthy plant. The indicator or the susceptible plants may be *Chenopodium* spp., or *Nicotiana* spp., and *Datura stramonium*. Inoculum is prepared in 0.02 M phosphate buffer pH 7 by grinding the samples in sterilized water with the pestle. After passing the extract through cheese cloth, it is rubbed on the leaves of the indicator plant (leaves may be dusted with carborundum powder as an abrasive). After inoculation, plants are washed with tap water to remove excess water fluid and are kept in insect free glass house and observed daily for symptom development.

DETECTION OF SEEDBORNE BACTERIA

Different methods such as growing on, plant injection, bacteriophage multiplication, direct isolation on agar media and serology have been used to assay for seedborne bacteria (Schaad, 1982).

Growing on

Seeds are placed on wet towels or on water agar in petri dishes and the resulting cotyledons are observed under a dissecting microscope for symptoms.

Plant Injection

This method includes the injection of seed comminutions or purified preparation of the isolated and purified bacterium into susceptible host plant for observing the symptoms.

Procedure: Grow culture in YDC medium for 23°C. Adjust to 0.1 OD at 6000 nm and dilute to 10^{-2}. Using a 2 ml syringe and 26 gauge needle, inject suspension into leaf blade, holding finger behind leaf, cover the plant with a plastic bag and put into 22°C incubator. A brown black lesion develops in 5-7 days.

Bacteriophage Multiplication

A method to identify bacteria directly in washing is the phage-plaque multiplication method of Katznelson (1950).

Procedure: Molten nutrient agar (45 - 50°C) is inoculated with an isolate which is to be identified. After the medium has solidified it is inoculated with one drop of the bacteriophage and incubated at 30°C for 24 hours. If plaques develop it means the bacterium is present.

Isolation on Agar Media

Specific agar media are used for presumptive identification of seedborne bacteria in seed washings.

Differential King et al. Medium B (KB) for fluorescent Pseudomonas

Proteose peptone #3 (difco)	20.0 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15.0 g
Glycerol	10.0 ml
Distilled water	1000.0 ml

after autoclaving add 2 ml cycloheximide.

YDC (Yeast extract dextrose calcium hydroxide) for Xanthomonas

Yeast extract	10.0 g
$CaCO_3$ light powder	20.0 g
Agar	15.0 g
*Glucose	20.0 g
Distilled water	1000.0 ml

* Autoclave separately

Semi Selective KBBC for Pseudomonas syringae

To autoclaved and cooled KB add the following:

Boric acid, 100 ml of sterile 1.5% (w/v) aqueous solution; cephalexin, 1 ml of a 10 mg/ml stock solution.

To prepare stock solution, dissolve 100 mg in 1 ml 75% ethanol and dilute to 10 ml with sterile water.

Cycloheximide 2 ml of a 100 mg/ml stock solution. For stock solution, add 1.0 g to 10 ml 75% methanol.

BSCAA and NSCAA for Xanthomonas campestris pv campestris (Schaad, 1970)

BSCAA

Soluble potato starch	10.0 g
K_2HPO_4	1.0 g
Glycerine	0.2 g
KH_2PO_4	1.0 g
Methyl green	0.2 ml
$MgSO_4$. $7H_2O$	2.0 g

Agar	15.0 g
Distilled water	1000.0 ml

After autoclaving add 2ml cycloheximide, 2 mg nitrofurantoin (0.4 ml) and 0.1 mg (0.4 ml) vanomycin per liter as described.

NSCAA (Nutrient, starch and cycloheximide agar) Randhawa and Schaad, 1984

Nutrient agar	23.0 g
Soluble potato starch (Baker)	15.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Divide each liter into two, 1 liter flask (500 ml/flask) and autoclave for 20 min. Remove from autoclave and add 2 ml of cycloheximide to each flask.

If difco NA is not available, use 3 g beef extract, 5 g Becto peptone and 15 g agar per liter.

Serology

Serology is the rapid and specific method for identification of seedborne bacteria. It includes the agglutination, agar double diffusion, ELISA and another one is immunofluorescence.

Immunofluorescence

It is a best known technique for the identification of *Xanthomonas campestris* (Schaad, 1978).
For recovery of *X. campestris* from seeds.
Place 40 g seed (10,000 seeds in a sterile 250 ml Erlenmeyer flask.
Add 75 ml of sterile saline washing solution (SWS).
Shake on a rotary shaker at 25°C for 3 minutes.
Filter through sterile, triple layer of cheese cloth and rinse with 25 ml of SWS into a sterile centrifuge tubes at 12,000 rpm for 10 minutes, at 20°C (10.000 rpm using Beckman SS-34 rotor) to pellet bacteria.
Discard supernatant and suspend each pallet in 1 ml of sterile saline.
Pool the two suspensions and transfer into a small sterile vial = washing sample.
Remove 0.5 ml and dilute serially to 10-2 using 4.5 ml saline.
Keep washing sample and each dilution.

Isolation of X. campestris

Pipette 0.1 ml of 0, 10-1, 10-2 dilutions into each of the three plates of NSCA, NSCAA and BSCAA. After adding sample spread the liquid using a flamed L_

shaped glass rod and a turn table (flame the rod after each plate).

Incubate the plates at 30°C.

Streak the control plate with stock culture of *X.campestris*.

After 2 days, record the number of colonies of *X. campestris* on NSCA and NSCAA (comparing colony type with control plate). Colonies of *X. campestris* will be small, yellow, clear and surrounded by a clear halo (starch hydolysis). The yellow color may be very light. Transfer several colonies to YD agar plates by streaking. Incubate at 30°C and observe yellow, mucoid colonies typical of *X. campestris* as a control plate for comparison. Return the NSCA assay plates to the incubator for 24 hour and read each plate as above.

After 5 days, read each BSCAA plate and transfer several suspected colonies of *X. campestris* to YDC agar. Colonies on BSCAA are clean to light green, mucoid and surrounded by zone of starch hydrolysis.

Identification of X. campestris by immunofluorescence (IF) staining

Grow suspected bacteria on YDC plates for 48 hours.

Make suspension of cells using 3-4 loops of bacteria in 3-1/2 ml 0.85% saline with about 0.3 ml formalin; let set 5-10 minutes and swirl before using microloop to put bacteria on slide.

Allow to air dry.

Flood slide with Kirkpatrick's fixative place between circles, slowly using pasteur pipette.

Place slide in petri dish with moist filter paper (moist chamber) for 3 minutes Rinse with fixative, drain and allow to air dry. (Hold slide and sue pasteur pipette).

Add one drop of *X. campestris* gamma globulin per well using 1 cc disposable syringe w/o needle. Incubate in moist chamber for 30 minutes in the dark.

Rinse slide in saline (few seconds) then phosphate buffered saline for 10 minutes.

Rinse slide with distilled water and air dry.

Repeat as step 6 (drop of gamma globulin) but use antirabbit globulin.

Place mounting solution between circles and add cover slip. Observe under Epi-fluorescence using 100 X objective. Observe control slide first.

DETECTION OF SEEDBORNE NEMATODES

The simplest and effective method to detect the nematodes from infested seeds is to submerge the seed in clean water for at least 30 minutes. Sometimes the seed tends to float in water surface that is quite normal for nematode infected seeds. Metal needles should be used to keep the seed in water long enough so that water

penetrates into the hull and it becomes soft. Two metal needles, one in each hand should be used to separate the hulls and the hull from the seed itself, and the process should be done under stereoscope. A large number of long, transparent, thread like individual nematode can be picked up for observation under a compound microscope.

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IDENTIFICATION OF BACTERIAL PLANT PATHOGENS

Mohammad Afzal Akhtar

INTRODUCTION

Bacteria are usually left to specialist for identification. Sometimes they are even ignored. This neglect of bacteria is due to lack of knowledge of phytobacteriology rather than the difficulties that may be encountered. This article gives information on a small number of tests, sufficient to allow a pathologist to decide whether a given bacterial culture is likely to be the pathogen he suspects, and also which organisms of those isolated from a diseased plant are worth further study as possible plant pathogens.

SYMPTOMS

The first step in diagnosis should be through examination and recording of the symptoms seen on the diseased plant. This is worth doing as it is often a great aid to future identification, and the information gained can save work by narrowing down the search to one or few likely organisms. Photographs and/or herbarium specimens are often worthwhile for future reference and may help other workers who receive the isolates for identification.

ISOLATION

For isolation of bacteria from plants, two methods are commonly used. The more usual, which should be employed whenever possible, differs from the method used for fungi. It is, in principle, the standard bacteriological technique of dilution plating. A small piece of diseased tissue is removed aseptically from the edge of a typical lesion at the boundary with healthy tissue or from the vascular system or other suitable place, depending on the disease. This is placed in a little sterile water (a few drops to a few ml depending on the size of the piece of tissue taken) and either teased apart with sterile needle, or ground with a sterilized pestle and mortar. Small quantities of the resulting suspension are then removed with a flamed wire loop and streaked out on to well dried agar plates, the aim being to separate the cells so that they produce individual colonies. It is sometimes an advantage to allow the suspension to stand for several hours before streaking. This gives the bacteria more time to free themselves from the tissues and to multiply. However, if the suspension is left too long, saprophytes will take over. Nutrient agar is usually a good medium for isolation, but occasionally carbohydrate is needed as, for example, with the sugarcane pathogens. The surface of the medium must be dried for successful plating, otherwise the bacteria will move in the surface moisture and a carpet of mixed growth will result instead of single colonies. If the medium is cooled to about 45°C before pouring, and the poured dishes kept at an even room temperature, they

usually dry in 24-48 hours. In very humid climate or in an emergency measure the poured dishes may be dried by placing them open and inverted in an incubator a little above room temperature for 2-3 hours. Care must be taken to avoid contamination.

After streaking, the dishes should be incubated at 25°C and examined daily for 4-5 days for most pathogens, or at intervals during 10-14 days if a slower growing pathogen is suspected. If the diagnosis based on the symptoms is born in mind, the expected pathogen can often be recognised and sub-cultured, even when numerous bacteria are present. With good material and well chosen pieces of tissue, the pathogen is often the only organism to grow. When more than one type of colony grows, the relative abundance of each should be noted. The most frequent organism and any that are consistently found in several suspensions of different origin, are worth examination unless they are recognized as saprophytes. They should be sub-cultured from well-separated colonies on to slopes of the same medium.

The other method of isolation is similar to that used for fungi. Pieces of infected tissue are placed directly on to the agar medium and bacterial growth occurs around them. If any saprophytes are present the growth is liable to consist largely of those organisms, which usually grow faster than pathogen and the latter are lost. For this reason this method should be used only for the few bacteria that cannot be isolated by the first method.

PURITY

All cultures should be checked for purity before tests begin. This is done by making a very dilute suspension of some of the cells in sterile water or saline and streaking a loopful on to dry agar. The streak is examined daily for several days to check that all colonies are identical. If there is any doubt about the purity of a culture at this stage, single colonies should again be sub-cultured until purity is certain. It is useless to work with mixed cultures.

APPEARANCE OF COLONIES

The streak tests for purity should also be used for observing the colony characteristics of shape, size, texture and markings of surface, elevations, type of margin, consistency, colour, translucency or opaqueness and rate of growth. The presence of pigments, precipitate or crystals in the medium should also be noted.

GRAM STAIN AND CELL MORPHOLOGY

Young, actively growing cultures (usually 24-48 h old) are used to prepare smears on microscope slides. Any of the well tried gram staining methods found in the literature will probably be satisfactory, but it is a good idea to keep to one method and become used to it. Jensen's modifications (Cruickshank, 1965 and

Schaad 1984) is in routine use at CDRI: cover the slide for one minute with aqueous crystal violet. This is poured off and washed away with 1% iodine solution in 2% aqueous KI. This is left for 1 minute, washed off with ethyl alcohol until the colour ceases to come away (a few seconds is enough) and the slide at once washed with water, counterstained with 0.5% aqueous safranin for about 3 min., washed again with water and dried. Gram-positive cells are dark purplish, gram-negative are red. During examination of the slides note is taken of the size, shape and arrangement (single, pairs, chains, angular or palisade) of the cells. Endospores may be visible if the stain is deep, but practice is needed to spot them.

PRELIMINARY SORTING

Sufficient information will have to be gained to allow elimination of organisms at this point. All cultures that have regularly spherical cells (Cocci) can be discarded, as also can any in which endospores are seen. Plant pathogens are not known in these groups. Size is useful differential characteristic, all plant pathogens are small rods, therefore, any bacteria greater than 2 Um in diameter may be eliminated as a non-pathogen. Gram negative rods constitute most of the plant pathogenic bacteria, these belong to the genera *Agrobacterium, Erwinia, Pseudomonas and Xanthomonas*; all are catalase positive. The plant pathogenic members are strict aerobe and oxidase negative, therefore, any facultative or oxidase positive isolates may be discarded. *Corynebacteria* are examined for club shaped and other irregular cells (pleomorphism), for banded or beaded staining and angular like arrangement of the cells caused by snapping type of division. Reference to published descriptions of *Corynebacterium* spp. (Elliot, 1951; Breed et al., 1957) should be helpful at this stage. Caution is needed, however, as undescribed saprophytic members of this group are frequently isolated from plants. As most plant pathogens are whitish, cream or yellow, colonies with other colours should be viewed with suspension and discarded early.

HEAT TEST FOR SPORES

This test is advisable because *Bacillus* spp. are common on plants, and their spores may not be shown by the gram stain, or may be so sparse that they are over-looked even when spore staining is done. Some species of *Bacillus* are gram-negative when examined in the usual way so that it is a good idea to heat test any gram-negative organism that may give doubt. The test is performed by preparing 2-3 ml of a very heavy suspension of cells in a thin walled sterile tube, and heated to 80°C for 15 min., in a waterbath. Aseptic technique is used to prevent entry of contaminating *Bacillus* spp., and loop full of suspension are streaked on to agar before and after treatment. If growth occurs after treatment spores are present and the culture may be discarded.

FLUORESCENT PIGMENT

The production of fluorescence, a yellowish green fluorescent pigment which

diffuses into the medium is characteristic of an important group with in the genus *Pseudomonas*. This group contains many important plant pathogens. The production of fluorescent pigment depends on cultural conditions and is not usually visible on nutrient agar. A visible amount is readily produced on medium B of King et al. (1954), containing agar 1.5% Difco proteose peptone No.3, 2%, Glycerol (A.R. grade), 1% K_2HPO_4 anhydrous, 0.15%; and $MgSO_4$ $7H_2O$,). The pH should be 7.2. The medium is sterilized at 15 lb for 15 min., and plates poured and dried as required. Several cultures may be spotted on to one plate if the surface is dry. The test is applicable only to gram-negative, whitish, grayish or pale organisms. Observations should be made daily as a culture will occasionally produce a very large fluorescent zone that will interfere with reading of adjacent cultures. Examination under UV light makes the fluorescence very clear, but it is not essential.

OXIDASE

The oxidase test of Kovacs (1956) may be applied with advantage to most gram-negative cultures. A young, usually 24 hr culture on nutrient agar is required. A small visible amount of the growth is taken with a sterile platinum loop and smeared on to a filter paper that has been dampened with a little freshly made 1% aqueous tetramethyl-P-phenylene diamine dihydrochloride. A dark bluish violet colour formed at the smear within 10 sec is a positive result. Some organism will not produce the colour even after 5 min. Many produce a whitish spot after/minute or so, which is seen against the pale blue background left by reagent. Organisms that fluoresce in Medium B and oxidase negative belong to groups 1 and 11 of Lelliott et al. (1966). They are likely to be plant pathogens.

ACID PRODUCTION FROM GLUCOSE

Various media and methods have been used to perform this test. One of the most suitable for plant pathogens is Hayward's (1964). The basal medium has the following percentage composition: Oxide bacteriological peptone 0.1%, NH_4. H_2PO_4 0.1%,KCl 0.02%, $MgSO_4.7H_2O$ 0.02%, bromothymol blue 0.003%, Oxide agar No.3 0.3%, it is adjusted to pH 7.2 with 40% NaOH, dispensed with 45 ml quantities and sterilized at 15 lb for 15 minutes. The glucose (other carbon sources may also be tested is sterilized separately as 5 ml portion added to a 45 ml portion of molten basal medium gives a final concentration of 1% glucose. This medium is dispensed into sterile tubes to a depth of 4 cm. These are inoculated in duplicate with a long wire stabed down to the base. One of each pair is then sealed with at least 1 cm thickness of sterile mineral oil to give anaerobic conditions. If glucose is oxidised aerobically, producing acid, the medium in the open tube turns yellow from the top. If glucose is fermented (anaerobic), the indicator in both tubes changes to yellow. Gas production is observed as bubbles trapped in semi-solid medium. If carbohydrate is not attacked the medium will often turn blue because of the alkali

released from peptone. Peptone is in low concentration to keep the effect of this alkali to a minimum.

NITRATE REDUCTION TO NITRITE

Most plant pathogens will grow well in the following medium which may be used for the nitrate reduction test. Oxide bacteriological peptone 1%, K_2HPO_4 0.5%, difco yeast extract 0.1%, KNO_3 0.1%, Oxide agar No 3 0.2%. The medium is sterilized at 15 lb for 15 min. Screw capped bottles containing about 10ml of medium are inoculated by stabbing a loopful of growth through to the base, and incubated for 2 days or more until reasonable growth has occurred. Bubbles trapped in the medium are presumed to be nitrogen, indicating denitrification. The reduction of nitrate to nitrite is tested as follows. A drop of gram's iodine is added to the culture to oxidise any hydroxylamine that may be present, 0.5ml of a 0.6% solutions of sulphanilic acid in 5N acetic acid (30% w/v) and 0.5ml of a 0.6% solutions of dimethyl- -naphtylamine in 5N acetic acid are then added. A red coloration develops at the surface in a few moments if nitrite is present. If no nitrite is detected a small amount of zinc dust (not more than 5 mg/ml of culture) is added from a scalpel point or a flattened needle. A red coloration after a few minutes indicates the presence of nitrate in the medium (the zinc has reduced some of it to nitrite, which has reacted with the reagents). No coloration indicates the absence of nitrate, which is presumed to have been reduced to nitrite and further reduced to nitrite. A second culture incubated for a shorter time should confirm this by giving a positive reaction for nitrite.

The nitrate reduction test is particularly useful for distinguishing *Xanthomonas* spp., which do not reduce nitrate, from many of the yellow saprophytes, which usually do.

SOFT ROT OF POTATO TISSUE

Well washed, firm potatoes are surface sterilized with alcohol, peeled aseptically and sliced into sections, 7 mm thick. These are placed in sterile petri dishes and a V-shaped groove cut across them. Sterile water is then poured over the slice to a depth of about 34 mm in dish. A very heavy inoculum is then streaked down the centre of the groove, and the dish incubated at 25°C. The slices are examined at 24 and 48 hr for soft rot by prodding with a loop. Note is also taken of the type of bacterial growth that may be visible and any pigment present.

Erwinia spp. of the soft rotting group produce massive areas of rot in 24 hr. Some *Xanthomonas* spp., also show pectolytic activity, as do some *Pseudomonas* spp. It should be remembered, however that among non-pathogen several *Bacillus* spp., show pectolytic activity. Many species of *Xanthomonas* have rather characteristic glistening honey coloured growth on potato.

There are many other tests that could have been suggested. A very important one whose omission may be rather surprising is flagella staining . This was purposely left out because, although it is of basic importance, it is not an easy technique to learn. If one is to adopt it, the test is well worth doing. It is the only way to distinguish some organisms with complete certainty.

OTHER TESTS

Besides morphological, biological and biochemical tests, some serological and bacteriophage tests are also used for identification.

SEROLOGICAL TESTS

1. Agglutination test
2. Double diffusion test
3. Tests with labelled antibodies

1. *Agglutination Test*

This test can be performed either on a slide or in a test tube. A bacterial suspension is prepared in PBS (Phosphate buffer saline) and a drop of antiserum of known origin is added and the mixture is allowed to react for a couple of hours at 35-40°C. If agglutination appears in the drop, this means that result is positive.

2. *Double Diffusion Test*

In a water agar plate 3 mm diameter wells are made in circular fashion with one in centre. The outer wells are filled with the antigen and the lid and incubated in moist chamber for 2-3 days at 25-28°C.

Immune precipitation lines will be visible in the agar, the precise position of which will depend on concentration and rate of diffusion of antigen and antibodies. The double diffusion method provides a very reliable tool for identification and for research on the specificity of antisera. The presence of *Pseudomonas solanacearum* in potato can be easily detected by this test.

3. *Test with Labelled Antibodies*

This group of serological tests is characterized by the use of antibodies labelled with a compounds such as fluorescence isothiocyanate (FITC). The best known methods for phytopathological work are:

i. IFM (Immunofluorescence microscopy)
ii. ELISA (Enzyme linked immunosorbent assay)
iii. RIA (Radio immuno assay)

ELISA and RIA are very complex and sophisticated but not extensively

studied in relation to pathogenic bacteria, whereas IFM test is very common in practice for identification of bacterial diseases of beans, peas, soybeans and brassica seeds. Positive as well as negative controls must be included in all serological test.

BACTERIOPHAGE TEST

Bacterial viruses called bacteriophages can attack and kill the bacteria. Structurally they resemble other viruses but difference is in choice of host cells. Morphologically, many bacteriophages have a tail through which they enter the host cell with their DNA. The bacteriophages can be extracted from soil or the same pathogen host, as it is very specific to particular species.

BRIEF PROCEDURE

Three ml molten nutrient agar (45-50°C) is inoculated with an isolate which is to be identified. After the plate has solidified, it is inoculated with one drop of the bacteriophages and incubated at 30°C for 24 hours. If plaques develop it means the bacterium is present for which the test was performed.

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PLANT NEMATOLOGY LABORATORY TECHNIQUES

M.A. Maqbool and Anjum Munir

INTRODUCTION

For the diagnosis and identification of nematode diseases it is necessary to have the knowledge about the methodology of nematode collection from soil and plant parts, isolation and preparation for examination. Following are techniques used in nematology labs.

NEMATODE COLLECTION

The first consideration which is of paramount importance in diagnosing related nematode collection is the timing of sampling of parasitic nematodes. Familiarity with life cycles of nematodes suspected is necessary in order to select ideal sampling times, particularly in case of annual crops. If nematode species are not known, however, sampling in late mid-season to near harvest is the best time for this exercise because the populations usually are near their highest. In contrast, sampling in spring gives the most useful data for relating numbers and kinds of nematodes to crop performance, but population levels are typically quite low compared to those near harvest. Objectives of sampling may include general surveys, diagnosis, advisory purposes and experimentation. The present discussion will be limited to sampling for disease diagnosis. Even for this purpose, the major components of the populations encountered in soil and plant roots and foliage must be assayed. Also consideration must be given to the expected life stages (eggs, juveniles, adults, or cysts in case of *Heterodera* and *Globodera* species) that may be present.

SAMPLE SIZES

Soil- about 250 g. Most of the plant parasitic nematodes are found in the soil around the roots. Care should be taken that soil sample is moist during transit.

Stem and leaves- 50-100 g if they show any apparent damage by nematodes.

Roots- 50-100 g including sand, partially attacked and well attacked roots. Particularly important is to have roots including the transition zone between rotten and healthy parts.

COLLECTION AND STORAGE

Polythene bags are excellent containers for collecting nematode infested material. Practically no loss of water occurs and the bags, being tough, are easily handled and can be sent through the post. Whole plants travel best, free from soil. Soil samples, with or without roots, travel well in these bags. Nematode infested plant material, and soil containing nematodes may be stored with little deterioration

in refrigerator at about 4°C. Samples from abroad are best sent by air.

ISOLATION

Baermann Funnel Technique

Basic requirements for this method are a funnel of a suitable diameter with a piece of rubber tubing attached to the stem and closed by a Hoffmann or spring clip. The funnel is placed in a support and is almost filled with tap water. Plant material containing nematodes is cut into small pieces, placed in a square of butter muslin which is then folded to enclose the material and gently submerged in the water. The nematodes emerge from the tissues, pass through the cloth and sink to the bottom of the funnel stem. After some hours, or overnight, a small quantity of water is run off; this contain the nematodes.

Sieving Technique

Equipment

Two 10-12 inch enamel basins; seven 7 inch sieves, made with copper, brass or phosphor-bronze screening, of 16, 25, 50, 100, 160, 250 and 400 meshes to the inch (these are equivalent to pore sizes of approximately 1mm, 690 μm, 240μm, 140μm, 65μm, 55μm and 30μm); one 6 inch pan; six 250 ml beakers; six 100 ml beakers (optional) and a piece of blotting cloth or silk.

Procedure

In pouring through the sieves, wash quickly and use as little water as possible, otherwise eelworms are lost. The finest blotting cloth may be substituted for the finest sieves; it is held between two pans whose bottoms have been out. It is important that the finer sieves should be thoroughly wet before use; filtering is helped by pouring a thin stream into them from some height. The finest sieve should be held at an angle and not square to the stream of water when pouring. The retention of nematodes appear to be better on such a sloping sieve.

Place the soil, or soil with roots, etc, in a large enamel basin and cover well with water. Allow it to soak, break down any lumps with fingers and remove any stones. In case of dry soil, soak for several hours.

Stir the muddy mixture and pour it through the 16 mesh sieve into the second enamel basin but do not pour the heavy bottom material. If a more complete assembly of nematodes is required, add more water and repeat the process; otherwise discard the reminder and wash the first basin.

Set the sieve down into the water which has been poured through it and rinse the material collected. Any residue on the sieve will contain only *Mermithida* or

other large rare eelworms. Discard the residue unless these forms are present.

Pour the contents, after stirring, of basin No.2 through the 25 mesh sieve into basin No. 1 (now cleaned), again leaving behind the heaviest debris. Repeat the rinsing of the sieve, then remove it and hold it on edge, tilted slightly forward, in the 6 in pan and rotate it slowly under a gentle stream of water which is allowed to trickle down the lower surface of the sieve. Transfer these washings to a 250 ml beaker labelled 25 mesh.

Repeat these operations through the remaining series of sieves, 50, 100, 160, 250 and 400 collecting the washings in each case into an appropriately labelled 250 ml beaker.

Allow the contents of the beakers to settle. Pour off the supernatant excess of water and transfer the remaining contents, if desired, to a series of 100 ml beakers. Again allow to settle and similarly reduce the bulk.

Root Incubation Technique

This has been used for obtaining specimens of *Radopholus similis* and *Pratylenchus*. Males of *Heterodera* and *Meloidogyne* are obtained by similar method.

The infested roots, when collected, are not allowed to dry. They are washed free from soil and then placed, still in the moist state, in closed jars or other containers where the atmosphere remains humid. The water drains to the bottom of the container and in it the nematodes are collected. This water is removed from time to time and replaced by spraying the roots from a wash bottle.

KILLING AND FIXING

Small Samples

Place a collection of nematodes in a drop of water on a plain or cavity glass slide. Hold the slide over a small flame, moving it about, for 5-6 sec. Watch the nematodes carefully either with the naked eye or under the stereoscopic microscope from time to time. The nematodes twist about and then suddenly straighten out. At this point heat rigor has occurred and the slide should be removed immediately. A little practice and experience is needed to achieve the best results. Overheating is to be avoided, for it leads to artefacts and obscurity of detail. Fix immediately after death either by transferring specimens with a handling needle to fixative, or by adding an equal sized drop of *double strength* fixative.

Large Samples

Nematodes collected in large numbers can be killed and fixed in bulk. The suspension is reduced in volume by spinning in a centrifuge at about 1500 rpm for 1-

2 minutes; or more slowly, simply by allowing the nematodes to settle under gravity. Then pour off the supernatant water so that the nematodes remain in a few ml only. Shake the centrifuge tube or other vessel to distribute the nematodes freely in suspension and plunge it into a beaker of hot water at 65°F for a least 2 min till the nematodes are dead. Then add an equal volume of *double strength* fixative.

Fixatives

Numerous fixatives have been recommended from time to time and most workers have their own particular choice. The one recently adopted in nematology at Rothamstead, is that described by Courtney, Polley and Miller (1955) called TAF. Its formula is:

Formalin (40% formaldehyde)	7 ml
Triethanolamine	2 ml
Distilled water	91 ml

The appearance of nematodes, after fixation in TAF is remarkably lifelike. The solution remains stable over a long period, the Triethanolamine neutralizes any free formic acid and, being hygroscopic, prevents specimens from drying up if they are left in the fixative and forgotten.

Other fixatives commonly used are:

Ditlevsens or F.A.A.

95% Alcohol	20 ml
Formalin (40% formaldehyde)	6 ml
Glacial acetic acid	1 ml
Distilled water	40 ml

As this contains alcohol a certain amount of shrinkage nearly always occurs. However, this quality is sometimes useful, particularly in helping to make plain such structures as incisors and annulations.

F.A. 4:10

Formalin (40% formaldehyde)	10 ml
Glacial acetic acid	10 ml
Distilled water	80 ml

Particularly no alteration in size occurs in this fixative. Its drawbacks are that there is a tendency for the stylet of *Tylenchus* to become transparent if left too long, Seinhorst (1954) observed that the cuticle of *Trichodorus pachydermus* swelled considerably in this fixative. However, this fixative is most widely used.

Formalin

Dilute solutions of 2-4% formaldehyde are commonly used for fixation,

particularly if no other fixative is available. Nematodes, so fixed, tend to appear granular if left for a long time.

PREPARATION FOR MOUNTING

Slow Transfer to Glycerol

After fixation, transfer nematodes to the following dilute glycerol solution in a glass staining block or small syracuse watch glass.

Glycerol	1.5 ml
Distilled water	98.5 ml

For use after Ditlevesen's fixative, Thorne recommends:

Glycerol	1.5 ml
7.5% ethanol	98.5 ml

A trace of copper sulphate or a little tymol must be added to prevent growth of molds. Place the dish containing the nematodes in a particularly closed Petri dish and transfer the latter to a desiccator. The dilute glycerol should then evaporate very slowly; with most nematode species, evaporation to pure glycerol takes about four weeks but the period varies. Some species can safely be processed more rapidly, others collapse if the process is not very gradual. Only experience can guide the worker to achieve the best results. Some workers prefer evaporation to take place in an oven at 55°C but the dish of nematodes must again be partially covered to ensure that it takes several days. Specimens should be mounted in absolute glycerine on slide supported by wax.

Vulval Cones of Heterodera spp.

The structure of vulva, fanestra and associated internal structures as well as the general shape of cyst are used to identify *Heterodera* species.

Soak dry cyst in water for about 24 hrs or overnight. Then place the cyst on a perspex slide on the stage of a stereomicroscope and cut the posterior end so that fenestral area is in the centre of that cut piece, trim if necessary so that it is not more then 5-10 times the fanestral area. Using very fine forceps (diamond tweezers) and an eyebrow handling needle, clean adhering body content. For thick walled and heavily pigmented species, bleaching for a few minutes in 90 vol H_2O_2 often makes the structure cleaner. Wash the cleansed vulval cones in distilled water and then pass through 70-95% and 100% ethanol to clove oil and mount in Canada balsam.

Permanent Slides of Perineal Pattern

Tease out females of *Meloidogyne* spp. from root tissues and fix in hot

lactophenol with acid fuchsin stain. Place a well developed specimen on a piece of celluloid. Pierce the cuticle on the anterior end of the body with sharp eye-knife or injection needle to relieve body pressure. Cut away the edge of the posterior third of the body, leaving only a small, almost flat piece bearing the perineal pattern. Clean the pattern under higher power of stereomicroscope. Complete cleaning by using a dental pulp file to hold down the pattern while brushing away the granular material with a eyebrow needle. Transfer the pattern to a drop of desiccated glycerin and repeat the process until at least half a dozen patterns are ready. Carefully arrange the patterns near the centre of the drop with three pieces of very fine glass rod for support, apply coverslip, tack down and finish with sealing the coverslip.

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IN-VITRO CULTURE OF PLANT PARASITIC NEMATODES

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INTRODUCTION

There are numerous techniques used in the culture of different nematode groups. It is difficult to assess their relative merit, and in practice the method selected often depends on personal preference.

This article deals rather briefly with some major techniques, additional information, however, can be obtained from the reviews by Hooper (1970), Zuckerman (1971) and Krusberg and Babineau (1979).

TERMINOLOGY

Terms commonly used are:

Gnotobiotic..... With known species (animal or plant), or none, associated with the primary species under culture.
Axenic .... No other associated species.
Monoxenic....... One other associated species.
Dixenic............ Two other associated species.
Xenic............... Unknown associated species.

CULTURE METHODS

1. Excised Root Culture

These cultures are so-called because the seed and shoot are excised from the root and discarded, after germination. The nutrients for root growth are contained in the medium. Some examples of successful nematode-host associations include:

Pratylenchus spp..Maize
Ditylenchus destructor..Clover, tomato
Heterodera oryzae...Rice
Hoplolaimus indicus..Sorghum
Meloidogyne incognita...Tomato
M. javanica..Tomato
Radopholus similis..Okra

GENERALISED TECHNIQUE

A) Media Preparation

Plant tissue culture media ingredients and formulations are given in Table 9.1.

Most media are commercially available ready made, however, they often need to be modified to suit a particular plant species.

Table 9.1. Plant tissue culture media - formulations (mg/ml).

Ingredients	1	2	3	4	5	6	7
$CaCl_2.2H_2O$	439.80	439.80	150.0	439.80	439.80	439.80	440.0
$Ca(NO_3)_2$(anhyd)							
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
FeNa EDTA	36.70	36.70	40.00	36.70	36.70	36.70	36.70
H_3BO_3	6.20	6.20	3.00	6.20	6.20	6.20	6.20
KCl							
KH_2PO_4	170.0	170.0		170.0	170.0	170.0	170.0
KI	0.83	0.83	0.75	0.83	0.83	0.83	0.83
KNO_3	1900	1900	3000	1900	1900	1900	1900
$MgSO_4.7H_2O$	370.60	370.60	250.00	370.60	370.60	370.60	370.0
$MnSO_4.4H_2O$	22.30	22.30	13.20	22.30	22.30	22.30	22.30
MoO_3							
Na_2SO_4							
NaH_2PO_4 $.2H_2O$		288.0	169.60	96.00	96.00	192.0	
Na_2MoO_4 $.2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25
NH_4NO_3	1650	1650		1650	1650	1650	1650
$(NH_4)_2SO_4$			134.0				
$ZnSO_4.7H_2O$	8.60	8.60	2.00	8.60	8.60	8.60	8.60
Sucrose	30000	30000		45000	45000	30000	
Inositol	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Folic acid							
Nictotinic acid			1.00	10.00	10.0		0.50
Thiamine HCl	0.40	0.40	10.00	30.00	30.00	0.40	0.10
Pyridoxine HCl			1.00	10.00	10.00		0.50
Glycerine							2.00
Biotin							
Adenine $SO_4.H_2O$	26.48			71.59		71.59	
IAA	1.00			0.50	10.00		
NAA		0.10				0.30	
Kinetin		2.00		10.00			
L-Tyrosine disodium salt				124.30	124.30		
2-i-P	10.00					3.00	
Agar	8000	8000		10000	10000	8000	10000

1 = Murashige's Begonia Multiplication Medium
2 = Murashige's Fern Multiplication Medium
3 = Gamborg's B5 Medium
4 = Murashige's Gerbera Multiplication Medium
5 = Murashige's Gerbera Pre-transplant Medium
6 = Murashige's Lily Multiplication Medium
7 = Murashige and Skoog Medium

Table 9.1 (cont). Plant tissue culture media - formulations(mg/ml)

Ingredients	8	9	10	11	12	13	14
$CaCl_2.2H_2O$	439.80	439.80	166.0	439.80	439.80	439.80	
$Ca(NO_3)_2$(anhyd)							208.50
$CoCl_2.6H_2O$	0.025	0.025		0.025	0.025	0.025	
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.001
FeNa EDTA	36.70	36.70	36.70	36.70	36.70	36.70	4.59
H_3BO_3	6.20	6.20	10.00	6.20	6.20	6.20	1.50
KCl							65.00
KH_2PO_4	170.0	170.0	68.00	170.0	170.0	170.0	
KI	0.83	0.83		0.83	0.83	0.83	0.75
KNO_3	1900	1900	950.0	1900	1900	1900	80.00
$MgSO_4.7H_2O$	370.0	370.60	185.0	370.60	370.60	370.60	720.00
$MnSO_4.4H_2O$	22.30	22.30	25.00	22.30	22.30	22.30	7.00
MoO_3							0.0001
Na_2SO_4							200.00
NaH_2PO_4 $.2H_2O$				192.0	192.0	18.70	
Na_2MoO_4 $.2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	
NH_4NO_3	1650	1650	720.0	1650	1650	1650	
$(NH_4)_2SO_4$							
$ZnSO_4.7H_2O$	8.60	8.60	10.00	8.60	8.60	8.60	3.00
Sucrose	30000			30000	30000	30000	
Inositol	100.0		100.0	100.0	100.0	100.0	
Folic acid			0.50				
Nictotinic acid			5.00				0.50
Thiamine HCl	0.40		0.50	0.40	0.40	0.40	0.10
Phridoxine HCl			0.50				0.10
Glycerine			2.00				3.00
Biotin			0.05				
Adenine $SO_4.H_2O$				71.59	71.59		
IAA				0.30	2.00	0.30	
NAA							
Kinetin					2.00	1.00	
L-Tyrosine disodium salt							
2-i-P				30.00			
Agar	8000	8000		8000	8000	6000	

8 = Murashige's Minimal Organic Medium
9 = Murashige and Skoog Plant Salt Mixture
10 = Nitsch's Medium H_3
11 = Murashige's Shoot Multiplication Medium
12 = Murashige's Shoot Multiplication Medium B_5
13 = Murashige's Shoot Tip Rooting Medium
14 = White's Medium[4] (Modified)

- Suscrose (2%) is the best carbon source for most dicotyledonous plants, while 2% glucose is often better for monocotyledonous plants.

- Use 0.57% or 1.0% agar

The ingredients are mixed and autoclaved, heat labile vitamins are added aseptically after the media has cooled. The media is then poured into sterile petri dishes under sterile conditions.

B) Seed Sterilisation

A few sterilants and their times for sterilizing seeds and tissues are given in Table 9.2.

Table 9.2. Sterilants and times for sterilizing seeds and tissues.

Sterilant	Concentration	Seed/tissue	Time
1) Conc. H_2SO_4			
		Lucern	35 Minutes
		Red clover	30 "
		White clover	30 "
		Celery	2 "
		Strawberry	7 "
2) Calcium hypochlorite			
	5%	Tomato	30 minutes
	20%	Hulled oats	30 "
3) Sodium hypochlorite			
	1%	Cucumber	15 "
	1%	Beet	15 "
	1%	Hulled rice	15 "
4) Mercuric chloride			
	0.01%	Carrot (tissue)	4 "
	0.01%	Beet	20 "
	0.01%	Peas	45 "
5) Ethyl mercury phosphate (327mg/l)			
		Oats	20 "
6) 0.1% HgCl :95% C_2H_3OH (in ratio 3:1)			
		Onion	4 "

After treatment, rinse seeds at least ten times in sterile distilled water.

C) Shoot Excision

The seed and shoot are excised after root growth has commenced. The timing is not critical but excision is usually carried out when the root is 3-4 cm long.

D) Nematode Sterilisation

·Again there are numerous techniques e.g.

i). Immerse in 0.1% malachite green for 10-15 minutes followed by several rinses in sterile distilled water.
ii) Immerse in 0.1% mercuric chloride for 2 minutes then in 1% streptomycin sulphate for a further 2 minutes.
iii) Hibitane diacetate 0.5%-1% for 25 minutes, then wash five times in sterile distilled water.
iv) Nematode eggs can be sterilised using 5% hydrogen peroxide for one hour.

Sodium hypochlorite dissolves nematode stylets and therefore should not be used.

Nematodes can either be transferred singly through sterilants using a micro-needle or mounted eyelash, or in bulk by consecutive settling and decanting in a suitable receptacle.

Plant parasitic *Dorylaimida*; *Trichodorus, Xiphenema, Longidorus* etc., are more difficult to sterilise. The above methods may be effective, alternatively, nematodes should be allowed to migrate through agar containing a sterilant.

E) Inoculation

Nematodes can either be transferred to the surface of the agar adjacent to the root tip using a sterile micro-needle, or inoculated in a drop of water. Nematodes can be trapped in surface water films, therefore, care should be taken to avoid this.

To start a culture, 20-30 nematodes per culture are usually sufficient. More nematodes should be added for amphimictic species.

F) Sub Culturing/Extraction

When a culture is exhausted, it can be sub-cultured by transferring sections of infested root and agar to fresh root cultures. New root cutures can be established from seed or by transferring uninfested lateral root tips to new media.

Nematodes can be extracted aseptically using the apparatus (or modification of) used by Webb (1975). Non-sterile extraction from roots and agar can be carried out in the usual manner.

G) Storage

1. Cultures should be stored at an appropriate temperature, usually 25°C.

2. *Callus Tissue Culture*

Callus tissue is a mass of undifferentiated and differentiated tissue which provides a food source for migratory nematodes. In general, nematode multiplication is greater on callus tissue than on normal tissue. Callus formation from root, shoot or embryo is stimulated by a strong auxin source, usually 2, 4-dinitrophenoxyacetic acid (2, 4-D). The media are basically the same as for excised root production, except for the auxin.

Lucern Callus

Lucerne callus is a suitable substrate for many nematodes, e.g., *Pratylenchus* spp. *Radopholus similis, Tylenchorynchus claytoni, Ditylenchus dipsaci, Aphelenchoides bicaudatus*.

Lucern seeds are sterilised in concentrated sulphuric acid. After germination on water agar, the whole seedling is placed on slopes of media containing 2mg/litre 2,4-D. Callus develops after about one week and should be inoculated with nematodes 10-14 days after establishment. Cultures may last up to two months and then can be sub-cultured by transferring infested callus onto fresh callus.

3. *Culturing Fungivorous Nematodes*

Some species of the genera *Aphelenchus, Aphelenchoides, Bursaphelenchus, Paraphelenchus, Ditylenchus, Neotylenchus, Hexatylus* and *Deladenus*, can be cultured on a variety of fungi.

Example. *Aphelenchoides besseyi* on *Botrytis cinerea*

Botrytis cinerea is readily maintained on potato dextrose agar (PDA). Nematodes are surface sterilised and transferred to fungal cultures in the usual manner. On maturity, browning cultures, the nematodes migrate onto the lid of the petri-dish, in drops of moisture. Nematodes required for experiments can thus be washed from the lids.

The cultures can be routinely sub-cultured by transferring a piece of agar containing both the nematodes and the fungus onto fresh media.

4. *Carrot Disc Culture*

Migratory endoparasitic nematodes *Pratylenchus* spp. and *Radopholus similis*, can be cultured on carrot discs placed on 1% water agar. Fresh carrots with tops need not be surface sterilised. Simply remove the tops, scrub the roots, and allow excess moisture to evaporate. Then working in a sterile environment, peel off the external tissue in a spiral pattern. As a precautionary measure, carrot tissue can be sterilised. Sterilised nematodes are added to the centre of the disc.

Advantages of sterile culture

1. Space saving (particularly of glasshouse space).
2. Maintenance of purity of species in culture.
3. Ease of examination e.g., for assessing population build-up.
4. Examination e.g., feeding observations, observations of root damage.

Possible disadvantages

In vitro culture may result in variations, or loss of pathogenicity of the nematode on its natural important host. Regular checks of pathogenicity should, therefore, be made. It is also advisable to maintain some stock pot cultures from which the original inoculum was derived.

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HISTOLOGICAL METHODS IN HOST-PARASITE STUDIES

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INTRODUCTION

Histological studies made with whole leaf preparations can provide a simple but effective means of quantitative determination of fungal development. They can reveal the earliest stage at which compatible and incompatible interactions are differentiated, and can be used not only as a supplemental means of screening for resistance but also an adjunct to interpreting biochemical and physiological research on the mechanism of resistance or aggressiveness of the fungal isolates.

PURIFICATION OF ISOLATES

To ensure the purity of the isolates they should be monospored. For this purpose the dilution plate method is used. Six test tubes containing 4.5 ml sterile water and 3 plates containing water agar (1.7 gram agar/100ml) are labelled for the preparation of dilutions and plating, respectively. Using 1 ml sterile pipette, 0.5 ml of spores from stock spore solutions (10^6 spores/ml) are transferred to tube 1. On a mixer, the tube is shaken well to ensure that spores are evenly distributed. Then 0.5 ml spore suspension is withdrawn from tube 1 and poured into tube 2 for making the next decimal dilution. Tube 2 is again shaken on a mixer and using a fresh sterile 1 ml pipette, 0.5 ml is withdrawn and proceed further as in the case of tube 1. The procedure is continued till all decimal dilutions are ready. Spread evenly 0.3 ml from the last three tubes on water agar plates using a sterile glass or metallic spreader. The suspension should be spread before it gets absorbed on the agar. Incubate the plates at room temperature for 24 to 36 hours (depending on the fungal isolate), pick the single spore colonies with a sterile needle and transfer it to plates containing PDA or any other suitable medium.

INOCULATION METHOD

To minimize the influence of environmental variation on disease expression, methods of preparing the inoculum and the inoculation process itself are standardized. Usually 7-day old cultures are used for harvesting spores in sterile distilled water and adjusted to the density to desired level (10^5 spore/ml) using a heamocytometer.

Inocultion of the plants: Fifteen to seventeen days old plants (5 plants), are sprayed with approximately 5 ml spore suspension (10^5 spore/ml in 0.05% Tween 80). Before inoculation, wax layer of leaves and stems is partially removed by gently rubbing. After inoculation, plants are transferred into a humid chamber for 24

hours, where humidity and temperature are maintained at 95-100% and 18 ± 2°C, respectively.

HISTOLOGICAL STUDIES

Light Microscopy

For light microscopy of whole mounts, infected leaflets are cut into 0.5 x 0.5 cm pieces, fixed and stained according to the method described by KOCH et al., (1983) and modified by Nasir et al., 1992. The 0.5 x 0.5 cm pieces are immersed into Lactophenol triphane blue. Lactophenol triphane blue is prepared as follows:

Phenol	10 gm
Glycerine	10 ml
Lactic acid	10 ml
Tryphane blue	0.02 g
Distilled water	10 ml

The solution containing the leaf sections is brought to boiling for 5 to 7 minutes. Rinsed with water and transferred to clearing solution, which can be prepared as follows:

The Chloral hydrate (2.5 gm/ml H_2O) solution containing stained leaf sections is brought to boiling for 3-5 minutes, rinsed with water and then mounted on a microscope slide in 50% glycerine. Leaflets are left for several weeks in the alcohlic tryphane blue without over-staining. This method is useful for phase contrast microscopy, as well as being useful for observations under ordinary light microscope. It is successful for histological host-pathogen studies in powdery mildews, cereal rusts and generally adaptable for other leaf pathogens. The method shows potential as a diagnostic tool in clinical plant pathology.

Electron Microscopy

For electron microscopy exise the small tissue pieces (3mm) in 0.1 M cacodylate buffer (pH 7.2) and prefix in 2.5% glutar aldehyde and 2% paraformaldehyde in the same buffer at room temperature. Rinse samples eight times and postfix in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) overnight at 4 C, rinse two times in buffer and distilled water. After dehydration in an acetone series (Table 10.1), embed the tissues in Spurr's medium (Spurr 1969, Table 10.2) in flat rubber molds and cure at 70 C for 24 hours. Ultra thin sections (ca 80 nm) cut on a Reichert-Jung ultramicrotome equipped with diamond knife, and mounted on Formvar-coated nickel and uncoated copper grids are stained in 4% uranyl-acetate, washed in distilled water, and poststained for 5 min in Reynolds lead citrate (Reynolds, 1963). These sections are now ready for electron microscopy.

Table 10.1. Dehydration series in acetone

Acetone	20%	10 minutes
Acetone	30%	10 minutes
Acetone	50%	10 minutes
Acetone	70%	10 minutes
Acetone	90%	10 minutes
Acetone	95%	20 minutes
Acetone	100%	20 minutes
Acetone	100%	20 minutes
Acetone	100%	20 minutes

Table 10.2. Spurr resin for embedding

Embedding material resin	VCD	(Serva 38216) (Merck 11477)	10 ml
Hardener	NSA	(Serva 30812) (Merck 11491)	26 ml
Flexibilizer	DER 736	(Sevra 18247) (Merck 11472)	6 ml
Accelerator	DMAE	(Serva 20130) (Merck 11474)	0.4 ml

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DETECTION AND IDENTIFICATION METHODS FOR PLANT VIRUSES

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INTRODUCTION

Viruses are infectious agents, invisible with light microscope, can pass through a bacterial filter. Virus particles (virions) are composed of nucleic acid surrounded by a protein coat (capsid). Nucleic acid is usually RNA but in some cases DNA too. Nucleic acid is infectious part of virus and protein coat provides protection to nucleic acid when the virus is outside the host. Size of virus particles varies from 18-20 nm (gemini viruses) to 1200 nm (Citrus tristeza virus).

Due to obligate parasitic nature of viruses, detection and identification methods used are quite different from those applied to detect fungi or bacteria. Identification of a virus disease, includes diagnosis, which still depends heavily on the scientific laboratory. However, there are some simple methods of virus detection which can further lead to the identification of the same. These methods can be divided into three groups: 1) Biological; 2) Serological; and 3) Physico-chemical.

1. BIOLOGICAL METHODS

a) *Symptomatology*

Viruses may produce symptoms visible or invisible to the naked eye, abnormalities in infected plants which are recognized as symptoms of disease and are of great value for quick orientation in field conditions, and identification with the help of test plants under control conditions. Symptoms induced by viruses are of two types, 1) systemic; and 2) local. Generally, all naturally infected plants in field condition produce systemic symptoms (virus is present throughout the plant). Local symptoms (infection is confined only at the site of virus entry) are produced when indicator/test plants are inoculated artificially. Symptoms are influenced/controlled by many factors including type and strain of virus, type and variety of host, age and developmental stage of host, presence of other viruses and pathogens and environmental and climatic conditions. Symptoms can be produced artificially through mechanical inoculation, insects (vector) transmission, grafting or through parasitic plants like Cuscuta.

b) *Mechanical Inoculation of Plant Viruses*

It is the application of plant extract containing viruses on the surface of leaves of healthy plant alongwith the abrasive material for the virus particle to penetrate

the cuticula and epidermis of a healthy leave. Not all the viruses can be transmitted mechanically. This barrier can be overcome through insect (vector), graft or dodder transmission. Plants that react promptly and characteristically to a particular virus, are called differential, indicator or test plants. In principle, any susceptible plant can be used as a test plant, but a number of species and cultivars are in use internationally. Test plants should be easy to raise in all seasons, should remain susceptible to a virus over a long period of their growth, should be easy to inoculate and should give uniform response under a wide range of environmental conditions.

Material

- Leaves of infected plants (take young infected tissue from glasshouse grown younger plants with primary symptoms).
- Use young healthy looking indicator (test) plants raised under shade in well-prepared and well-fertilized mixture of soil, sand and peat.
- Phosphate buffer.
- Mortar and Pestle
- Carborundum 600 mesh
- Labels

Preparation of 0.05 M phosphate buffer: (pH 7.0)

KH_2PO_4 = 6.80 gm/lit
K_2HPO_4 = 8.70 gm/lit

Adjust the pH to 7.0 by mixing both the solutions with the help of pH meter.

Stabilizating Additive

Many plants contain inhibitors that may inactivate the virus, decrease or inhibit its infectivity, or interfere with its transmission. Some compounds when added to the inoculum are known to have a stabilizing effect on viruses in plant extracts and also have a stabilizing effect on unstable viruses. The selection of compound and concentration depends on the particular virus/host plant system. Some of the commonly used stabilizing agents are: Thioglycol acid, Ethylenediaminetetraacetic acid, 2-Mercaptoethanol, Sodium diethyldithiocarbamate, Ascorbic acid, Sodium sulfite and Bovin serum albumin.

Preparation of Inoculum

In chilled pestle and mortar grind infected tissues along with buffer in 1:1 ratio or more till a fine homogenate is obtained. Filter the extract through cheese cloth.

Procedure

Dust leaves inoculated with 600 mesh carborundum and then apply in

inoculum with a folded cheese cloth, cotton swab, glass spatula or with the fingers of your right hand. Rinse them with water immediately after inoculation. Lable each plant with date and name of inoculated virus/isolate. Observe every day for symptoms to appear and describe them.

c) Graft Transmission

This method is applied when viruses cannot be sap transmittable. Most viruses which are systemic in their host can be transmitted in this way but through the plants that are closely related. When the plants are incompatible transmission usually does not occur. This will also not happen when virus is localized. Mostly vascular union between stock and scion is required to allow a virus (or mycoplasma) to pass together with assimilates through the union.

d) Transmission through Dodder (Cuscuta)

Some plant species do not graft successfully, dodder (*Cuscuta*) may be used to establish vascular contact between virus donor and acceptor plant. Mainly *Cuscuta campestris* and *C. subinculusa* have been used to transmit the viruses.

2. SEROLOGY AND SEROLOGICAL METHODS

When an animal is injected with a pathogen, whether it be a virus or bacterium or a foreign protein, specific proteins are produced in the blood serum of the animal. These proteins are known as "antibodies" and combine specifically with the substance injected, known as antigen. Blood serum containing antibodies is called antiserum. Antibodies in the serum will bind with antigen to produce a precipitate and this is the basis of serological tests for viruses. Following are the serological techniques which are commonly used for virus detection and identification.

Tube Precipitation Test

Precipitation is used to describe the insolubilization of macro molecules. It is widely believed that precipitation is caused by the formation of a lattice or antigen and antibody molecules that grow in size until it is so large that the complex becomes insoluble.

In this test, various dilutions of antigserum and antigen (virus) are mixed and kept in a water bath at 30-40ºC in small glass tubes in such a way that half the contents are immersed in the heated water, convection currents are produced that speed up precipitate formation. The type of precipitate obtained depends on the shape of the antigens. Elongated virus particles produce bulky flocculent precipitates, whereas isometric virus particles or disrupted elongated particles give dense granular precipitates. Reactants (antiserum/antigen) are generally diluted in 0.85% NaCl or phosphate buffer saline. Usually precipitate forms after 2 hrs at 30-40ºC and readings are taken by holding the tubes over a light box in front of a black

background and again after overnight incubation at 4°C. The titre of antisera can be increased considerably by precipitating the antibodies with polyethylene glycol and subsequently resuspending them in saline.

Ouchterlony Double Diffusion Test

In this test the gel initially contains neither of the reactants (antibody/antigen). Then they are added to wells cut in gel and are allowed to diffuse towards each other in petri dish. The point where reactants meet in optimal proportions precipitation lines are formed. The smaller, isometric particles diffuse through the gel without pre-treatment but as the size of particles increases the rate of diffusion in the gel decreases. To overcome this, pre-treatment of larger rod-shaped viruses is required. They may be broken down by chemical treatment or physically by ultrasonic treatment.

Material:

- Agar
- Flat bottom petri dishes
- Suction pump
- Pasteur pipet, Cork borer or template
- NaN_3
- 0.02 M phosphate buffer, pH 7.0

Preparation of Gel:

a) Gel for viruses not exceeding 300 nm:

Agar	=	0.75 gm
NaN_3	=	0.02 gm
1.4 M NaCl	=	10 ml
0.2 M buffer pH 7.6	=	5 ml
Make volume	=	100 ml

b) Gel for elongated viruses:

Agar	=	0.8%
SDS (Sodium lauryl sulfate)	=	0.5-0.8%
NaN_3	=	1%

c) Gel for Cucumber mosaic virus:

		(For 1 lit.)
Agar	=	7.5 gm
K_2HPO_4	=	1.86 gm

NaN_3	=	0.2 gm
Na-EDTA	=	0.2 gm

Procedure

After autoclaving pour gel in to Petri dishes (12:5 ml each), cover the Petri dishes and allow gel to set and cut the wells in agar using hollow straw tubes, cork borer or a template and remove the plugs with a pipette attached to a suction pump. Load reactants (antigen and antiserum) in the wells. Precipitation lines often become apparent within 24-48 hrs, but prolonged incubation may be needed in some cases.

Ultrasonic Treatment (Sonication)

- Put (1-2 ml) partially purified or purified antigen into thick walled conical sonication tubes.
- Fill sonicator with ice cold water.
- Fix antigen containing tube in a way that the meniscus should be at the same level as water in sonicator.
- Switch on sonicator for 10-15 minutes
- Antigen is ready for use in gel.

Note:

- Always use healthy plant extract as control.
- Avoid excess heating of agar.
- Sodium azide is poisonous and may form explosive compound with metals.

Latex Agglutination Test

This test is a modification of micro-precipitation. It involves first coating the antibodies on the latex balls (they are polystyrene balls 810 nm in diameter). The test is performed in plastic microtitre plates by mixing 50 ul of the antigen preparation (either purified or present in the leaf juice) with 25 ul of sensitized latex and then plates are placed on the rotary shaker for 1 hr at 130 turns per minute Aggregation takes place and indicates antigen - antibody interaction. The latex balls are much larger than antibodies and the reaction is visible to the naked eye. The main advantages of the latex test are the easy availability of the carrier particles and long storage life of the sensitized reagent.

ELISA - Enzyme-linked-immunosorbent Assay

The sensitivity of detection of antigen - antibody reactions can be increased by attaching to either of the two reactants a label that can be detected in minute quantities such as enzyme. The use of enzymes for labelling antibodies was used for

localizing antigens. It was soon found that, when the reactants were attached to a solid phase, enzyme immuno-assay was particularly suitable for the quantitative measurements of antigens and antibodies. As a result, the method known as Enzyme-linked-immunosorbent Assay (ELISA) was developed and rapidly adopted for the measurement of very low concentration of virus and specific antibodies. This method combines rapidity of serological virus assay with sensitivity of biological enzymatic reaction.

The presence of bound enzyme is revealed by a chromogenic substrate, which is initially colorless but yields a colored product after enzymatic degradation. Besides a simple visual scoring, for field screening of virus-infected sample, accurate quantitative readings can be made with a spectrophotometer. Two types of ELISA procedures are generally performed:

a) *Direct ELISA*

In this test virus specific immunoglobulins (IgG) extracted from antiserum are used for coating the plates and enzyme conjugate has to be prepared with same IgG for the detection of antigen. Since antigen is sandwiched between IgG and IgG enzyme conjugate the method is generally known as double antibody sandwich method. This method is strain specific. Enzyme conjugate prepared against one strain of virus will not necessarily react with closely related virus strains. Thus making it unsuitable for large scale diagnostic work and in case when adequate quantities of antisera are not available for IgG purification and conjugation. This method is useful to distinguish between different strains.

b) *Indirect ELISA*

The problem of strain specificity can be overcome by using so called indirect ELISA. In this test, plates are first coated with antigen followed by virus specific IgG coating of plates produced in rabbit. The enzyme is conjugated with goat or chicken's IgG produced by injecting them with rabbit serum, then antirabbit globulin enzyme conjugate is added to the plate. Finally, substrate is added to the plates. This method is not very specific but allows the detection of wider range of serologically related viruses.

Preparation of ELISA buffers

Coating buffer for:

		1000 ml	500 ml	250 ml	100 ml
Na_2CO_3	=	1.5 gm	0.75 gm	0.375 gm	0.15 gm
$NaHCO_3$	=	2.93 gm	1.465 gm	0.732 gm	0.293 gm
NaN_3	=	0.2 gm	0.1 gm	0.05 gm	0.02 gm

PBS Tween buffer for:

		1000 ml	500 ml	250 ml	100 ml
NaCl	=	8.0 gm	4.0 gm	2.0 gm	0.8 gm
KH_2PO_4	=	0.2 gm	0.1 gm	0.05 gm	0.02 gm
$NaHPO_4$ $12H_2O$	=	2.9 gm	1.4 gm	0.7 gm	0.29 gm
KCl	=	0.2 gm	0.1 gm	0.05 gm	0.02 gm
NaN_3	=	0.2 gm	0.1 gm	0.05 gm	0.02 gm
Tween-20	=	0.5 ml	0.25 ml	0.125 ml	0.05 ml

Sample extraction buffer:

PBS-Tween	=	1000 ml
PVP (Polyvinylpirrolidon)	=	20 gm
Eggalbumin	=	10 gm

Generally, buffer is used in 1:10 ratio or more (w/v) i.e., in 1 gm tissue 10 ml extraction buffer. Add 1% egg albumin when testing potato tubers or sprouts. Use 1 M urea for Potato virus A (PVA) in extraction buffer.

Conjugate buffer:

PBS-Tween	=	100.0 ml
PVP (Polyvinylpyrolidon)	=	2.0 g
Eggalbumin	=	0.02 g

Substrate buffer:

Prepare 10% diethanolamin pH. 9.8

Diethanolamine	=	10 ml
Dist. water	=	90 ml

Adjust pH with concentrated
HCl

Direct ELISA test procedure (Double antibody sandwich form - DAS)

- Coat ELISA plates with 200 l IgG diluted in coating buffer.
- Incubate for 1 or 2 hrs at 37°C.
- Add 200 l sample to be tested to each well.
- Incubate the plate over night at 4°C or 6 hrs at 37°C.
- Add 200 l conjugate diluted in conjugate buffer.
- Incubate the plate for 1-2 hrs at 37°C.

- Pour off the enzyme conjugate and add 200μl substrate (p-nitrophenyle phosphate (0.5-1 mg/ml) prepared in substrate buffer to each well.

- Incubate plates at room temperature for 30-60 min. In case of positive reaction substrate after hydrolysis turns yellow (p-nitrophenyle phosphate is converted to p-nitrophenol).
- Plates can be scored visually or in ELISA reader at 405 nm.
- Samples giving double or more ELISA value compared to healthy controls are considered to be diseased (positive).

Indirect ELISA Test Procedure

- Coat paltes with antigens mixed in coating buffer
- Incubate plate for 1 hr at 37°C.
- Add serum containing antibodies.
- Incubate the plate at 37°C for 1-2 hrs.
- Add enzyme labelled IgG conjugate.
- Incubate for 1-2 hrs at 37°C.
- Add substrate to the wells.
- Incubate at 37°C for 30-60 min.
- Plates can be scored visually or with ELISA reader.

NOTE:- After each step plates are washed thrice with PBS-Tween.

Serological Specific Electron Microscopy (SSEM)

It is the method in which serology and electron microscopy are combined. Along with ELISA, SSEM is the most sensitive method currently in use for the detection of plant viruses. The method can result in a 1000-fold or more increase in sensitivity over conventional electron microscopy in detecting viruses and especially it is very useful when the infection is mixed. With SSEM, one can visualize virus/antibody complex on electronmicroscopy grid. Generally, grids for SSEM are prepared in the following ways:

Trapping: Grids are first coated with antiserum and virus containing fluid is applied on the grid followed by negative staining.

Clumping of virus particles: In this method, virus containing extract is mixed with antiserum and then the mixture is applied on the grid. The grid is washed and negatively stained. By this method, 10-fold increase in number of particles on the grid is achieved.

Antibody coating: Carbon coated grid is floated on the virus containing extract of diseased plant, then the grid is treated with antiserum followed by negative staining. This method is also known as decoration method.

3. PHYSICAL METHODS

Electron Microscopy

The electron microscopy (EM) is one of the most important and powerful tool for modern virus research. The device has brought viruses in the range of human and photographic vision. The electron microscope gives a rapid idea of the type of particle involved in infection and these morphological properties of a given virus are mainly useful for assigning the virus to a particular virus group.

Leaf Dip Method

Materials:

- Fine forcep.
- Copper grid (support film coated).
- Blotting paper.
- Sample leaf.
- Clean glass slide.
- Pasture pipettes.
- Stain (2% phosphotungstic acid or 2% uranyle acetate).

Procedure:

- Clamp the grade into forcep.
- Select a small piece of tissue 4-10 nm2
- Squash it on glass slide with 2 drops of buffer.
- Using the fine pasture pipette place be drop of squashed leaf on coated side of the grid.
- Hold it for 1-2 min.
- Remove excess of samples with the help of blotting paper.
- Place a drop of suitable stain on the same side of the grid.
- Hold it for 1-2 min.
- Air dry the grid.
- Observe it under electron microscope.

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