INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY RESEARCH AND TECHNOLOGY
ISSN 2582-7358
Impact Factor 6.328
Peer-Reviewed Journal

Cultivation and Identification of Pathogens for Plant Disease Diagnosis

Dr. Shalini Saxena

Lab. of Cytogenetics, Department of Botany, Bareilly College Bareilly

Abstract

Diagnosis of plant pathogens is one of the critical aspects in effective plant disease management. Much of the study of pathogens depends on its ability to grow in the laboratory, and this is possible only if suitable culture media are available for the growth of pathogens. Plant pathogens can be isolated from various sources such as diseased leaves, roots, fruits, stem and soil, water etc., following some standard guidelines and procedures. During isolation, pure culture of the actual pathogen needs to be obtained from a mixed culture, as a diseased plant part may contain many species. By transferring a small sample into a new, sterile growth medium in such a way as to disperse the individual cells across the medium surface leading to thinning effect, the propagules may be separated out, before inoculating the new medium. Most commonly used methods for obtaining pure cultures of fungi are streaking technique, spore suspension dilution technique and single hyphal tip culture. For bacteria, commonly used methods for obtaining pure cultures are streak plate method, pour plate method and spread plate technique. Finally, various approaches need to be conducted for proper and accurate identification of the pathogen associated with any disease, *viz*., classical approach for identification of microorganisms as well as biochemical, serological and nucleic acid based tests in the aid of identification.

Keywords: Cultivation, disease diagnosis, identification, microorganisms, pathogen

Introduction

Disease diagnosis requires thorough investigation, following aseries of standard procedures, which start from study of symptom expression in diseased plants. Symptoms, though give vital information on the probable cause of the disease, it cannot be conclusive as many similar symptoms are produced by many different pathogens and abiotic stress. Along with symptoms, signs are also studied which are actual parts of the pathogen visible to naked eye such as spores of fungus, dormant structures such as sclerotia present on host surface or bacterial ooze coming out from host tissues. Further, dissection and observation under compound microscopes for specific spores and fruiting bodies give crucial leads in identification of possible disease agents. Sometimes neither symptoms nor signs and microscopic observation provide enough specific or characteristic information to decide the cause of an infectious plant disease. In such cases, it may be necessary for further laboratory tests to isolate and identify the causal agent.This requires specialized skills for cultivation of pathogens through isolation and pure culture facilitating accurate identification. Furthermore, isolation and pure culture isamandatory step in pathogenicity test for confirming association of a pathogen in case of a new disease, in cases where the organism has not been reported to be a pathogen on that particular host. Furthermore, microbial cultures are fundamental for downstream studies on genetic diversity as well as molecular biology.

Cultivation of Pathogens/ Microorganisms

Cultivation of plant pathogens by isolation and pure culture, enables study of various morphological, cultural, physiological and biochemical parameters facilitating proper identification of genus and differentiation among closely related species of pathogens. Facultative parasites and facultative saprophytes have the ability to grow in artificial media and can be isolated and maintained in appropriate media for characterization of these pathogens. But obligate parasites such as downy mildew, powdery mildew and rust fungi and viruses, viroids, phytoplasmacannot be cultured in artificial media and have to maintained in live hosts plants by adopting different transmission methods.

Culture Media

When microorganisms are cultivated in the laboratory, a growth environment called a medium is used. A typical culture medium must contain all the essential nutrients and growth factors necessary for proper growth and development of microorganisms. A culture media can be solid, semi solid or liquid (broth). For solidifying any media, a jellifying material generally agar-agar, a complex polysaccharide derived from red algae, is added into it. Agar has a unique physical property as it acts as inert material in media which melts at 96°C and remains liquid until cooled to 40-45°C, the temperature at which it solidifies. In a semi-solid media agar is added at a concentration of 0.5% which is generally used for micro aerophillic bacteria. Due to great variation in nutrient requirement of different pathogens, the composition of the media used for their cultivation also varies. Media rich in carbohydrates and slightly acidic in nature (pH 6-6.5) favour the growth of fungi, whereas bacteria prefer neutral or slightly alkaline pH. A media must be sterilized prior to inoculation with microorganisms.

Types of Media Based on Chemical Composition

On the basis of their composition, there are three main types of culture media: (i) natural or empirical culture media; (ii) semi-synthetic media; and (iii) synthetic or chemically defined culture media. The exact chemical composition of anatural medium is not known. The natural culture media include milk, vegetable juices, meat extracts, rice grain and infusions. Those media whose chemical composition is partially known are called semi-synthetic media. Potato dextrose agar (PDA), Nutrient agar (NA), CzapekDox agar, Oat meal agar, Beef peptone agar are some of the semi-synthetic media. Semi-synthetic media are routinely used in laboratory. Synthetic or a chemically defined mediumis one whose chemical composition is precisely known such as Mineral glucose media, Richard’s solution, Raulin’s medium, Martin’s rose Bengal medium etc. Synthetic media are costly and take more time to prepare than semi-synthetic and natural media. The choice of the media depends on purpose and requirements of the experiment.

Types of Media Based on Application or Function

Based on specific function of obtaining pure culture, media may be of mainly two types: (i) Selective media (ii) Differential media.

The selective media are those which permit the growth of some specific group or type of organisms while preventing or retarding or inhibiting the growth of others, thus facilitating microbial isolation.This selectivity is achieved in several ways. For example, organisms that have the ability to utilize a given sugar are screened easily by making that particular sugar the only carbon source in the medium for the growth of the microorganism. Like-wise, the selective inhibition of some types of microorganisms can be studied by adding certain dyes, antibiotics, salts or specific inhibitors that will affect the metabolism or enzymatic systems of the organisms. For example, media containing potassium tellurite, sodium azide or thallium acetate at different concentrations of 0.1 - 0.5 g/l will inhibit the growth of all Gram-negative bacteria. Also, media added with crystal violet dye becomes selectively bacteriostatic for Gram-positive bacteria. Eg. Phenylethyl alcohol agar, Mannitol salt agar and Hektoen enteric agar.

A differential medium is that which will cause certain colonies to develop differentially from other closely related organisms present by producing characteristic change in the bacterial growth or the medium surrounding the colonies. These media are used for distinguishing among morphologically and biochemically related groups of microorganisms.Certain dyes or chemicals (such as phenol red, eosin or methylene blue) are added in the media due to which the organisms will produce characteristic changes or growth patterns that are used for identification or differentiation.Unlike selective media, differential media does not kill organisms but it gives an indicationif the target organism is present.

Isolation of Plant Pathogens

Plant pathogens can be isolated from various sources such as diseased leaves, roots, fruits, stem and soil, water etc. In general,attempt to isolate the plant pathogen is

done from the margins of the diseased tissue where the pathogen in active form is more abundant or active, whereas in recently killed tissue saprophytes are more numerous.The infected tissue may contain many saprophytesin the necrotic areasand these saprophytes may outgrow the plant pathogen on the culture medium, thwarting accurate identification of the pathogen. To avoid this, plant tissues are surface sterilized with 10% Clorex or1% Sodium hypochlorite solution or 70% ethyl alcohol for 30 seconds to 2 minutesfollowed by thorough washing with sterilized distilled water. The tissue sections are blotted in sterile paper towel to remove any traces of surface sterilant. Thereafter, surface sterilized tissue sections are placed on various nutrient media and the organism that grows out of this tissue in the medium, is then isolated and subsequently pure cultured.Bacteria are often isolated by chopping up surface sterilized infected tissue in a small amount of sterile waterallowingbacteria to ooze out or byimmersion of cut ends of stem of wilted plants in a beaker containing distilled water. This water:bacteria suspension is then streaked onto 3-4 petri plates containing bacteriological medium such as nutrient agar with the help of sterilized inoculating loop without recharging the loop. In cases where a specific plant pathogen is suspected, a medium selective for the pathogen may be used. For example, TTC media for *Ralstoniasolanacearum*, King's B agar media for *Pseudomonas spp.* These plates are incubated at 25 - 30°C for 4 to 10 days.

In case of fungal pathogen, the pathogen grows out of the host tissue and a small segment of this culture is further transferred to a fresh sterilized culture plate of nutrient medium and incubated for 7 days for further purification. In case of bacteria, colonies are developed in nutrient media which are to be picked up and purified.

Purification of Plant Pathogens

Microorganisms isolated from diseased plant tissues under controlled condition in a medium may contain the pathogen in association with other microbes such as secondary saprophytes. Also, microorganisms isolated from various sources such as soil, water, food, sewage etc. may contain billions of individuals. From the isolated mixed culture, it

is essential to obtain a culture containing only one kind of microorganism called pure culture. Pure culture**,** in microbiology, is a laboratory culture containing a singlespeciesof organism. A pure culture is usually obtained from a mixed culture,comprising of many species, by transferring a small sample into a new growth medium so as to disperse the individual cells across the medium surface, which leads to thinning of the sample many fold before inoculating the new medium. Both methods separate the individual cells so that, when they multiply, each will form a discrete colony, which may then be used to inoculate more medium, as a result only one type of organism will be present in that media. Isolation of a pure culture may be improved by providing a mixed inoculum with a medium favouring the growth of one organism in comparison to the others. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another. Pure cultures are essential in order to study the biochemical characteristics, morphology, staining, immunological and genetic properties. A culture which contains more than one kind of microorganism is called a mixed culture and if it contains only two kinds of microorganisms that are deliberately maintained in association with one another, it is called two-member culture or dual culture.

Most Commonly Used Methods for Obtaining Pure Cultures of Fungi Are

Streaking Technique

This technique involves purification of fungus cultures by taking a small portion of fungus on a loop and streaking it over an agar surface in a petri dish. As streak dissipates, the spores become more and more separated until eventually individual colonies are obtained arising from single spores or clumps of spores. Streaking out a suspension of spores in sterile water can improve the prospects of obtaining single spore colonies.

Spore Suspension Dilution Technique

Spore inoculum is placed in a tube of 10 ml of sterile water, from which a clean sterile pipette is used to transfer an aliquot (e.g. 1ml) of this to a tube with 9 ml sterile water. A fresh sterile pipette is used to mix this and transfer 1 ml of this to another tube containing 9 ml sterile water. This is continued for as many dilutions as required and 0.1 ml is plated out each dilution separately in petri dishes by spread plate or pour plate technique.

Single Hyphal Tip Culture

In this method, the growth of spore is allowed on a plain agar surface in a petri dish for 24- 48 hours and hyphal tip coming out from the single spore or single cell of multiseptae spore are marked and transferred.

Most Commonly Used Methods for Obtaining Pure Cultures of Bacteria Are

Streak Plate Method

Loopful of bacterial suspension is streaked over the surface of nutrient agar medium in plates by to and fro motion of inoculating loop. Two more plates are streaked without recharging the loop with bacterial suspension. Plates are labelled and incubated in an inverted position at 25°C. Most bacteria develop colonies within 4 to 5 days, but some of them may take as long as 10 days. Single colonies are usually obtained in second and third plate.

Pour Plate Method

In this technique, successive dilutions of the inoculums (serially diluting the original specimen) are added into sterile Petri plates to which is poured melted and cooled (42-45°C) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify. After incubation, the plates are examined for the presence of individual colonies growing throughout the medium. The pure colonies which are of

different size, shape and colour may be isolated/ transferred into test tube culture media for making pure cultures.

Spread Plate Technique

The spread plate technique is used for the separation of a dilute, mixed population of microorganisms so that individual colonies can be isolated. In this technique microorganisms are spread over the solidified agar medium with a sterile L-shaped glass rod. Alternatively, sterile glass beads may be used for uniform spreading of the cells. Some of the cells will be separated from each other by a distance sufficient to allow the colonies that develop to be free from each other.

After incubation in steak plate, pour plate or spread plate techniques, appearance of the discrete, well separated colonies would be observed. Sometimes, more than one type of colony may develop and in such case the colonies which are more abundant and constantly found in several suspensions from affected tissues are selected. Also, colonies which come up slowly are likely to be pathogenic. For confirmation, it is desirable to select two or three types of colonies, streaked on nutrient agar medium and tested for pathogenicity.(Vishunavat and Kolte, 2005)

The next step is to subculture the colonies to separate agar-slants with a sterilized needle or loop. Sub-culturing is the procedure of transferring of microbes from their parent culture to a fresh one or from one medium to another. Live cultures on a culture medium can be successfully stored in refrigerators or cold rooms maintained at 4°C, where the metabolic activities of microorganisms will be greatly slowed down at this temperature but not low enough to stop metabolism completely. Hence, regular sub-culturing is required at an interval of 2-3 weeks in bacteria and 3-4 months in fungi.

Identification of Microorganisms / Pathogens

Upon successful isolation of amicroorganism from a infected plant tissue, the organism must be identified. There are estimated some 1.6 million fungal species most of

which are non- infectious. Many fungi and bacteria have never been isolated and identified. The characteristics upon which their identification is based are often complex. Diagnosticians with experience are often able to identify the most commonly isolated organisms. The fact that scientists can identify so many kinds of organisms including microbes with scientific names is because they have been described and ordered by a system of classification and nomenclature that is internationally accepted. This science devoted to identifying, naming and classifying organisms is called taxonomy.In case of fungus and bacteria, when an unknown organism is isolated in the laboratory in the pure form, it is usually identified by a combination of information from microscopic observation of morphology, cultural characteristics, biochemical tests, serological reactions and genetic relationship.

Classical Approach for Identification of Microorganisms

Identification of Bacteria

The classical approach for bacterial identification involves a series of studies such as morphology and arrangement of cells, cultural characteristics on agar and in broth, gram staining and other staining reactions, the absence or presence of motility and endospores, biochemical and molecular tests etc.Bergey'sManual of Determinative Bacteriology serves as a practical guide for identification of bacteria which contains key and tables describing additional criteria for identification of genus and species of bacterium. Morphological studies such as shape, arrangement, differential staining reactions, and cultural characteristics such as form, margin, elevation, density provides vital keys(Arneja., 2012). The identification and characterization of bacterial isolates based on its expression of the "chemical identity" through testing their biochemical and physiological properties are fairly stable and reliable techniques of bacterial differentiation. The ability of a bacterium to utilize C or N sources such as Levan production, carbohydrate fermentation test, hydrogen sulphide, indole production or to produce various enzymes and gases such as catalase test, casein hydrolysis test are

determined which serves as the basis for bacterial differentiation. Additional tests may include analysis of fatty acids, carbohydrate utilization (*i.e.* BIOLOG test), and enzyme activity testing (*i.e.* pectinase, isozyme patterns) helps in bacterial identification (Borah *et al*., 2015).

Identification of Fungi

Identification of fungus, as soon as it is isolated in a pure form, is often made by recognition of characteristic structures seen in culture which includes colonial morphology: waxy, leathery, velvety, fluffy; hyphal characters: coenocytic or septate; asexual spores: zoospers, sporangiospores, conidia, blastospores; sexual spores: ascospores, basidiospores, oospores or zygospores; reproductive bodies: ascocarp, basidiocarp, pycidia, acervuli and arrangement of conidia: solitary, masses, chains (acropetal and basipetal). Also, dimensions of various structures *i.e.* hyphae, spores, reproductive bodies is calibrated using ocular micrometer. After recording the microscopic details of a culture it is identified up to generic/ specific level consulting various taxonomic books and monographs available on various groups of fungi. Further, the field of chemotaxonomy including isozyme analysis and fatty acid profile provides a reliable tool for species identification (Arneja, 2012)

Identification of Biotic Causal Agents

A major problem in identification of biotic causal agents is the inability of some infectious pathogens to grow on artificial media. Viruses, as well as some fungi (e.g. powdery and downy mildew causing agents) and some prokaryotes (e.g. phytoplasmas), require a living host in order to grow. In cases where the plant pathogen is difficult or impossible to grow on artificial media, other methods may be used for their detection, such as the use of serological tests for viruses. Viral identification is often accomplished utilizing ELISA (enzyme-linked immunosorbent assay) which is based on the binding of an antibody produced to a specific virus with the virus in the infected plant material. More tests are currently being developed using the polymerase chain reaction (PCR) for

detection of specific organisms. These types of reactions take specialized equipment and reagents, and the tests are not commonly done outside diagnostic and research laboratories. Other techniques used for the identification of viruses include negative staining and electron microscopy to view the viral particles in plant tissue or suspensions. PCR and ELISA tests, as well as other serological and nucleic acid based laboratory tests, may be used for organisms that grows on artificial media as a confirmatory tool (Riley *et al*., 2012).

Serological Tests in the Aid Of Identification

These test forms an important step in microbial identification. Serological tests, involving the reactions of microorganisms with specific antibodies, are useful in determining the identity of strains and species, as well as relationships among organisms. Slide agglutination, ELISA, and Western blotting are examples of serological tests. It usually involves detection of antigens by enzyme or fluorescence immunoassays. Serology is also used to confirm identification obtained by other methods.

Nucleic Acid Based Tests in The Aid Of Identification

Among the tools available for pathogen detection, nucleic acid (NA)-based techniques are widely recognized as some of the most powerful. NA-based detection techniques, particularly those that rely on the polymerase chain reaction (PCR), typically are rapid, specific, and highly sensitive. Other (NA)-based techniques such as Restriction fragment length polymorphisms (RFLP) of DNA representing selected genes can be used to identify pathogen species. Amplified fragment length polymorphism (AFLP), a modification of the RFLP technique, has been used for species identification and more commonly to examine genotypic diversity within a population. For fungus and bacteria, ribosomal RNA genes and intervening sequences are common targets for PCR amplification. The internal transcribed spacer (ITS) regions of ribosomal genes are especially useful targets for species-specific primers for fungal identification(Vincelli and Tisserat, 2008). Similarly, the 16S rRNA gene consists of highly conserved nucleotide

sequences, interspersed with variable regions provides targets for genus- or species-specific primers for bacterial identification. PCR primers targeting the conserved regions of rRNA can amplify variable sequences of the rRNA gene enabling bacterial identification by analysis of the PCR product followed by comparison of sequences with known sequences stored in a database (Jenkins *et al*., 2012).

Conclusion

Accurate and timely diagnosis of plant diseases can help to formulate disease management strategies in the right direction, whereas improper identification of the disease and the disease-causing agent, management practices adopted can be a waste of time and money and can lead to further disease spread. Therefore, for proper diagnosis of any plant disease, very good observation skills, sound training and education is very much important. Various diagnostic kits available, identification of causal organism(s) associated with the disease(s) by adopting various conventional and advanced techniques at laboratory and field level along with Artificial Intelligence (AI) technology is going to help further in early detection and prompt action on disease management.

References

Aneja, K.R. (2003). Experiments in Microbiology, Plant Pathology and Biotechnology. Fourth edition New Age International (P) Limited, Publishers, New Delhi.

Bora, L.C., Kataki, L. and Talukdar, K. (2015). Practical Bacteriology. Assam Agricultural University, Jorhat.

Jenkins, C., Ling, C.L., Ciesielczuk, H.L., Lockwood, J., Hopkins, S., McHugh, T.D., Gillespie, S.H. and Kibbler, C.C.(2012). Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. *Journal of Medical Microbiology*.61: 483–488. doi: 10.1099/jmm.0.030387-0.

Riley, M.B., Williamson, M.R. and Maloy, O. (2002). Plant disease diagnosis. *The Plant Health Instructor*. doi: 10.1094/PHI-I-2002-1021-01.

Vincelli, P. and Tisserat, N. (2008). Nucleic Acid-Based Pathogen Detection in Applied Plant Pathology. *Plant Disease*. 92(5): 660-669. doi:10.1094/ PDIS-92-5-0660.

Vishunavat, K. and Kolte, S.J. 2005. Essentials of Phytopathological Techniques. Kalyani Publishers, New Delhi.