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Mini Review

General Research Methods to Deal with *Phytophthora infestans* in Laboratory Conditions

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Abstract

The plant destroyer known as *Phytophthora infestans* (Mont) de Bary) causes of potato late blight is very difficult to be isolated and purified on artificial media in lab conditions. Therefore, according to research experience on the this specific pathogen in past 4 years, the general research methods have been summarized in this paper which includes the symptomology, method of sample collection, isolation protocols, culture storage and specific media preparation.

Keywords: *Plant; Late Blight; Isolated; Media; Methods*

Introduction

Potato late blight is one of the most devastating plant diseases worldwide (Frobes, 2012). It is caused by Oomycete *Phytophthora infestans* (plant destroyer) that infects members of the Solanaceae family [1]. It was first reported in the 1830s in Europe and in the US. It is famous for being the cause of the 1840s Irish Potato Famine, when a million people starved and a million and a half people emigrated [2]. Late blight continued to be a devastating problem until the 1880s when the first fungicide was discovered [3]. In recent years, it has reemerged as a problem. It is favored by cool, moist weather and can kill plants within two weeks under favorable conditions [4]. Keeping in mind the devastating nature of the pathogen we tried our best to present an overview of all the methods which we used in laboratory of Plant Pathology, College of Agriculture, Sargodha.

Symptomology

Leaves: The earliest symptoms of leaves begin as small, pale to dark green, irregularly shaped spots. These spots are not bordered by veins and often have pale green to yellow rings surrounding them. Lesions begin more frequently at leaf tips and margins. Under favorable conditions, these spots grow rapidly into large brown to purplish black areas and give the typical appearance of "V" shape. A white mildew can be seen at the lower side of the leaves which is the distinguish character of this pathogen.

Stem: Black leaves expand length wise on the petioles and stem which may cause death of plant parts above the lesion.

Tuber: Superficial and irregular discoloration and brown necrotic lesions can be seen on potato tubers.

Method of sample collection

The leaves with typical symptoms (white mildew was visible) were selected from major potato growing areas. We collected the single lesion leaves in polythene zipper bags. The other method which we used after inoculated the small pieces of infected leaves onto potato cut tubers and then tubers were sealed with adhesive tape. The samples were brought to laboratory of Plant Pathology College of Agriculture, Sargodha for further experimental work.

Isolations protocols

Blighted leaves were incubated overnight or longer in humid boxes in daylight at 15 - 20°C (we used petri plates lined with damp tissue paper) to encourage sporulation. The other technique which we used was cutting of leaves into small pieces; put it under tuber slices of a susceptible variety (e.g. Desiree or any other). Make sure the petri-plates are air tight (cover with parafilm and incubate at 18-degrees centigrade). Mycelia grow through the tuber slices between 4 - 7 days depending on the aggressiveness of the different isolates.

When sporulation was developed, we isolated onto antibiotic rye agar media. This can be done by cutting small pieces of infected tissue and placing on the agar (or even under the agar), but the method we found most effective was to cut small cubes of antibiotic rye agar (approx. 2 x 2 mm) using a sterile scalpel and use these to pick up sporangia by lowering them gently onto the sporulation trying to avoid directly touching the infected tissue as far as possible.

These agar cubes were then placed onto fresh plates of antibiotic rye agar and the plates incubated at 18°C (light isn't needed) checking regularly for growth of *P. infestans* hyphae from the cubes into the agar (using a lower powered microscope). When small colonies was developed these were sub-cultured onto fresh antibiotic rye agar plates (Personal communication with Dr. Louise Cooke).

Baiting technique

We observed the alternate method to isolate *Phytophthora infestans* from soil is to use apple fruit trap. In order to get that you have to choose apple fruit, wash it carefully, then sterilize it in suitable solution, wash it again in sterilized distilled water then immersed it until its half. Irrigate the soil and leave the fruit till appearance white mycelium growth in area of the fruit between soil and air, then you can isolate on suitable medium.

Bacterial contamination problem and solution

We observed the problems with bacterial contamination even on selective media. Therefore, we tried to put infected leaves or sporangiophores/sporangia between air-dried potato tuber slices (ca. 0.5 - 0.8 cm thick) or in a hole made with cork borer in a clean and surface disinfected young tuber of a susceptible cultivar. Keep the slices in moist chamber at 20°C degrees with no contact between the slices and paper towel (mycelium will grow through the slices and you can easily take it from the surface). Regarding the whole tuber, you can slice it when symptoms develop near the inoculation point or wait until you see sporangia on the surface.

Purification of isolates

The specific media (detail mentioned) was poured into sterilized petri plates and plug of white colony of pathogen was removed from previously inoculated plates as well as from tuber slices onto fresh antibiotic media plates and put at 18 - 20°C in incubator for 7 - 10 days for pure cultures. Isolates were sub cultured when required for experiments by removing a section of mycelium from the actively growing edge of a colony through cork borer or a scalpel and transferring to specific media. Isolates were routinely sub cultured at two-three-weekly intervals.

Culture storage

Refrigeration

Pure cultures of isolates were stored at almost 0 - 4°C either in room temperature or refrigerators. This protocol was worked for short duration up to 3 - 4 months for fungi because the metabolic actions of the microorganisms were slowed down but was not stopped.

It has been observed that the nutrients was utilized and ultimately waste products was released in nutrient medium resulting is the death of the microbes after sometime

Cryopreservation

We cut mycelia plugs of 1 cm with cork borer from a 2 week old culture (fresh plate) and place 1 ml 10% glycerol in each cryotubes tube. Almost put 3 - 4 plugs in each tube to make sure that glycerol covers everything and transfer tubes to freezer box and place immediately in the - 80°C freezer for 30 minutes. The same procedure was followed to store the cultures of *P. infestans* in 15% DMSO.

Preparation of inoculation

Newly formed sporangia were collected by needle and add 10ml of distilled water to each peri dish and spore suspension were filtered through cheese cloth by making concentration of 60000 sporangia/ml through haemocytometer. These collected spores were refrigerated at 4°C for 2 hours to release zoospores for use of inoculation [5].

Identification of pathogen

The pathogen belongs to class Oomycetes and genus Phytophthora. The vegetation is mycelium characterized by absence of cross walls. Both sexual and asexual reproduction present while sporangia emerged at asexual reproduction phase. Microscopic views revealed the lemon shaped sporangia with approximately 21 - 40 µm × 10 - 20 µm. Sporangia can be seen at the end margins of sporangiophores. The asexual spores known as oospores were orange red, round shaped with measurement of approximately 30 - 34 µm. The branched sporangiophore, with swellings at the points where sporangia were attached is the distinctive for *P. infestans* and useful for identification of this pathogen.

Culture media preparation

Some media recipes according to the description of renewed scientists those are working on this aspect for making specific media for isolation of *Phytophthora infestans* are described mentioned below: However, according to our experience carbohydrate is very necessary for sporulation. Therefore, we observed in our experimental findings that corn meal agar, carrot agar media and rye agar media are very important media for further study.

Potato dextrose agar media

We prepared potato starch by boiling 200g sliced; unpeeled potatoes in 1 liter distilled water for 30 minutes. Filter through cheesecloth and add dextrose (15g), Agar (20g) and made volume upto 1 liter after adding distilled water. Autoclave for 15 minutes at 121°C for further use [6].

Water agar media

We suspended 20.00 grams in 1000 ml distilled water and heat to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After autoclave, cool media up to 45 - 50°C. Mix well and pour into sterile petri plates for further uses [7].

Rye agar media

The Rye grass seeds about 200 - 300 taken and washed them using tap water and them soaked seed for a while. Add more water and then boil seed for an almost half an hour. After boiling we extracted the seeds and use only extraction after adding 20g sucrose and 20g agar and then complete it to 1L. Autoclave this media for 15 minutes at 121°C. After autoclave, cool media up to 45 - 50°C. Mix well and pour into sterile petri plates for further uses (Caten and Jinks, 1968).

Pea agar media

Frozen garden peas (160g) were blended and made volume up to 1L with 10g of agar added. The medium was autoclaved for 15 minutes at 121°C. After autoclave, cool media up to 45 - 50°C. Mix well and pour into sterile petri plates for further uses (Hollomon, 1965).

Carrot agar media

Two hundred grams of frozen fresh carrots were boiled in 600 ml of distilled water and blend and drained through muslin cloth. Medium was then made up to 1L with 9.6g of agar added and autoclaved for 15 minutes at 121°C. After autoclave, cool media up to 45 - 50°C. Mix well and pour into sterile petri plates for further uses [8,9].

Corn meal agar media

We suspended almost 17 grams in 1000 ml distilled water and boiled to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After autoclave, cool media up to 45 - 50°C. Mix well and pour into sterile petri plates for further uses [10-12].

Conclusion

Potato is incredibly significant crop worldwide especially in Pakistan. Therefore, keeping in mind its significance we tried our best to find out some isolation and identification techniques during our ongoing project in which we are studying genetic diversity of *Phytophthora infestans* in potato growing areas of Punjab, Pakistan. Hence, the general research methods which gave best results have been summarized in this article.

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Conflict of Interests

The authors declare that there is no conflict of interest to publish the article.

Bibliography

1. Fry W. "Phytophthora infestans: the plant (and R gene) destroyer". *Molecular Plant Pathology* 9.3 (2008): 385-402.
2. Turner RS. "After the famine: Plant pathology, Phytophthora infestans, and the late blight of potatoes (1845-1960)". *Biological Sciences* 35.2 (2005): 341-370.
3. Haas BJ., *et al*. "Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans". *Nature* 461.7262 (2009): 393-398.
4. Schumann GL and D'Arcy CJ. "Late blight of potato and tomato". The Plant Health Instructor (2000).
5. Pliakhnevich M and V Ivaniuk. "Aggressiveness and metalaxyl sensitivity of Phytophthora infestans strains in Belarus". *Zembdirbyste Agriculture* 95.3 (2008): 379-387.
6. Murray B., *et al*. "Manual of clinical microbiology, 9th edition". American Society for Microbiology, Washington, D.C (2007).
7. Atlas RM. "Handbook of Microbiological Media". 2nd Edition, CRC Press, New York (1996).
8. Erselius L J and Shaw D S. "Protein and enzyme difference between Phytophthora palmivora and P. megakarya: evidence for self-fertilization in pairings of the two species". *Transactions of the British Mycological Society* 78.2 (1982): 227-238.
9. Kumbar B. "Standardization of specific media for Phytophthora infestans". *Global Journal of Bioscience and Biotechnology* 6.2 (2017): 374-376.
10. Salfinger Y and Tortorello ML. "Compendium of Methods for the Microbiological Examination of Foods, 5th Edition". American Public Health Association, Washington, D.C (2015).

11. Medina MV and Platt HW. "Comparison of different culture media on the mycelial growth, sporangia and oospore production of Phytophthora infestans". *American Journal of Potato Research* 76.3 (1999): 121-125.

12. Young GK. "Competitive selection within Phytophthora infestans populations from Northern Ireland and Michigan". Ph. D. Thesis, The Queen's University of Belfast (2007): 300.

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