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A simple method for diagnostic of *Phytophthora infestans* (Mont.) de Bary from potato agricultural fields of potato

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A B S T R A C T

A correct detection and appropriate identification of causal pathogens associated with crop plants or seeds are considered to be the most important issue in designing the proper management plans for plant diseases. This study was designed to detect *Phytophthora infestans* inoculum from potato grown soil. A high detection rate of *P.infestans* was obtained from the naturally infested soil of potato fields. Naturally soils were firstly moistened in a plastic pots and then pre-incubated at ±18°C for 3 days, baiting with potato tuber slice for 24, 48, and 72 h. The baits were then thoroughly washed, flooded with 10–15 ml of distilled water in Petri-dishes and incubated under continuous darkness in chamber ±18ºC. Sporangia started to emerge from the margins of potato tuber slice. They were easily observed under the stereomicroscope. Pure culture of the fungus was obtained by isolating from baited tubers on a Rye Agar medium. This is the first report of recovery of *P. infestans* from naturally infested potato growing soils using susceptible potato tuber (K. Bahar) as bait in India. All isolates were determined to be A2 mating type.

Introduction

Potatoes are susceptible to a range of pests and diseases that have large negative impacts on yield and tuber quality. Late Blight of Potato is a number one disease. Early detection in seeds, planting materials, or ensuring disease-free planting materials through rapid diagnostics are likely the effective means of reducing bacterial disease incidence. Potato (*Solanum tuberosum* L.) is a member of the night shade family and is the third most important non-cereal food crop in the world after wheat and rice which is consumed by more than billions of people worldwide. A third of potato production takes place in developing countries, and over 1 billion people have potato as their staple diet. Global damage and control costs exceed US$6.2 billion per year, with over $1 billion spent on fungicides alone (Haverkort et al. 2008). Baiting techniques use plant material to isolate selectively the pathogen from soil or diseased roots. The baiting technique is commonly used for detection of *Phytophthora* occurrence in soils (Erwin and Ribeiro 1996, Mohammadi et al. 2008). The technique involves floating pieces of susceptible tissue on a soil water slurry with a high water/soil ratio (Erwin and Ribeiro 1996). Zoospores formed by *Phytophthora* in the sample infect the baits which can be detected by plating baiting tissue onto selective agar containing antibacterial and antifungal antibiotics allowing outgrowth of *Phytophthora* from the tissue (Reeser et al. 2011). *Phytophthora* species growing out of the bait can be identified on the basis of colony morphology, mycelial characteristics, cardinal growth temperatures, and production, morphology, and dimensions of sporangia, oogonia, and antheridia, or DNA sequence analysis (Lamour and Kamoun 2009). Depending on the time of year at which the sample is taken, the efficiency of detection ranges more than 90%. The host species from which the bait tissue is derived also influences the efficiency of detection (O'Brien et al. 2009). Although the bait tissue is derived from a host species that is susceptible to the pathogen, different host species give very different efficiencies of detection (Erwin and Ribeiro 1996). Isolation of *Phytophthora* from the infected bait requires a considerable time, the use of selective media and considerable knowledge of the genus (Yamak et al. 2002). A major problem is the presence of fast growing organisms such as *Pythium*, which tend to inhibit growth of the target species (Canaday and Schmitthenner 1982). The infection of the bait can be a limiting factor. The efficiency of detection of *Phytophthora* in soil by the baiting technique can be improved by seveing out the soil and rewetting it (double baiting demonstrating that, as with tissue sections, the

pathogen, although present, will not always grow out of the sample (Canaday and Schmitthenner 1982). Davison and Tay (2005) found that double baiting increased the recovery of positive samples. The quality of the water used can also significantly affect the outcome as zoospores are very sensitive to toxic ions present in unpurified water (Tsao 1983). The objectives of this study were to develop a rapid and sensitive baiting method for isolation of *P.infestans* from the soil carried with transported potato tuber seeds or in production fields, and to develop a rapid assay to detect the pathogen in infected potato growing fields.

Materials and Methods

Soil sample Collection, Soil Bating and Isolation

Soil samples were collected from potato growing fields from Western U.P, H.P and Karnataka states (one field from each district), India, during growing seasons. Five individual samples were taken arbitrarily to a depth of 15 cm using a hand trowel and were well mixed to represent a single composite sample for each field, with a final volume of approximately 100 cm^3. All soil samples were sieved, and then stored in plastic bags at incubating chambers at ±18ºC. *P.infestans* isolation from soil was made different from as described by Lacey et al. 1965 potato tuber slice baiting method. Soil samples were moistened, (Potato tuber slices where dipped for 5 min. in 200ml of distilled water containing dissolved 1mg (each Nystatin, Rifampicin and Benlat), dried for 5 min. on filter paper, tuber slice were inserted in pots, pre-incubated at 18ºC for four days, and then taken out from plastic pots, washed with running water and incubated at 18±ºC in BOD dark chamber (Fig.1). Sporangias emerging from the edge of the infected potato tuber slices were observed under a stereomicroscope after 48 h of incubation. Tuber slice with sporangia of *Phytophthora* were placed in another Petri dish with 10 ml of sterile distilled water. The entire set of Petri dishes (*i.e.* two plates each) was placed in a refrigerator at 9ºC for 30 min to complete the process of zoospore release. Cultures were grown on Rye Agar medium agar and maintained with regular transfers. Vegetative and reproductive stages were examined in cultures grown for 1 to 4 weeks. Isolates were scored for appearance of hyphae, size, and shape of sporangia and chlamydospores, and the presence or absence of oospores. Observations were compared to published descriptions for *Phytophthora* species (Ho 1981, Erwin and Ribeiro 1996, Ranjbaran et al. 2006, Gallegly and Hong 2008). The mating types of isolates were determined by pairing known A1 and A2 testers of *P.infestans* on Rye Agar medium. The unknown isolate was placed on one side of the 10-mm-diameter Petri dish and the known A1 or A2 tester was placed on the other side. The Petri dishes were incubated in the dark at ±18ºC for two weeks or until oospores were formed. The mating type was identified for unknown isolates based on the presence of oospores. If pairing with the known A1 tester produced oospores, then the unknown isolate was determined to be A2, and vice versa. Inoculums for pathogenicity tests were prepared by growing isolates on Rye agar in Petri plates at ±18ºC for 2 weeks. Inoculations were performed by the standard detached leaf method (Singh and Bhattacharyya, 1995), using 5 mm pieces of mycelia plug, and the wound was covered to prevent desiccation of the inoculums and host tissue. After 24hrs, the infection caused by *P.infestans* zoospores starts to appear in the potato detached leaves.

Results

The baiting bioassay detected *P. infestans* in naturally infested potato growing soils from India. While during baiting method for detection of *Phytophthora*, many other contamination were caused by other microorganisms. The percentage of potato tuber slice detecting *P.infestans* was consistently the highest and usually was significantly greater than those for the other baits tested. Duration of baiting affected both detection of *P.infestans* and incidence of contaminants.After 48 hr, more infection of *P.infestans* was observed (Fig.1). The temperature during baiting has a dramatic effect on the detection of *Phytophthora* or incidence of contamination. Five isolates were obtained belonging to *P.infestans.* The phenotypic characteristics of these isolates were fluffy cottony mycelium with slightly striated pattern; slow growth rate on the Rye agar medium (Fig.2). Both hyphal swellings and chlamydospores are absent. None of the isolates grew at 5ºC but they showed growth at 28ºC.Sporangiosphores was branched compound, sympodial, with a small swelling at the base of each branch. This type of sporangia are more frequently observed on the potato plants than in culture while sporangia were terminal or lateral, ellipsoid, ovoid or limoniform, semipapillate (<3.5µm); deciduous, pedicelless than 5 µm long. Their size 17-59x 12-31 µm, av. 27-38 x 18-22 µm:LxB 1.6-1.7; discharge pore 7 urn or less in diameter. Zoospores are biflagellate, 8-10 um encysted. In sexuality it is heterothallic, rarely homothallic (Fig.3). Oospores formed in pairings. Oogonia have double wall (thicked) smooth, 28-50 µm diameter, av. 38 µm (Fig.4). Oospore are Aplerotic, 25-35 pm diameter, av. 30 µm, Antheridia, when present, amphigynous, up to 22 µm long, av. 17 x 16 µm, cylindrical.

Figure 1 Soil baiting method for A) detection of *Phytophthora infestans* from potato growing soil, B) incubated baited tuber with mycelia growth on tuber.

Figure 2 Isolation of *P.infestans* from baited tuber on Rye agar medium.

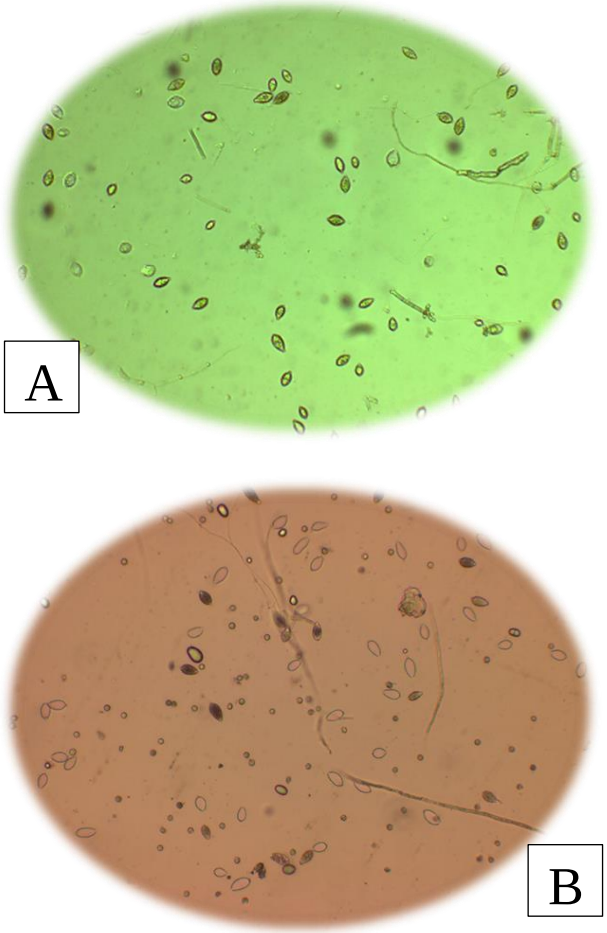

Figure 3 Microscopic view of A) Sporangiosphores from incubated baited potato tuber flake, B) Zoosporangias released form the spornagiosphores

All isolates obtained from potato plants belonged to the mating type A2. All isolates showed spherical, aplerotic oospores, which ranged from 27.5 to 28.8 µm in diameter and with an oospore wall of 2.0 to 2.2 µm thick. Potato tuber flakes died (including bacterial contamination) five days after inoculation. Pathogenicity tests showed that the isolates were pathogenic on detached leaves of potato (Fig.5).

Discussion and Conclusions

Plant pathogens are a serious problem for seed export, plant disease control and plant quarantine. Seed export is a major agricultural industry worldwide with a total of 57 countries exporting vegetable seed, accounting for 106 thousand metric tons and contributing to $2,851 million in 2010 (www.worldseed.org, last accessed in November 2012). Identification and detection of plant pathogens is an important issue, especially when quarantine pests are involved. A zero tolerance is required for this organism because of which export of planting material is not possible. A number of techniques are used for the detection of multiple species of *Phytophthora* within a sample (Gallegly and Hong 2008). Potato tuber slice is a specific baiting method to *P.infestans only*. Despite this host specificity, these baits also attract *Pythium* species. This study showed that potato tuber flakes baiting were effective for isolating *P.infestans* from potato growing regions. Pre-wetting of soil for various periods of time before submersion in water favours the production of sporangia. Other studies showed that pre-wetting soil before dilution plating or baiting with pepper leaf disks favoured the detection of oospore inoculums of *P. capsici*. Double baiting increased the recovery of positive samples from 1.9 to 2.5% and 6.3 to 7.5% of samples taken from the centre and margins respectively of disease fronts in Western Australia (Davison and Tay 2005). Five new isolates from *Phytophthora* isolates that were detected from infected potato growing soils from India were *P.infestans*. This is the first record of *P.infestans* from potato growing soil, through potato tuber flakes, in India. This data showed that *P.infestans* is the major causal agent of Late blight of potato in India. All isolates were detected as being the A2 mating type. Information on distribution of A1 and A2 mating types is critical to determine the genetic variability within the field pathogen population. The relative ratios of A1 and A2 mating types could suggest the level of sexual recombination occurring in natural field conditions, as well as the potential for oospore production. Predominance of a both mating type in the majority of India potato fields may indicate genetically highly diverse populations. The study has also demonstrated that A2 mating type is rapidly displacing A1 mating type population. This is alarming information because the new population is more competent and aggressive in attacking the crop and causing the damage to potato tuber not only in field but also in cold storage houses. *P.infestans* isolates were sent to another research group in India. Their results with specific primers (A2) were in agreement with our morphological observation and detected them as *P.infestans*.

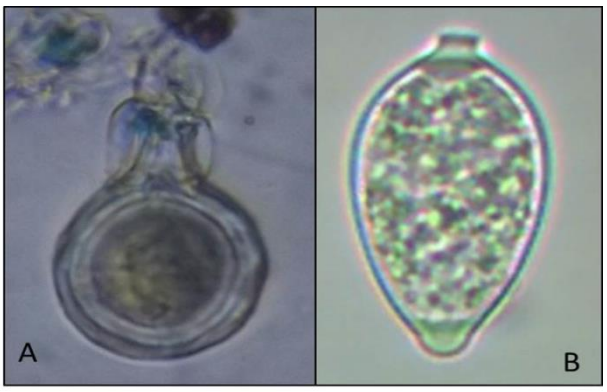

Figure 4 Closed view of *P.infestans* A) sexually formed Oospores through mating type test, B) Single Sporangia.

Figure 5. Detached leaf method to determine the pathogenicity of isolated *P.infestans* from soil.

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