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ORIGINAL ARTICLE

A quantitative Real Time PCR based method for the detection of *Phytophthora infestans* causing Late blight of potato, in infested soil

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Abstract A fast and simple polymerase chain reaction method has been developed for detection of *Phytophthora infestans* oospores, the causal agent of Late blight of Potato in soil. The method involves the disruption of oospores by grinding dry soil, using abrasive properties, in the presence of glass powder and skimmed milk powder within a short time. The latter prevents loss of DNA by adsorption to soil particles or by degradation and reduces the co-extraction of PCR inhibitors with the DNA. After phenol/chloroform extraction; the DNA is suitable for direct PCR amplification without a precipitation step. This amplification leads to detection of pathogen in infested soils before planting of crop. The real-time PCR assay we describe is highly sensitive and specific, and has several advantages over conventional PCR assays used for *P. infestans* detection to confirm positive inoculum level in potato seeds and elsewhere. With increasing amounts of standard DNA templates, the respective threshold cycle (Ct) values were determined and a linear relationship was established between these Ct values and the logarithm of initial template amounts. The method is rapid, cost efficient, and when combined with suitable internal controls can be applied to the detection and quantification of *P. infestans* oospores on a large-scale basis.

1. Introduction

Potato Late blight is the most important and destructive disease of potato worldwide. The oomycete pathogen *Phytophthora infestans* (Mont. de Bary) is one of the most widely known emerging plant pathogens till today. It initially emerged in the early 1840s in the United States and Europe and rapidly spread across potato-growing regions, leading to the Irish potato famine. Global potato crop losses caused by late blight are estimated at €5.2 billion annually (Haverkort et al., 2009).

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It is still considered a major threat to global food security (Kox et al., 2002). Potato, after rice and wheat, is the third most important food crop in the world as well as in India from human consumption point of view. India is the second largest potato producer in the world after China; with the highest potato productivity among the top four potato producers in the world (Singh et al., 2011) and a critical alternative to the main cereal crops for feeding the world's population (Haas et al., 2009), besides being used as an animal feed (Dancs et al., 2008). The disease caused by the *P. infestans* is the greatest threat to the potato crop, accounting for significant annual losses in hilly areas in India (Fig. 2). Tuber late blight results in tuber rotting both in the field and later in storage either in tubers intended for seed or consumption (Bonde and Schultz, 1943;Johnson and Cummings, 2009; Kirk et al., 2009; Lambert and Currier, 1997; Murphy and McKay, 1924, 1925; Olanya et al., 2009). Seed tubers infected with *P. infestans* will either rot in storage, after planting in the field or survive and initiate new epidemics of potato late blight (Doster et al., 1986; Dowley and O'Sullivan, 1991; Kirk et al., 2009; Stevenson et al., 2007). Considering the economic importance of potato, early pathogen detection in the field and storage facilities is crucial because a few contaminated plants are enough to spread the infection and severely compromise production (Hussain et al., 2005; Jyan et al., 2002; Judelson and Tooley, 2000; Trout et al., 1997). In India, losses are more in hilly regions where crop is grown under rain-fed conditions as compared to the plains. Of late, this disease has become a major biotic factor in limiting crop production across the country including the plains. Nuclear rDNA including the small and large subunits, 5.8S, and the internal transcribed Spacer region, proved an ideal target for specific PCR primers, as each sequence is variable at the family, genus, or species level (White et al., 1990). A PCR assay was developed for the detection of late blight of potato in many parts of the world, based on small nucleotide differences in internal transcribed spacer sequences between 18S, 5.8S and 28S ribosomal DNAs (Hussain et al., 2005; Jyan et al., 2002; Judelson and Tooley, 2000). PCR also can be performed as real-time PCR where accumulation of PCR product is measured using fluorescence. Fluorescent detection of PCR products can be accomplished by use of either nonspecific DNA-binding of the fluorescent dye SYBR green or by sequence-specific hybridization with a fluorescent probe (Kong et al., 2003; Livak et al., 1995). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above a threshold value indicates detection of accumulated PCR product. This real-time detection of PCR products further reduces the analysis time, enabling molecular identification within 3 h. More recently, real-time PCR (qPCR) has been used for the fast, accurate and culture independent quantification of a variety of pathogens (including *Pythium spp.*) from plant tissues (Schaad and Frederick, 2002; Schena et al., 2004) and soils (Kernaghan et al., 2007; Livak et al., 1995; Okubara et al., 2005; Schroeder et al., 2006). Although a variety of chemistries have been developed for quantitative real-time PCR (Hayden et al., 2006), SYBR green intercalating dyes represent the most flexible approach to the analysis of species assemblages. The availability of a specific and sensitive diagnostic method for the detection and identification of *P. infestans* could be very useful for potato growers. Rapid detection of the pathogen, where it is harboured, how it may have entered the production system and how it can spread are crucial to formulating effective, long-term management strategies. Conventional and real-time PCR are important tools for the diagnosis and study of phytopathogenic fungi and have contributed to the alleviation of some of the problems associated with the detection, control and containment of plant pathogens (Bates et al., 2001; Filion et al., 2003; Schena et al., 2004). Furthermore, fluorescent detection of PCR products in real-time PCR assays makes this technique more suitable for automation and high throughput testing. Although PCR and isolation are the standard in diagnosis, these techniques are relatively expensive and depend on the availability of equipment and expertise. However, the role of oospores in the epidemiology of late blight is poorly understood (Andrivon, 1995). Since the work of Drenth et al., 1995, only few efforts have been made to clarify the role of oospores in initiating late blight epidemics (Andersson et al., 1998; Pittis and Shattock, 1994). Volossiouk et al. (1995) demonstrated the ability of skimmed milk powder to prevent DNA degradation and its adsorption to soil particles during the extraction. Indeed, they showed that skimmed milk powder efficiently replaced the more expansive ingredients of Denhartdt's solution: bovine serum albumin, Ficoll and Polyvinlypyrrolidine. Compounds such as polyphenols, fulvic and humic acids are commonly present in soil and have been shown to co-precipitate with DNA (Tsai and Olson, 1991). These compounds are powerful inhibitors of the Taq-Polymerase (Tsai and Olson, 1991). Holben et al. (1983) subsequently modified the procedure by using polyvinylpyrrolidone to remove soil organic matter from the cell preparations and repetitive caesium chloride density gradient centrifugation to purify the DNA. While these approaches have been effective, they remain very labour-intensive. The direct extraction of DNA from soil organisms without prior purification or culturing clearly would provide an attractive alternative. In our own study and efforts to apply PCR diagnostics on a large scale to the detection of the *P. infestans* oospores in soil, we have exploited the advantages of direct extraction without a need for DNA purification (Robb et al., 1994). Tissue grinding in glass powder was combined with a simple sodium dodecyl sulphate (SDS) buffer–phenol extraction method to provide extracts which could be subjected directly to PCR amplification. In the present study, we have adapted this approach to soil samples, providing for a simple extraction protocol which can be used directly with PCR amplification without additional DNA purification. The simplicity, speed, and low cost of this approach make it especially attractive for large-scale studies in diseased soil. The aim of this study was to extend the use of this method to the detection of late blight oospores in artificial soils and to develop a simpler and more rapid method than those currently available.

2. Materials and methods

2.1. DNA extraction from mycelium

Twenty isolates were used for DNA extraction (Table 1). Mycelia of *P. infestans* isolates were obtained by growth in pea broth (120 g frozen peas per litre sterile distilled water) at 18 °C for at least 2 weeks. Mycelia were harvested by filtra-

Table 1 Isolates of *Phytophthora infestans* tested in polymerase chain reaction.

Isolates	Mating type	Place and year of collection
H.P.-1	A1	Shimla Hills, 1999
H.P.-2	A1	Shimla Hills, 2000
H.P.-3	A1	Shimla Hills, 2002
H.P.-4	A1	Shimla Hills, 2001
H.P.-5	A2	Shimla Hills, 2003
H.P.-6	A2	Shimla Hills, 2004
H.P.-7	A2	Shimla Hills, 2004
H.P.-8	A1	Shimla Hills, 1998
H.P.-9	A1	Shimla Hills, 2005
H.P.-10	A2	Shimla Hills, 2007
U.P.-1	A2	Western U.P. region, 2007
U.P.-2	A1	Western U.P. region, unknown
U.P.-3	A1	Western U.P. region, unknown
U.P.-4	A1	Western U.P. region, 2009
U.P.-5	A1	Western U.P. region, 2007
U.P.-6	A1	Western U.P. region, 2009
U.P.-7	A1	Western U.P. region, 2009
U.P.-8	A1	Western U.P. region, unknown
U.P.-9	A1	Western U.P. region, 2010
U.P.-10	A1	Western U.P. region, 2010

tion and frozen at −20 °C. DNA was extracted from mycelia according to a modification (Murray and Thompson, 1977) of the Cetyltrimethylammonium bromide procedure (CTAB). Frozen mycelia were ground to powder, placed in sterile ookargide tubes containing pre-warmed extraction buffer and beta mercaptoethanol and these ookargide tubes were incubated at 65 °C for an hour, with occasional stirring and equal volume of Chloroform: Isoamyl alcohol (24:1 v/v) was added, mixed gently. Centrifuged at 10,000 rpm for 10 min. Upper aqueous phase transferred into fresh tubes followed by adding 0.6 vol of ice cold Isopropanol, incubated at −20 °C overnight. Centrifuged at 12,000 rpm for 10 min at 4 °C. DNA pellets were separated from supernatants. Pellets washed twice with 70% ethanol, recentrifuged at 10,000 rpm for 10 min then dried at room temperature until smell of ethanol was removed from the tubes. Pellets were dissolved in TE buffer and stored at −20 °C in small aliquots. Isolated DNA was purified by using 1 µl RNAs A (5 µg/ml). DNA yield and purity were determined by Nanodrop 2000 spectrophotometer (Thermo scientific).

2.2. Oospore extraction

Oospores formed at the interface of interacting A_1 and A_2 colonies were extracted by modifying the method of Forster et al. (1983). The mycelium at the interface of the paired isolates was extracted by scrapping with the help of a spatula. The agar scrappings containing oospores were ground in distilled water using an electric grinder. The resultant suspension was sonicated in a sonicator (Branson Sonifer 450, USA) at 20 duty cycle for 10 min, in order to separate the oospores from the mycelium. The sonicated suspension was centrifuged at 10,000 rpm for 10 min. The pellet was separated from the supernatent and the supernatent was discarded. The pellets containing oospores were wrapped in nylon cloths (pore size 20 µm) and pouches of appropriate size were prepared (Pittis and Shattock, 1994). Mycelial plugs of each of two *P. infestans* mating types were transferred on to Rye B agar medium plates.

2.3. Oospore quantification

A screening method was developed to collect oospores from infected soil collected. Based on the size of *P. infestans* oospores 200, 300 and 400 mesh screens of 20 cm in diameter were used to eliminate most of the soil. And a 15 mesh, 20 cm diameter screen was used to collect the oospores. In addition, 20 g of soil was first macerated in 300 ml of water in a 500 ml beaker for 30 min, stirred with a glass rod for 10 min, and then added to series of screens (200, 300, 400 and 600 mesh from top to bottom). The screens were rinsed with copious amounts of water, and the material remaining on the 15 mesh screen was collected for DNA extraction and staining of oospores. Oospores formed on leaves have 30 um (24–35 um) diameter on average and the diameter of those formed on culture media ranges between 24 and 56 um (Fig. 6).

2.4. Oospores viability test

The numbers of viable oospores in soil samples were determined using Sutherland and Cohen (1983), with some minor modifications. A MTT staining method was used to determine whether the oospores recovered from the soil were alive or dead. First, 0.05% MTT was added to the material gathered from the 15 mesh screen. The sample was incubated for 48 h at 35 °C and then observed under a microscope. The oospores that germinated stained blue, and those in dormancy appeared pink, but dead oospores were either stained dark or not stained. Impurities such as pieces of soil remained unstained (Fig. 6).

2.5. Extraction of DNA from artificially inoculated soil

Typical farm soils were taken from CPRIC, Modipuram. The optimized protocol developed in this study was based on previously described direct extraction method (Nazar et al., 1991). As indicated in Fig. 1, in the basic procedure, 0.25 g of soil sample is ground with fine glass powder by using a mortar and pestle for about 10 min or until a fine powder remains. The powdered soil is suspended in 0.5 ml of skim milk powder solution (0.1 g of milk powder in 25 ml of H_2O) by vigorous vortexing; The soil and debris are removed by centrifugation at 4 °C (12,000 rpm, 15 min), and the supernatant is mixed with 2 ml of extraction buffer (0.3% SDS in 0.14 M NaCl, 50 mM sodium acetate [pH 5.1]) by vortexing. An equal volume of Phenol:Chloroform:Isoamly alcohol (25:24:1) is added; the phases are mixed by intermittent vortexing for 2 min at room temperature and then separated by centrifugation (12,000 rpm, 10 min). The nucleic acid in the aqueous phase is precipitated with two volumes of 100% ethanol for several hours or overnight when convenient. The precipitate is collected by centrifugation at 12,000 rpm, 20 min 4 °C, and the pellet is washed with 70% ethanol with centrifugation between rinses, and dried. The dry pellet is dissolved in 50 µl of water. The DNA quantification was performed using Nanodrop 2000 (Thermoscientific) and was analysed on 0.8% Agarose gel in 1X TAE buffer (Ameresco). The DNA samples were stored at −20 °C until it is assayed.

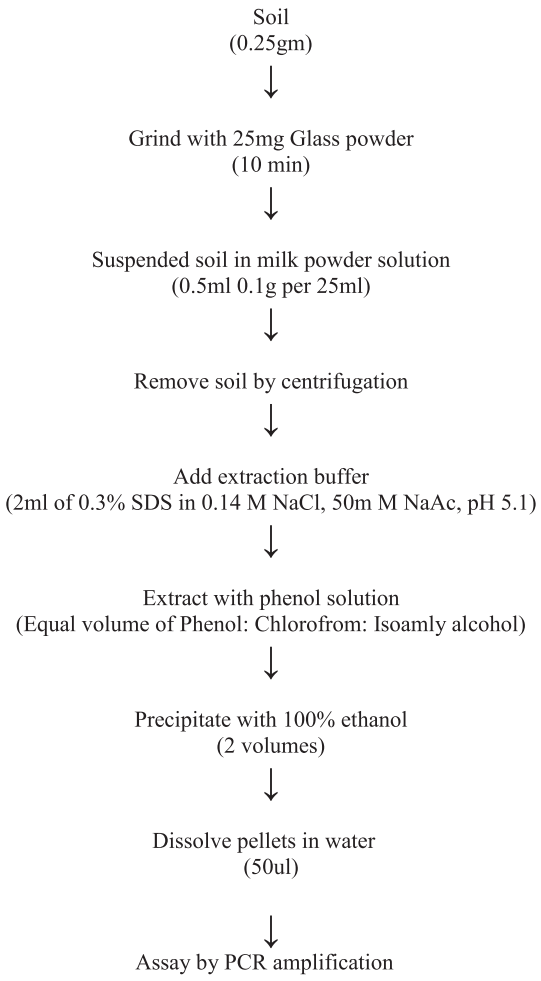

Figure 1 Outline of the direct DNA extraction protocol for *P. infestans* oospores present in soil.

Figure 2 Late blight infected field of potato crop.

2.6. PCR amplification of soil DNA

5 µl of DNA extract was assayed; usually, the extract was first diluted 10-fold to reduce or avoid inhibiting substances. PCR amplification normally was conducted by using 25 µl of the PCR reaction mixture containing 10x DreamTaq buffer with 1.5 mM $MgCl_2$, 2 Mm dNTPs (Fermentas), 0.1 mg of bovine serum albumin (BSA 100 mg/ml), 10 pmol of each *P. infestans*-specific oligonucleotide primer (Taqf/Taqr), 0.2 U/µl of *Taq* DNA polymerase (Fermentas), and double distilled water for volume make-up. The primers were synthesized by Imperial Life Sciences, (Gurgaon, India). The amplification was performed in a programmable block (Eppendorf Mastercycler, Germany) by using 30 reaction cycles, each consisting of a 2-min denaturation at 94 °C, followed by denaturation at 30 s, a 30 s annealing at 60 °C, and a 1-min elongation at 72 °C final elongation at 72 °C for 10 min in the end at 4 °C. The products of PCR amplification were analysed after fractionation by 1.5% Agarose gel electrophoresis in 1X TBE buffer, stained with Ethidium bromide.

2.7. Real time PCR

Real-time PCR was performed in Micro Amp optical 96-Well plates in automated ABI Step one Plus Version 2.2. 25 µl reactions consisted of 9.5 µl of HPLC water, 1 µl each of the two primers (based on ITS region) (300 nm), 1 µl of 1:5 dilutions of the template DNA and 12.5 ul of 2x SYBR™ Green dye "ready to use" master mixer (Applied Biosystem). Wells with no DNA served as no template controls (NTC). The amplification conditions used were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Five *P. infestans* DNA standards, serially diluted with HPLC water and photometrically quantified were tested. For each well, a fluorescent light emission was recorded in real time and data were analysed automatically with ABI Stepone Plus Real-Time System SDS software (Version 2.2) (Fig. 4).

2.8. Preparation of DNA standards

If not otherwise mentioned the genomic DNA was used for the standard curve by using 5 ng as the initial concentration and using the five dilutions by making 1:5 dilution series for genomic DNA. Each standard dilution was tested in triplicate to ensure repeatability. Absolute Real-time PCR data were analysed with SDS 2.4 Software (Applied Biosystem). All analyses were based on the Ct values of the PCR products, which is the PCR cycles at which the fluorescence measured between each cycle exceeds a threshold determined by background fluorescence at baseline and is placed in the exponential phase of the amplification curve. The baseline was set automatically by software. The SDS software calculated the standard curve for each run based on the Ct for the standard (Fig. 3).

3. Results and discussion

Traditionally, identification of species of *Phytophthora* has been based on morphological characteristics and growth on selective media, which can be time consuming and laborious. In addition, considerable expertise is needed to clearly differentiate among closely related species. When dealing with quarantine pathogens, suspicious material should be evaluated quickly and reliably for the presence of the quarantined organism. Quick evaluation methods will avoid deterioration of product quality as well as expensive storage of arboriculture trade materials. Reliable evaluation methods are essential to take adequate phytosanitary measures, discussion, trade and forestry, and may prevent the spread of quarantine organisms to new areas. More and more molecular techniques are being

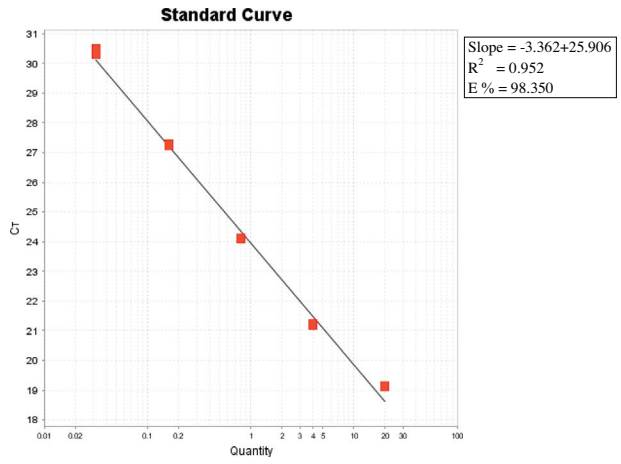

Figure 3 Standard curve of cycle threshold (Ct) values calculated from serial dilutions of DNA from *Phytophthora infestans* using SYBR Green dye assay. Ct values shown are mean values for triplicate reactions. Plots represent cycle threshold (Ct) versus the Log of DNA concentration *P. infestans* DNA was diluted from concentrations of 5–0.0005 ng/μl.

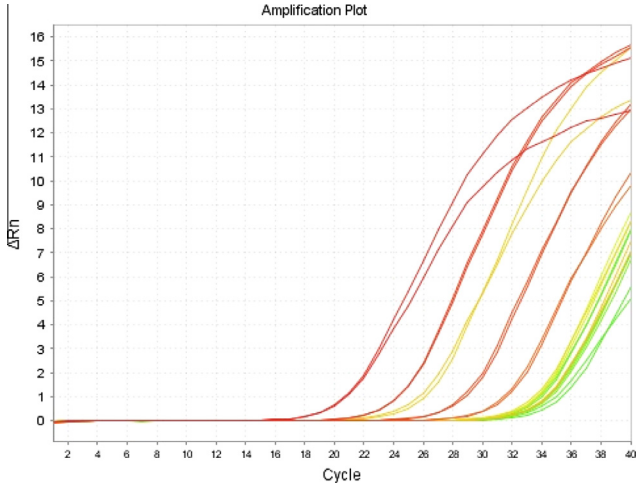

Figure 4 Real-time polymerase chain reaction of *P. infestans* assayed with SYBR green targeting the ITS gene.

deployed for rapid detection and identification of plant pathogens (Cooke et al., 2000; Lee et al., 1993). Nucleic acid amplification via the polymerase chain reaction (PCR) also can be used to detect unviable organisms; therefore, it can be surmised that the rate of false negatives is much lower for this technique. This we showed in a previous study (Pennisi, 2010), in which the diagnostic sensitivity (proportion of true positives correctly identified by the test) of PCR was higher than that of culture based morphological identification (92% and 78%, respectively). Moreover, in contrast to morphological identification, PCR methods can be applied directly to the plant material without the need for culturing, making diagnosis possible on the day of receipt of the sample. PCR products are detected by agarose gel electrophoresis and ethidium bromide staining, which is laborious and time consuming. Technical limitations have continued to limit the large-scale use of PCR with soil samples primarily because extraction techniques have been labour intensive and often unreliable. Over the last two decades, methods for the extraction of DNA from soil samples for DNA analyses of all types have been markedly improved. The target organisms almost always have been separated from soil samples and sometimes even cultured. For example, Faegri and colleagues (1977) used differential centrifugation followed by lysis of the cells, extraction of the nucleic acid, and purification of the DNA by hydroxyapatite column chromatography. For *Phytophthora* spp., ITS sequences of ribosomal DNA have been shown to be useful for species identification, although some related species share identical ITS sequences (Flier et al., 2002) as shown in Fig. 5, the amplification was always better using 5 ul of soil extract before amplifications, than when the soil DNA extract was diluted 10-fold before addition to the PCR Mix. One of the main barriers to successful amplification of DNA extracted from soil samples is the presence of humic acids and related compounds produced by the degradation of organic matter. These compounds are known to be powerful inhibitors of the Taq polymerase (Tsai and Olson, 1991). In our study, results presented here strongly suggested that skimmed milk prevents the co-purification of the inhibitiors with DNA. When DNA was extracted with the same procedure, soil DNA extracts were a dark brown colour, presumably due to the presence of humic compounds. But when skimmed milk was added, samples were colourless or faint brown. Another factor which affected detection was the efficiency of DNA extraction from the soil, which could also explain the difference in results for the different types of soil. The fungal walls were disrupted by grind in dry soil, which would be much more effective in sandy soil, as sand is naturally abrasive, leading to a better yield of DNA and consequently to more PCR product. For a given extraction method, PCR conditions can greatly affect the results. The first reaction was followed by dilution step and a second amplification reaction using an internal pairs of primers. All the steps during the extraction have been simplified in order to produce a rapid and simple method. Thus, liquid nitrogen, ion exchange columns and expensive chemicals were all found to be unnecessary. This simplified method (Fig. 1) worked successfully in oospores of different concentrations which were well established. Oospores remain dormant for different lengths of time and thus maintain low and continuously infective populations (Weste, 1983). Thus, this method would be of use to determine the occurrence of the late blight in natural soil. This would help to detect whether to avoid sowing potato in such infested soil, when highly susceptible cultivars are available; or making a choice of the appropriate cultivar if resistance is available (Navas-Cort'es et al., 1998). The basic strategy for using this method is therefore to assess the type of soil to be tested. Soils which contain a low level of sand will help in disruption of the oospore, and therefore up to 30% wt/wt of acid-washed sand could be added. Using this protocol system it should be possible to adapt this for use with any of nested primers to a range of different DNA targets such as ribosomal RNA genes or specific genes. Real Time PCR diagnosis can results in appropriate control measures and/or eradication procedures more rapidly and accurately than the conventional methods of pathogen isolation and quantification. Nevertheless, additional efforts are required to allow successful implementation for quantitative soil diagnosis. In consideration that PCR can also amplify DNA from dead or non-active organisms, detection of non-viable propagules

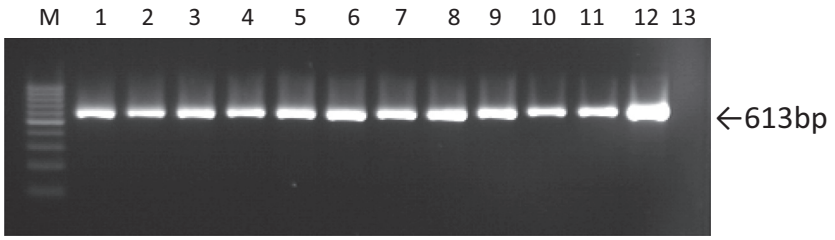

Figure 5 PCR detection of *P. infestans* isolates from different potato growing regions in northern India. Lanes 1–6 = H.P regions, 7–10 = U.P regions, 12 = Positive control, 13 = Negative control, *M* = 100 bp DNA marker.

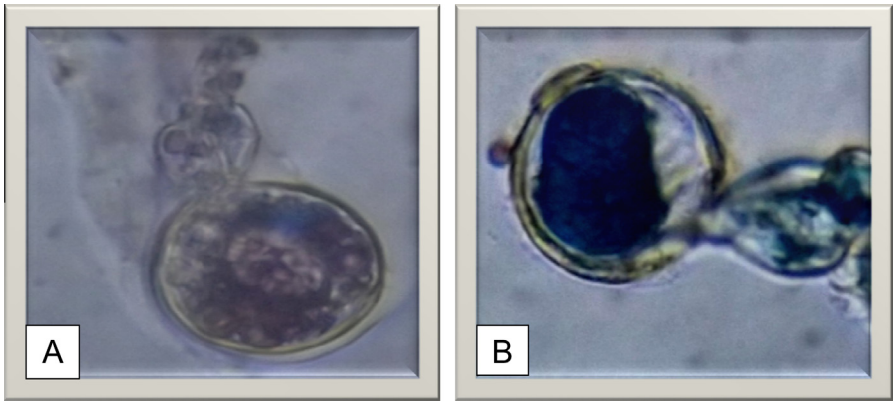

Figure 6 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) staining of oospores recovered from the soil by the screen method. (A) Dormant oospore; (B) germinating oospore.

may not be ruled out. In addition, sampling methods require special attention when using this highly sensitive technology. Finally, also a link between pathogen DNA concentration and threshold levels needs to be established. The correlation between the Ct-value and the target DNA concentration was high (R^2 = 0.952) with an Efficiency of 98.350. Standard curves obtained demonstrated that the selected primer was highly accurate over a linear range of magnitude. The eventual goal of this study was to quantify pathogen DNA in biological samples (Fig. 3). In summary, therefore, a rapid and cost-effective method to extract DNA directly from soil samples which can be utilized with PCR amplification to effectively detect specific soil organisms has been developed. Many PCR-based assays for specific organisms have already been developed and many more are certain to follow. The extraction procedure which is defined by this study should be applicable to many, if not all, of these specific assays, providing for accurate and efficient monitoring of these target organisms in soil. Extracts from samples containing large amounts of clay are less effective, but qualitative analyses are possible and the use of internal control templates should permit quantitative analyses as well.

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