Optimization of Sample Size and DNA Extraction Methods to Improve PCR Detection of Different Propagules of *Phytophthora infestans*

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ABSTRACT

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The plant pathogen *Phytophthora infestans* causes a destructive blight of potato tubers and foliage. A rapid polymerase chain reaction (PCR) assay has been developed for detection of *P. infestans* in potato tubers. In this study, the effect of method of DNA extraction on different propagule types and the minimal number of propagules of *P. infestans* detectable by PCR were assessed using the PINF and internal transcribed spacer (ITS)5 primers. Sensitivity of the primers for PCR was high, and DNA was detectable at concentrations as low as 10 pg/ml. Zoospores and oospores responded differently to different extraction methods, whereas all extraction methods worked equally well for sporangia. Freeze-thaw DNA lysis, in which propagules were frozen at –80°C and thawed at 65°C three times for 15 min each, or direct PCR, in which propagules were placed directly in the reaction mix, were effective methods for PCR detection of sporangia or zoospores but were not effective methods for PCR detection of DNA in oospores of *P. infestans*. DNA from a single sporangium or oospore could be amplified by PCR after hexadecyltrimethyl-ammonium bromide (CTAB) or NaOH lysis extraction methods, whereas DNA from a single zoospore could be amplified by CTAB or direct PCR methods. "IsoCode" Stixs, used in forensic applications, were used to collect the pathogen from leaf and tuber lesions and provided another simple method to extract template DNA. PCR detection of the pathogen in infected tubers using PINF and ITS5 primers was compared to tissue isolation or visual observation. The probability of detection of *P. infestans* in infected tubers at 7 days post inoculation using the PCR assay, tissue isolation, or visual observation was 0.90, 0.80, and 0.75, respectively. The PINF and ITS5 primers provide a powerful tool for rapid and sensitive detection of zoospores, sporangia, and oospores of *P. infestans* when used with appropriate extraction methods, and could easily be deployed to reduce spread of the pathogen in potato tubers.

Phytophthora infestans (Mont.) de Bary, the causal agent of late blight disease, is one of the most devastating pathogens of potato and tomato worldwide and was responsible for the Irish potato famine in the 1840s (12,13). Late blight causes a major foliar disease and tuber blight that can limit yield, tuber quality, and marketability of the crop in the United States and worldwide (18,26). Late blight epidemics, if left uncontrolled, can cause losses up to 100% when weather conditions are favorable (26). The disease is reemerging in many areas of the United States and is responsible for costly fungicide application (6,10,11). Epidemics on potato and tomato crops have become severe due to the widespread occurrence of new aggressive and fungicide-resistant strains of *P. infestans* (6,10,11,14,15).

The primary inoculum for late blight in fields is likely associated with infected seed potatoes (4,29). Global migrations of the pathogen have occurred via transport of infected seed tubers (13). During the growing season, tubers can become infected by contact of zoospores carried into the soil by rain (21). Sources of initial inoculum for potato late blight disease in the fields include infected tubers that are used as seed, infected tubers that are discarded prior to or during the season (cull piles), or infected tubers that are not harvested and survive in the soil (1,2,4,11,18). Tubers also can come in contact with infected haulms and contaminated soil at harvest. In storage, healthy tubers can become contaminated with sporangia of *P. infestans* through contact with diseased tubers (7,16). In addition, infected seed tubers cut in the same machine as healthy tubers can spread sporangia to seed pieces and cause disease in fields (20). Improved detection of the pathogen in tubers could reduce spread of the pathogen. Diagnostic laboratories at either state or federal agencies could deploy the polymerase chain reaction (PCR) assay to screen seed potatoes prior to shipping and planting to reduce the spread of new and virulent forms of the pathogen in infected seed potatoes.

Repetitive DNA polymorphism analysis (19,25), oligonucleotide hybridization to amplified ribosomal DNA spacers (23), and PCR amplification of ribosomal DNA and internal transcribed spacer (ITS) regions (19,24,27,28,30,31) have been used to identify the pathogen. PCR-based identification has many advantages over other molecular and traditional isolation methods because the procedure is rapid and less time-consuming (17,34). In plants that can be infected simultaneously by two or more *Phytophthora* spp., use of PCR can accurately differentiate individual species (8,24,30). Several methods for PCR detection of *P. infestans* in plant material for rapid diagnosis of late blight have been developed (19,24,30,31). In our laboratory, a PCR primer, the PINF primer, was constructed from ITS region DNA and used with ITS5 primer to yield an approximately 600-bp product specific to *P. infestans* (31). In our previous work, the specificity of the primers for *P. infestans* was confirmed using 15 *Phytophthora* spp. and other pathogens known to infect potato (31). On potato, the primers only amplified DNA of *P. infestans* and not other species of *Phytophthora* known to infect potato, or host DNA (31)

The specific objective of this study was to optimize DNA extraction methods for zoospores, sporangia, and oospores of *P. infestans* and determine the minimal number of propagules detectable by PCR. In addition, the sensitivity of the PINF and ITS5 primers was evaluated. The minimal time required for detection of *P. infestans* in infected tubers after inoculation, the probability of detection of infected tubers in an artificially inoculated seed lot, and detection of the pathogen in naturally infected tubers from the field also were evaluated. The probability of detection of *P. infestans* by PCR, visual observation, and tissue isolation was calculated and used to optimize sampling strategies. The overall goal of this work is to provide tools that will reduce the risk of spread of *P.*

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infestans in tubers. A portion of this work has been presented previously (33).

MATERIALS AND METHODS

Pathogen isolates. *P. infestans* isolate 94-1 (US-1 genotype) was collected from diseased tomato in North Carolina in 1994 and used as an A1 mating type for oospore formation. Isolate 93-4 (US-8 genotype) was collected from diseased potato leaves in North Carolina in 1993 and was used as an A2 mating type isolate for oospore formation and also for sporangia and zoospore production, and potato inoculation. Isolates were maintained on Rye B agar (20 g of sucrose, 15 g of agar, 0.05 g of β-sitosterol, rye B broth at 60 ml per liter, and distilled water added up to 1 liter) and stored longterm in 10% dimethylsulfoxide in cryogenic storage in liquid nitrogen.

DNA extraction. Isolates of *P. infestans* cultured on Rye B agar were transferred to pea broth (120 g of frozen peas per liter of distilled water) and grown for at least 1 week at 18°C. Mycelia were harvested by filtration and frozen at –20°C. DNA was extracted using a hexadecyltrimethyl-ammonium bromide (CTAB) procedure (22,31). Briefly, the frozen mycelia (10 mg) was placed in sterile 1.5-ml microcentrifuge tubes to which 150-µl of extraction buffer (0.35 M sorbital, 0.1 M Tris, 0.005 M EDTA, pH 7.5, 0.02 M sodium bisulfite) was added, and tubes were vortexed. After the addition of nuclei lysis buffer (150 µl) containing 0.2 M Tris, 0.05 M EDTA, pH 7.5, 2.0 M NaCl, 2% CTAB, and 60 µl of 5% sarkosyl (N-lauryl sarcosine), tubes were vortexed and incubated at 65°C for 15 to 30 min. After incubation, one volume (300 µl) of chloroform:isoamyl alcohol (24:1) was added to each tube and centrifuged for 15 min at 13,000 × *g* at room temperature. The aqueous phase was removed to a new tube and chloroform extraction was repeated. DNA was precipitated overnight at –20°C in 0.1 volumes of 3 M sodium acetate (pH 8.0), and two volumes of cold 100% ethanol. The supernatant was discarded, and pellets were washed with 70% ethanol, then dried under vacuum centrifugation. DNA was suspended in TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

PCR conditions. PCR was conducted in a 50-µl reaction volume by the procedure of Trout et al. (31). PCR was conducted in thin-walled 0.2-ml tubes. Briefly, 1 µl of DNA template (1 ng quantified with a spectrophotometer) was added to a 49-µl master reaction mixture containing 5 µl of 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, and 500 mM KCl, pH 8.3), 36.6 µl of sterile distilled H_2O, 1 µl of 10 mM $MgCl_2$, 2 µl of 2 mM dNTPs, 2 µl of 10 µM ITS5 primer, 2 µl of 10 µM PINF primer, and 0.4 µl of *Taq* polymerase (Boehriger Mannheim Biochemicals, Indianapolis, IN). All reactions were overlaid with sterile mineral oil prior to thermal cycling. The sequences of the PINF and ITS5 primers were 5′-CTCGCTA-CAATAGGAGGGTC-3′ and 5′-GGAAG-TAAAAGTCGTAACAAGG-3′ (31,34), respectively. Thermal cycling parameters were initial denaturation at 96°C for 2 min, followed by 35 cycles consisting of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min followed. Electrophoresis of amplified products was conducted on 1.6% agarose gels containing ethidium bromide at 0.5 µg/ml with 1× Tris-borate EDTA running buffer. A 100-bp DNA ladder was included in each gel as a molecular size standard.

Sensitivity of PINF and ITS5 primers. DNA of *P. infestans* was extracted from mycelium using the CTAB method and was quantified using a spectrophotometer. The DNA was diluted to 1, 5, 10, 50, 100, 500, 1,000, and 10,000 pg/µl. One microliter of each dilution was used for PCR reactions as described above.

Effect of DNA extraction method on PCR of different propagule types. *P. infestans* was grown for 2 weeks on Rye B agar at 18°C in constant light to produce sporangia and zoospores. Water (10 ml) was added to each petri dish containing sporangia and the agar surface was gently rubbed with a glass rod to dislodge sporangia. The sporangial suspension was chilled at 12 to 16°C for 80 min to release zoospores. A cross between isolates 94-1 (A1) and 93-4 (A2) was used to produce oospores. Agar plugs of the A1 and A2 isolates were placed on Rye B agar medium and incubated in the dark at 18°C for 2 to 3 weeks. Oospores were removed from the agar medium after grinding in 20 ml of sterile distilled H_2O using a blender. Oospore suspensions were digested with lysing enzyme (1 ml of lysing enzyme and 10 ml of oospores in H_2O [product no. L2265; Sigma-Aldrich, St. Louis]) at 50 mg/ml and incubated at 18°C for 20 to 24 h to lyse sporangia and mycelia in the suspensions. Oospores were harvested by centrifugation and washed with sterile distilled H_2O until the oospore suspension was determined to be clear of lysed propagules by microscopic observation.

Experiments were conducted to determine the effect of different DNA extraction methods, including CTAB, NaOH lysis, freeze-thaw, and direct PCR (no extraction), on zoospores, sporangia, and oospores and to determine the minimal number of each propagule type detectable by PCR. Suspensions consisting of either zoospores, sporangia, or oospores were prepared as described above. Experiments were conducted with a series of dilutions containing 1, 10, 100, or 1,000 propagules of each propagule type per PCR reaction mixture. Propagule densities in the initial suspension were quantified on a haemacytometer and appropriate dilutions were prepared to give 100 and 1,000 propagules per tube per PCR reaction. Sporangia (1 or 10) were directly removed from culture plates to tubes with the tip of a needle under a stereomicroscope. Suspensions of zoospores and oospores were spread on water agar plates and individual propagules were selected with the tip of a needle for 1- and 10-propagule treatments. Propagule numbers were confirmed by directly counting extra subsamples of each propagule type and concentration with a microscope.

Experiments were conducted to determine the effect of different extraction procedures, including CTAB, NaOH lysis, and freeze-thaw lysis, or direct PCR (no extraction) on release of DNA and PCR detection of different propagule types of *P. infestans*. The CTAB extraction procedure was used as describe previously (31). For the NaOH lysis method, propagules were ground in 10 µl of 0.5 N NaOH with plastic pestles, after which 5 µl was transferred immediately to a sterile 1.5-ml tube containing 495 µl of 100 mM Tris, pH 8.0 (32). The suspension (1 µl) was added to 49 µl of master mix for PCR. For the freeze-thaw lysis method, tubes containing propagules suspended in H_2O were centrifuged and H_2O was discarded. Tubes containing propagules were alternatively frozen at –80°C and thawed at 65°C in a water bath three times for 15 min each. For PCR, 49 µl of master mixture was added into each tube. For direct PCR, 49 µl of master mix was added directly into each tube containing propagules of the pathogen in 1 µl of water.

All DNA extraction experiments were conducted separately for each propagule type. There were three replicate reaction tubes per extraction procedure at each of four propagule levels. The entire experiment was conducted three times. Appropriate nontemplate controls were included in each experiment. Data are presented as the percentage of positive detections by PCR for each propagule level and extraction method. The data for each propagule type was analyzed separately as a factorial with two factors (DNA extraction method, four levels; and number of propagules, four levels). Data were analyzed by analysis of variance using the statistical analysis system (SAS, Inc. Cary, NC).

Experiments also were conducted to determine whether DNA of *P. infestans* could be detected using a simplified extraction method on IsoCode Stixs (Schleicher & Schuell, Keene, NH). IsoCode Stixs are commonly used in crime lab forensic applications for blood and saliva samples and contain paper impregnated with reagents that bind PCR inhibitors, thus eliminating the need for lengthy DNA extraction procedures. The method described here was from the manufacturer's instructions. A small piece of tissue (1 cm^2) containing the late blight pathogen was removed from an infected leaf or tuber

lesion containing *P. infestans* and the sample was ground in a few drops of water with a Konte pestle. The tissue was placed onto the end of one of the four triangular sample areas of the IsoCode Stix (Fig. 1A). A pure culture of the pathogen was used as a positive control. Samples were dried at 80°C for 5 to 10 min in a hot-air oven. Each sample triangle was placed over a sterile microcentrifuge tube, and the lid was closed while pulling the end of the stick to detach the sample triangle directly into the tube. Then, 50 µl of sterile distilled water was added into each tube and the sample was washed by pulse-vortexing three times for at least 5 s each. The water was removed with a sterile pipette after washing and the tube was centrifuged for 5 s to remove residual droplets of water. Subsequently, the sample was resuspended in 50 µl of sterile distilled water and the tube was incubated at 95°C for 30 min and gently mixed after incubation to release the DNA from the membrane. The water containing the template DNA was removed to a new tube, and 5 to 10 µl of DNA were used for each PCR reaction. Experiments were repeated twice.

Detection of *P. infestans* in infected tubers by PCR, visual observation or tissue isolation. An experiment was conducted to determine the minimum time period before infections could be detected by PCR, visual observation, and tissue isolation. Small pieces of mycelium of *P. infestans* were removed from 3- to 4-week-old petri dishes containing Rye B agar media and placed into wounds cut into potato tubers with a sterile cork borer. Wounds were sealed by replacing the cut in the tuber with the plug. Inoculated tubers were incubated in moist chambers at 18°C. Tubers were observed daily for 7 days, and visual symptoms were recorded. Three different tubers were destructively sampled daily for a 7-day period. A sample of infected tissue approximately 2 cm from the inoculation site was removed and each sample was divided into three pieces. One piece of tissue was extracted using the NaOH lysis method and used for PCR. The other two pieces of tissue were used for isolation of *P. infestans*. Prior to isolation, tissue was surface disinfected with 0.05% sodium hypochlorite for 1 min, washed two times with sterile water, and placed on Rye B medium amended with 200 mg^{-1} of vancomycin, 10 mg^{-1} of pimaricin, 100 mg^{-1} of pentachloronitrobenzene, and 5 mg^{-1} of hymexazol. Experiments were conducted three times.

An experiment was conducted to determine the probability of detection of the pathogen in an infected seed lot of 100 tubers. One hundred tubers were inoculated with mycelium of *P. infestans* as described above. All tubers were sampled 7 days after inoculation from visible lesions approximately 2 cm from the inoculation site. Tissue was rated for visual evidence of disease and then divided in half and used for either PCR assays or tissue isolations as described above. Tissue (20 mg) was sampled from lesions on each tuber, and NaOH lysis was used to extract the DNA as described above. The experiments were repeated two times.

In each experiment, the probability of detection of infected tubers in the infected seed lot (Pd) was calculated as follows: Pd_i = number of positive tubers detected containing *P. infestans*/100. The average of the two experiments, $Pd = (Pd_1 + Pd_2)/2$, along with its standard error (SE) is reported. Using the Pd, we can compute the probability of detecting at least one infected tuber from any lot of tubers as a function of the number of tubers in the lot (N), the number of infected tubers in the lot, and the sample size (n). The number of infected tubers that we would expect to detect is N_1 = number infected × Pd. The probability that a sample of n tubers from this lot contains at least one detectable infected tuber is found from the hypergeometric distribution, and is Pr(detect at least one infected tuber) = $1 - ([N_1/0][N - N_1]/n)/(N/n)$ (5). We constructed a table of sample sizes needed to be able to detect at least one infected tuber with at least 80 or 90 % probability.

Detection of *P. infestans* in naturally infected tubers. Four potato seed lots were collected from potato growers in North Carolina prior to planting in February 1997 and were used to determine whether *P. infestans* was present in seed potatoes. The seed potato sampled originated from various seed-growing regions of the United States and Canada, including Maine, Wisconsin, and New Brunswick. Approximate 100 tubers collected randomly from each of four seed lots (cv. Atlantic) were used in subsequent experiments.

Tubers were washed, blotted dry, and incubated in moist chambers at 18°C for 2 weeks. Visible late blight symptoms were not observed on tubers at the time of sampling. Tissue (20 mg) was randomly sampled from each tuber, and NaOH lysis was used to extract the DNA as described above. One PCR assay was conducted on each of the sampled tubers to identify infected tubers. Visible lesions were not present on the tubers; therefore, samples were taken randomly on the tuber. Experiments were repeated twice.

Tubers also were sampled from the National Late Blight Fungicide Trial conducted in 1997 by Mary Powelson at Oregon State University. The fungicides Acrobat MZ (2.52 kg/ha), Tattoo C (2.69 liters/ha), and Curzate (0.23 kg/ha) + Manzate (1.96 kg/ha), were evaluated for abil-

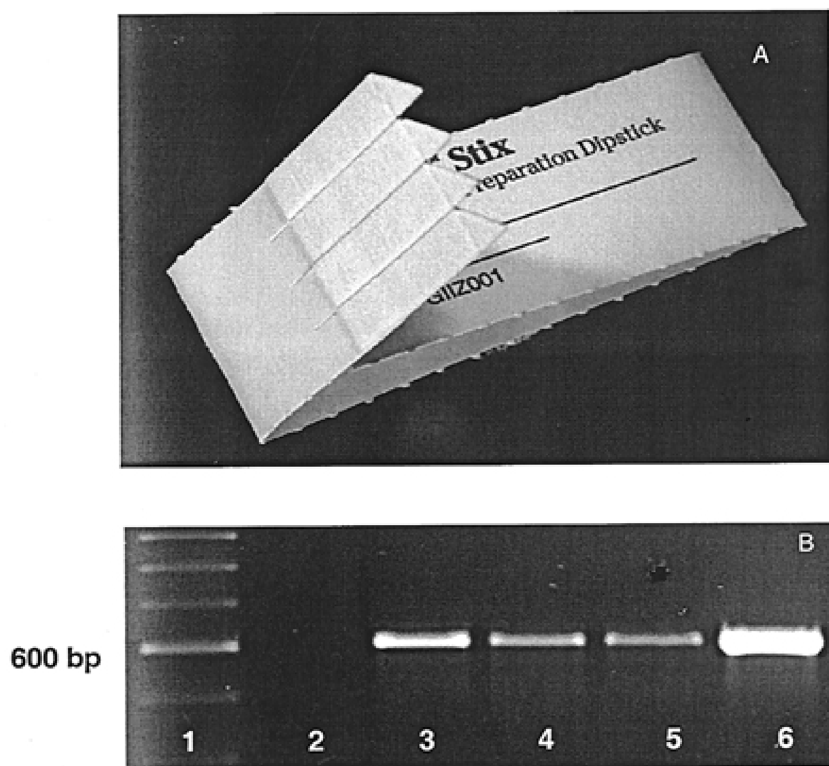

Fig. 1. A, Sample collection matrix of IsoCode Stixs designed to isolate DNA to be used as template for polymerase chain reaction (PCR). The base paper for IsoCode is grade 903 paper composed of cotton linter (Schleicher & Schuell, Keene, NH). The papers are impregnated with reagents that bind PCR inhibitors and release DNA in a simple water solution. **B,** PCR amplification of *Phytophthora infestans* DNA by IsoCode Stix extractions. Lane 1, 100-bp DNA ladder; lane 2, no template control; lanes 3–5 contain amplified DNA products from Isocode Stixs extracted from mycelium of *P. infestans*, tubers, and leaf lesions, respectively; lane 6, DNA product derived from PCR of *P. infestans* DNA from hexadecyltrimethyl-ammonium bromide extraction (positive control).

ity to control late blight of potatoes on the Lewis Brown Horticultural Research Farm near Corvallis, OR. Potato was planted and inoculated with *P. infestans* (US-8) by applying an aerosol suspension of the pathogen in the spreader rows. Fungicide applications were initiated 15 days after inoculation. A subsample of 50 tubers from each of three fungicide treatments and a nontreated control were shipped to North Carolina State University (NCSU) after harvest. The tubers were washed, blotted dry, and incubated in moist chambers at 18°C for 2 weeks to encourage lesion development. Tubers then were observed for visual evidence of disease and a sample (1 cm^3) was removed from the lesion and tested by the PCR assay in our laboratory after NaOH lysis of the tissue (32). Tubers also were sampled from a second, similar fungicide test conducted in 1998 at Upper Mountain Research Station in Laurel Springs, NC. Tubers were sampled from the same fungicide treatments. Tubers were incubated as described above and evaluated by the PCR assay and visual inspection. Tubers in the Laurel Springs test did not have symptoms of the disease.

RESULTS

Sensitivity of PINF primer. The targeted DNA of *P. infestans* was amplified by PCR using PINF and ITS5 primers at concentrations of template DNA as low as 10 pg/μl (Fig. 2). DNA was not amplified when the template DNA was reduced to concentrations of 5 or 1 pg/μl (Fig. 2).

Effect of DNA extraction method on PCR detection of different propagule types. The percentage of positive PCR detections varied with extraction method and number of propagules for zoospores and oospores of *P. infestans* (Table 1, Fig. 3). DNA from single zoospores of *P. infestans* was successfully amplified by PCR after CTAB extraction or direct PCR but not by NaOH lysis or freeze-thaw lysis methods (Fig. 3A). In contrast, the percentage of positive PCR detections for DNA extracted from sporangia was not affected by extraction method but increased as the number of sporangia in the sample increased (Fig. 3B, Table 1). Extraction method had a large effect on the percentage of positive PCR detections from oospores. DNA from single oospores was detected by PCR after CTAB and NaOH lysis, but DNA from oospores was not detected by PCR after freeze-thaw lysis or by direct PCR at any level of oospores (Fig. 3C). The latter methods may not have successfully lysed the oospore walls to release DNA.

DNA template of *P. infestans* from either infected tubers, leaf lesions, or mycelium was successfully amplified by PCR from IsoCode Stixs using the simple methods provided by the manufacturer and the PINF and ITS5 primers (Fig. 1).

Detection of *P. infestans* in infected tubers by PCR, visual observation, or tissue isolation. Symptoms did not become obvious until 3 days after inoculation. *P. infestans* was positively detected in only 22, 22, and 44% of asymptomatic, artificially inoculated tubers 3 days after inoculation by either PCR, visual observation, or tissue isolation, respectively. However, 4 days after inoculation, the percent positive detections were 100% by both PCR and visual observation and 89% by tissue isolation. After 4 days, when symptoms became apparent, detection was 100% by all methods.

Symptomatic tissue from 100 artificially inoculated tubers was assayed 7 days after inoculation by PCR assay, visual observation, and tissue isolation, and the probability of infected tubers containing *P. infestans* was estimated to be 0.90 (SE = 0.007), 0.75 (SE = 0.014), and 0.80 (SE = 0.071), respectively. *P. infestans* was detected at significantly higher levels by PCR than by visual observation or tissue isolation.

Using a Pd of 90%, we computed the Pd of at least one infected tuber in an infected seed lot 7 days after inoculation for lots with different numbers of infected tubers and different sample sizes. For a lot of 400 tubers, the sample size needed to detect at least one infected tuber with a probability of 90% is given in Table 2.

Detection of *P. infestans* in naturally infected tubers. None of the tubers assayed from North Carolina growers' stored seed lots were positive for *P. infestans* by PCR or visual observation. However, infected tubers from the National Late Blight Fungicide Trail conducted in 1997 at Oregon State University were tested with the PINF and ITS5 primers and the pathogen was detected in some of these tubers (Table 3). Tubers from control plots with no fungicide treatment had the lowest percentage of infected tubers as indicated by both visual observation and PCR assays. The incidence of disease was significantly higher in tubers from fungicide-treated plots than in tubers from nontreated control plots ($P < 0.05$). The percentage of infected tubers determined by visual observation and PCR were not significantly different. However, the incidence of disease in tubers as determined by visual observation was slightly higher than the incidence of disease determined by PCR. No infected tubers were found by either visual observation or PCR assay in the asymptomatic tubers from the 1998 field test conducted in Laurel Springs, NC.

DISCUSSION

DNA from *P. infestans* was detectable at a concentration as small as 10 pg/μl using the PCR assay with PINF and ITS5 prim-

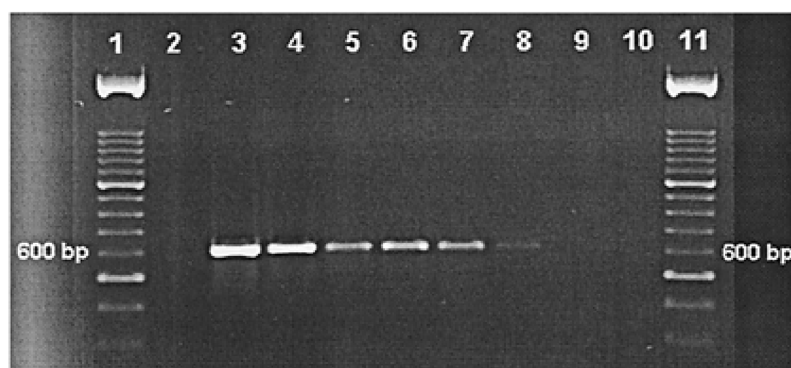

Fig. 2. Detection of *Phytophthora infestans* DNA using the PINF and internal transcribed spacer (ITS)5 primers. Lanes 1 and 11, 100-bp DNA ladder; lane 2, no template control; lanes 3–10, *P. infestans* template DNA at concentration of 10,000, 1,000, 500, 100, 50, 10, 5, and 1 pg/μl, respectively.

Table 1. Analysis of variance of the effect of DNA extraction method and propagule number on polymerase chain reaction (PCR) detection of DNA from sporangia, zoospores or oospores of *Phytophthora infestans*

		Probability > F[w]		
Source of variation	**df**	**Sporangia**	**Zoospores**	**Oospores**
DNA extraction method (extract)[x]	3	0.9437	0.0218*	0.0001*
Number of propagules (number)[y]	3	0.0001*	0.0001*	0.0001*
Number × extract	9	0.8582	0.0079*	0.0002*
Rep[z]	2	0.0291*	0.3332	0.0547

[w] $P > F$ is the probability value greater than the f statistic in the analysis of variance; * indicates the values are significant at $P < 0.05$ or less.
[x] DNA extraction methods included hexadecyltrimethyl-ammonium bromide, NaOH lysis, freeze-thaw lysis, and direct PCR (no extraction).
[y] Numbers of propagules from each propagule type consisted of 1, 10, 100, and 1,000 propagules.
[z] There were three replicate tubes per DNA extraction method at each level of propagules.

ers. Others have used our assay and reported detection of 1 pg/µl when a colorimetric assay was used to detect the amplified products (Jim Beck, Novartis Chemical Company, *personal communication*). The PINF and ITS5 primers were as sensitive as the primers reported by Tooley et al. (30) for detection of *P. infestans* but not as sensitive as the primers recently reported by Judelson and Tooley (19). Niepold and Schöber-Butin (24) also developed primers for detection of *P. infestans* from repetitive DNA sequences and studied their sensitivity. However, their results cannot be compared with ours because they did not quantify DNA template concentrations. Detection of lower concentrations of DNA and increased sensitivity with the PINF and ITS5 primers could be achieved if southern blot hybridization, colorimetric assays, or nested PCR assays were used (3,9,30).

Extraction methods were optimized in our study to improve PCR detection of specific propagule types of *P. infestans*. Our data clearly indicate that the lower threshold level for detection of DNA from various propagule types by PCR is dependant upon the DNA extraction method used and the propagule type. Some propagules, such as oospores, are more recalcitrant to DNA extraction and require full CTAB DNA extraction or lysis with NaOH before PCR is possible. When extraction methods were optimized, single propagules of all spore types could be detected using the PINF and ITS5 primers. It is important, from a biological perspective, to consider the effects of extraction methods used on subsequent PCR rather than only the minimum sensitivity levels of various primers (19), because extraction method can have a large impact on the quantity and quality of DNA and the success of the PCR reactions. In plant tissues, infections could potentially contain multiple propagule types of the pathogen, and DNA concentrations in

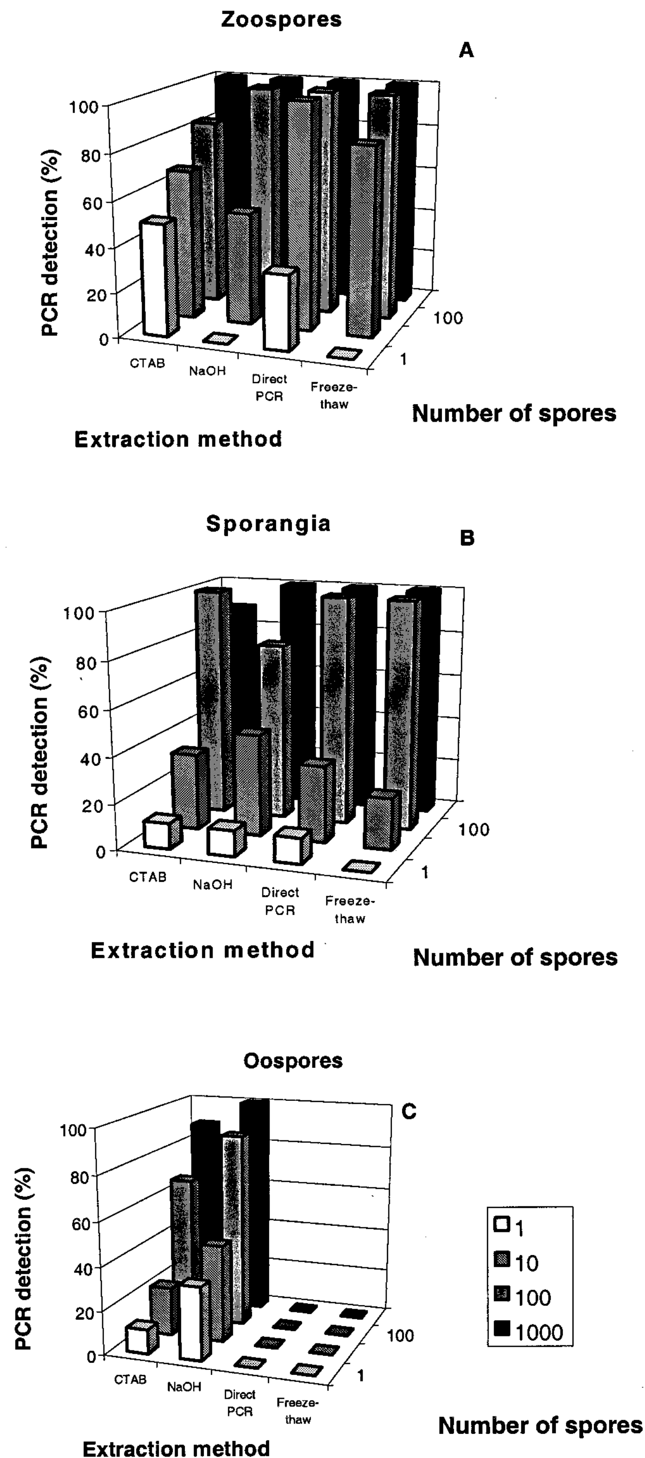

Fig. 3. Detection of *Phytophthora infestans* DNA extracted from **A,** zoospores, **B,** sporangia, and **C,** oospores using the PINF and internal transcribed spacer (ITS)5 primers over a range of propagule numbers. DNA templates were derived from hexadecyltrimethyl-ammonium bromide, NaOH lysis, freeze-thaw lysis, or direct polymerase chain reaction (no extraction) methods.

Table 2. Sample sizes needed to detect at least one tuber infected with *Phytophthora infestans* with a probability of 80 or 90% in a potato seed lot with various number of infected tubers[z]

No. of infected tubers	Sample size per probability	
	80%	**90%**
5	110	147
10	65	90
15	43	60
20	34	48
25	27	38
30	23	32
35	19	27
40	17	24

[z] Lot size = 400 tubers, probability of detection = 0.90.

Table 3. Effect of fungicide treatment on the incidence of potato tubers infected with *Phytophthora infestans* by visual observation and polymerase chain reaction (PCR) assay

Treatment	Infected tubers (%)[y]	
	Visual observation[z]	**PCR assay**
Control	0 b	1.67 b
Acrobat MZ	36.38 a	27.37 a
Tattoo C	51.67 a	40.84 a
Curzate + Manzate	44.00 a	35.00 a

[y] Means followed by the same letter are not significantly different according to Fisher's protected least significant difference ($P < 0.05$).

[z] A subset of 50 naturally infected tubers from each of four fungicide treatments were sent to North Carolina State University by Mary Powelson from the National Late Blight Fungicide Trial conducted at Oregon State University in 1997. Visual observation of infection in tubers was evaluated on the same tubers that were subjected to the PCR assay.

well-colonized tissue would be expected well above the 10 pg/µl threshold of our assay.

The PINF primer was useful for detection of single propagules of either zoospores, sporangia, or oospores of *P. infestans* when used with the appropriate extraction method. DNA from sporangia was easily extracted using all methods, but DNA from zoospores and oospores was more sensitive to extraction method. DNA from oospores was not detected by direct PCR or freeze-thaw lysis methods. These methods may have failed to lyse the oospore walls sufficiently to release DNA. NaOH lysis and CTAB extraction methods were an efficient method for DNA extraction from oospores. In contrast, direct PCR and freeze-thaw lysis methods were useful for detection of zoospores and sporangia at higher inoculum levels. There are advantages to the direct PCR and freeze-thaw lysis methods, because they are simple, require no chemicals, and all DNA is retained in the sample tube. The NaOH lysis is less laborious than CTAB extraction but is not as well suited for longterm maintenance of DNA samples.

ITS DNA from single oospores, sporangia, and zoospores of *P. infestans* was amplified using PCR with the PINF and ITS5 primers. PCR is known to be extremely sensitive and others have used the technique to amplify ribosomal DNA (rDNA) from single spores of *Neurospora tetrasperma* (22). Amplification and analysis of DNA from single propagules could be useful for studies of recombination frequencies in propagules that are difficult to germinate, such as oospores of *P. infestans*. Resting spores of *Plasmodiophora brassicae* in potting mix soils were detected using PCR and the NaOH lysis method of DNA extraction (9). Detection of oospores of *Phytophthora infestans* in soil also could be possible using the methods described in this paper.

IsoCode Stixs provided a useful method for DNA collection and extraction from late blight–infected tubers and leaf tissues from field samples. DNA templates prepared from IsoCode Stixs extractions were adequate for PCR amplification of rDNA. Any gene of interest, including mitochondrial DNA, could be amplified from samples with the simplified methods and other primers. The IsoCode Stixs have advantages for sample collection from fields, because the cards are simple and easy to handle, require no chemicals, and eliminate the need to bring infected tubers or tissues into the laboratory. Air drying tissue for 3 h on the IsoCode Stixs in the field can be substituted for the hot-air oven drying of samples and is also described in the manufacturer's instructions (*unpublished data*). We did not test the sensitivity of detection of DNA on the IsoCode Stixs, but their wide application in human forensic work suggests that very low levels of DNA may be detectable and justifies further evaluation of this method of sample collection for DNA analysis in plant pathogen studies. The obvious limitation of the method is that, because the pathogen is not obtained in pure culture from tissue samples, repeated extractions or assays that require large amounts of DNA, such as restriction fragment length polymorphism, analysis are not possible.

The Pd of *P. infestans* in an artificially infected seed lot by PCR assay was higher than the Pd by tissue isolation or visual inspection. Traditional isolation of the pathogen on culture media is time consuming because the pathogen grows slowly and contamination is frequent. Visual observations of disease may be misleading because other pathogens can cause similar symptoms in tubers. The PCR assay was sensitive and, using statistical calculations, we were able to predict the optimum sample size to detect at least 1 infected tuber in a seed lot of 400 tubers using the Pd by PCR as 0.90. With increased number of infected tubers in the lot, the sample size required to detect infected tubers decreases (Table 2). There is a need for further testing and deployment of PCR detection methods for seed potato certification to reduce the transmission of new and more aggressive strains of *P. infestans* in tubers (14). Currently, potato tubers are visually inspected for evidence of late blight, and symptoms of the disease can be misdiagnosed or masked by other tuber rotting pathogens.

The PCR assay was useful for detection of the pathogen in naturally infected tubers from the Oregon field trial. In naturally infected tubers with visual symptoms, detection by visual observations was slightly higher than by PCR. We incubated the tubers for 2 weeks before the assay to obtain clearly visible lesions. Overestimation of disease by visual observation may have been possible in our tests. On the other hand, underestimation by PCR may have occurred due to sampling variation or presence of bacteria that degrade DNA in the samples. Detection of infected tubers by PCR is more accurate than visual observation, because other potato pathogens may rot tubers and form lesions that can be misdiagnosed or symptoms may be absent altogether (30).

Tuber blight was not controlled with foliar fungicides in the tests conducted at the Lewis Brown Horticulture Research Farm in Oregon in 1997. The length of sporulation time and potential for propagules to wash down to the soil were greater in plants from fungicide-treated plots than in plants from nontreated control plots, because the rate of epidemics was slower in fungicide-treated plots. Inoculum was produced over a longer period of time in the fungicide-treated than in nontreated control plots. Plants in control plots that were not treated with fungicides died before tuber infection occurred. These factors could explain why tubers from control plots had a lower percentage of infected tubers than tubers from fungicide-treated plots (Mary Powelson, *personal communication*). In contrast, in an NCSU study in 1998, late blight was found on aboveground plant parts but was not observed on tubers using either visual observation or PCR assay. These results may be due to the differences in soil types, microorganisms in soil, and rainfall levels during the experiment, which can affect the spread of *P. infestans* inoculum from leaves to tubers in soil (1,21).

The PCR detection method described here for *P. infestans* could be used as a practical screen for detection of latent infections caused by the pathogen in potato seed lots prior to storage, shipping, or planting. Rotted tubers in storage are difficult to diagnose by traditional methods. The accurate diagnosis of late blight in seed tubers could assist certification agencies in the potato seed industry which currently do not certify for the pathogen. Tubers that are pathogen-free should reduce movement of late blight pathogen between fields and, ultimately, improve disease management.

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