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ORIGINAL ARTICLE

Rapid and sensitive detection of *Phytophthora colocasiae* associated with leaf blight of taro by species-specific polymerase chain reaction assay

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Abstract The failure to adequately identify plant pathogens from culture-based morphological techniques has led to the development of culture-independent molecular approaches. The timely and accurate detection of pathogens is a critical aid in the study of epidemiology and biology of plant diseases. In the case of regulated organisms, the availability of sensitive and reliable assay is essential when trying to achieve early detection of pathogens. We developed and tested the PCR assay for detection of *Phytophthora colocasiae*, an oomycetes pathogen of leaf blight of taro and of rotting of taro tubers. The method described here is specific for *P. colocasiae* when tested across fungal, bacterial, and other *Phytophthora* species. In conventional (single-round) PCR, the limit of detection was 20 pg DNA for both primer sets, whereas in nested PCR the detection limit for both was 0.2 pg. In sampling studies, *P. colocasiae*-specific primers were used to detect leaf blight in infected leaves and tubers of taro cultivar. The causal pathogen *P. colocasiae* was detected by PCR from artificially infected tubers after 16 h of post inoculation, before any visible symptoms were present. The method was also tested to detect fungal DNA in infected leaves and infested soils. The PCR assay and direct tissue extraction methods provide tools which may be used to detect *P. colocasiae* pathogens in taro planting material and thus limit the transmission and spread of new, aggressive strains of *P. colocasiae* in taro-growing regions.

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Introduction

Leaf blight of taro (*Colocasia esculenta*), caused by *Phytophthora colocasiae*, a hemibiotrophic oomycete plant pathogen, is the most destructive disease of *Colocasia* (Raciborski 1900). The life cycle and infection process of *P. colocasiae* are well known (Misra et al. 2008). Infection generally starts with motile zoospores that swim on the leaf surface encyst and germinate directly by producing one or more germ tubes. Germ tubes form an appressorium and a penetration peg, which pierces the cuticle and penetrates an epidermal cell to form an infection vesicle. Branching hyphae with narrow, digit-like haustoria expand from the site of penetration to neighboring cells through the intercellular space. Later, infected tissue necrotizes and the mycelium develops sporangiophores that emerge through the stomata to produce numerous asexual spores called sporangia. In addition to leaf blight, *P. colocasiae* causes a serious post-harvest decay of corms (Jackson et al. 1979). The abundant production of zoosporangia, zoospores and cysts make *P. colocasiae* a devastating pathogen to the economically important tuber crop taro.

Besides *P. colocasiae*, few other species of *Phytophthora*, viz., *P. araceae*, *P. palmivora*, *P. parasitica*, *P. nicotiana* (Umabala and Rama 1972), have been reported to infect taro. However, the role of these species in the severity and damage or epidemiological aspects is not known. Disease management of *P. colocasiae* is heavily geared towards prevention of infection through the production of clean planting material, disease-free soil, high levels of nursery

hygiene, and improvment of drainage and soil health in the field. Since hygiene is a relevant part of integrated disease management of *P. colocasiae,* it is thus important to accurately determine the absence of *P. colocasiae* in planting material, potting mixture, and soil samples. The use of disease-free planting material is important in halting the spread of *P. colocasiae* from nurseries to fields and also highly relevant to limiting the spread of it across the globe.

Conventional diagnostic tests are based on isolating *Phytophthora* from diseased plants using culture media containing a cocktail of antibiotics (Erwin and Ribeiro 1996; Drenth and Sendall 2004), induction of spore formation, and subsequent microscopic examination of the morphology of spores. Other characters such as the presence of chlamydospores, hyphal swelling, and structures associated with the formation of oospores are also taken into account when identifying *Phytophthora* to the species level. The major limitations of these methods are the reliance on the ability of the organism to be cultured, the time-consuming and laborious nature, and the requirement for extensive taxonomical knowledge, altogether often complicating timely disease management decisions. Molecular techniques can circumvent many of these drawbacks, especially if they make use of the polymerase chain reaction (Lievens and Thomma 2005). In general, these methods are much faster, more specific, more sensitive, and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise (Lievens and Thomma 2005). Obviously, the specificity of nucleic acid-based techniques is determined by the sequences that are targeted. In general, there are two approaches to select target sequences. The first, and most common, strategy involves targeting ubiquitously conserved known genes carrying sequence variation that can be exploited. Currently, the primary target in fungal molecular diagnostics is the internal transcribed spacer (ITS) regions and nuclear ribosomal DNA (rDNA), which have been extensively used in molecular phylogenetic studies (White et al. 1990). A large amount of rDNA sequence data is available in public databases, which aids the design of a diagnostic assay. These extensive sequence data allow comparison of sequences which permits, in turn, determining diagnostic regions harboring the required specificity. Apart from the discriminatory potential, the high copy number of rDNA genes in any genome permits a highly sensitive detection.

The second strategy to select target sequences for detection of plant pathogens involves the screening of random parts of the genome to find diagnostic sequences. This can be achieved by several techniques, including random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and amplified fragment length polymorphism (AFLP) (Vos et al. 1995) technology. Nevertheless, since the location of possible useful sequences in the genome is a priori unknown, there are often few sequence data available for comparison to other organisms in order to guarantee specificity. As a consequence, extensive screening is required to ensure specificity of the potential marker.

Successful primer design for detection of pathogen requires that the target region be unique to the organism of interest and conserved across populations of the organism of interest. The ITS region has been shown to be conserved between *Phytophthora* spp. but to differ across species (Cooke et al. 2000). Most importantly, sequence information is available in this region for nearly all known species of *Phytophthora*. The species most similar to *P. colocasiae* in ITS region are *P. meadii* (99% sequence homology), *P. citrophthora* (99% sequence homology), and *P. capsici* (98% sequence homology). Since ribosomal sequences do not reflect sufficient sequence variation to discriminate between these *Phytophthora* species, and thus for designing PCR-based diagnostic primers for *P. colocasiae*, some other conservative genomic regions are required.

An objective of this study was to develop *P. colocasiae* species-specific PCR primers based on its conserved DNA sequence to detect and distinguish *P. colocasiae* from all other species of *Phytophthora*. The potential applications for the designed PCR primers are discussed.

Materials and methods

Biological materials

Taro (*Colocasia esculenta*: var. Telia) plants grown in pots containing soil and *Trichoderma*-enriched compost from tubers were used for the control. Isolates of *P. colocasiae* used in this study were isolated from mature leaves of taro showing typical symptoms of taro blight. The regions of India representing the known range of this fungus were given preference for this study (Table 1). *Phytophthora colocasiae* was confirmed in all 14 isolates by comparing their morphology with several other accessions of *P. colocasiae* maintained in the Indian Institute of Spices Research (IISR, Calicut, India) collections. Typically, *P. colocasiae* is characterized by the production of ovoid, ellipsoid, or fusiform, semipapillate sporangia that are caducous and with a medium pedicel (3.5–10 μm). For isolation, leaf tissue segments of 2–3 cm from leaf blight-infected area were excised from lesion margins. The leaf segments were sterilized in 1% sodium hypochlorite for 2 min, rinsed twice with sterile distilled water, and placed onto *Phytophthora*-selective media (rye agar amended with 20 mg/L rifamycin, 200 mg/L vancomycin, 200 mg/L ampicillin, 68 mg/L pentachloronitrobenzene, and 50 mg/L 50% benlate). Segments were incubated in Petri dishes for 4–5 days at 20°C, and mycelia were then transferred and

Table 1 Confirmed isolates of *Phytophthora colocasiae* along with their region of collection, used in PCR amplification by designed PCSP Primer

No.	IISR number	Place of collection
1	02–03	RC CTCRI, Bhubaneswar, Orissa
2	02–04	Salepur,Bhubaneswar, Orissa
3	02–05	Malikpur, Faizabad, Uttar Pradesh
4	02–06	Gopigand, Varanasi, Uttar Pradesh
5	02–07	Nayagarh, Orissa
6	02–08	Nadia, West Bengal
7	02–09	Anandpur, Orissa
8	02–10	Khandpara, Orissa
9	98–35a	Chelavoor, Calicut, Kerala
10	98–35b	Chelavoor, Calicut, Kerala
11	PC-53	Jaipur, Orissa
12	PC-71	Khairabad, Uttar Pradesh
13	98–111	Sikkim, Assam
14	PC-Tvm.	CTCRI, Trivandrum, Kerala

maintained on potato dextrose agar medium (PDA; 250 g/L potato, 20 g/L dextrose and 20 g/L agar). All voucher specimens were deposited in IISR. To take into account in planta detection of *P. colocasiae*, a comparable portion of leaf blight symptomatic tissues were excised from the margins of necrotic area of taro leaves.

Authenticated cultures of *Phytophthora* genus (*P. capsici*, *P. citrophthora*, *P. parasitica*, *P. araceae*, *P. meadii*, *P. nicotianae*, *P. infestans*, *P. cryptogea*, and *P. palmivora*), *Pythium aphanidermatum*, fungus (*Fusarium solani*, *Colletotrichum capsici*, *Rhizopus oryzae*, *Botryodiplodia theobromae*, and *Rhizoctonia solani*) and bacteria (*Erwinia carotovora*) were also used to test the specificity of designed primers.

Genomic DNA preparation

Phytophthora colocasiae strains and all other *Phytophthora* and fungal strains were grown on PDA medium. For DNA isolation, small blocks of actively growing cultures were used to inoculate Erlenmeyer flasks (250 mL) containing 100 mL of autoclaved potato dextrose broth. The cultures were placed on a rotary shaker (Innova-4230, USA) at 100*g* and incubated at optimum temperature (25–28°C) for the species concerned. After 5–10 days, depending on the growth rate of the species, mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels, and stored at −20°C until ready for DNA isolation. For each strain, 30 mg of freeze-dried mycelium were ground with glass beads and the DNA was isolated according to the method of Mishra et al. (2008a). DNA was extracted from healthy and diseased leaves of taro using the method described by Wang et al. (1993). Bacterial cultures were grown overnight at 37°C in Luria-Bertani broth, and genomic DNA was extracted by the method of Sambrook et al. (1989). DNA was extracted from soil according to Mishra et al. (2008a) with few modifications. Briefly, 0.25 g of soil sample was ground with a mortar and pestle until a fine powder remained. The powdered soil was suspended in 0.5 ml of skimmed milk powder solution (0.1 g of milk powder in 25 mL of H_2O) by vigorous vortexing. The soil and debris were removed by centrifugation at 10,000*g* for 10 min and the supernatant was mixed with SDS extraction buffer (Tris-HCl, 200 mM; EDTA, 25 mM; NaCl, 1 M; SDS, 4% and proteinase K, 0.05 mg/mL; pH 8.0). Samples were vortexed and incubated at 65°C for 30 min. After incubation, samples were centrifuged at 10,000*g* for 15 min and supernatant was transferred to a fresh tube. To the supernatant, 150 µl of 3 M guanidine hydrochloride was added and incubated at −20°C for 10 min. Samples were centrifuged at 10,000*g* for 10 min and the precipitation was carried out with isopropanol. The pellet obtained were washed with 70% ethanol, air dried, and dissolved in 50 µl of TE buffer (Tris-HCl, 10 mM, pH 8; EDTA, 1 mM). The nucleic acid dissolved in TE buffer was treated with 3 µl of RNase A (10 mg/mL), incubated at 37°C, and stored at −20°C until use. DNA was quantified by spectrophotometric measurement of UV absorption at 260 nm (Shimadzu UV-260). DNA was also quantified by means of 0.8% agarose gel electrophoresis followed by ethidium bromide visualization using a 1.5-kb DNA ladder as DNA size marker.

PCR amplification

The genomic region of three *P. colocasiae* isolates (02–08, 98–111, and PC-Tvm) defined by the *Phytophthora* spp.-specific oligonucleotide primers Phyto 2 and Phyto 3 was amplified by PCR (Hayden et al. 2004). Each 25 µL of PCR reaction consisted of 200 ng of template DNA, 100 µM each deoxynucleotide triphosphate, 20 ng of each primers, 1.5 mM $MgCl_2$, 1x Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01% gelatin), and 1 U of Taq DNA polymerase (Promega). Amplifications were performed in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). The PCR reaction mixtures were heated at an initial step of 94°C for 2 min and then subjected to 35 cycles of following programme: 94°C for 30 s, 60°C for 1 min, 72°C for 1 min 45 s. After the last cycle, the temperature was maintained at 72°C for 8 min. Amplified products were resolved on a 1.5% agarose gel containing 0.5 mg/mL ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Alpha imager; Alpha Innotech, USA). The single amplified band was eluted using QIAquick Gel extraction kit (Quiagen, Tokyo, Japan) and cloned into the pGEM-T® vector

(Promega). The cDNA clones were transformed and amplified in *E. coli* DH5α cells. The positive transformants were selected by blue/white screening and sequenced by using T7 or SP6 promoter primers. The nucleotide sequence analysis with a BLAST (NCBI), showed 100% homology among three isolates of *P. colocasiae*, and the sequence of isolate 98–111 was submitted to the GenBank at the National Center for Biotechnology Information (accession no. EU600194).

Development of *Phytophthora colocasiae* specific primers and PCR conditions

DNA from three isolates of *P. colocasiae* (02–08, 98–111, and PC-Tvm) was amplified with PCR primers, Phyto 2 and Phyto 3. The obtained genomic region sequences was analyzed with a BLAST (NCBI) and were further aligned with available data sequences of *Phytophthora* and fungal species by using the BioEdit sequence alignment clustal program (Hall 1999). Regions of dissimilarity of 12 *Phytophthora* and 10 fungal species and similarity in *P. colocasiae* were used to design and construct *P. colocasiae* specific primers, named the PCSP primer.

The standard PCR protocol kept with 100 ng of template DNA was used for testing oligonucleotide PCSP primers. Amplifications were performed in an Eppendorf thermal cycler. The following gradient-PCR cycling regime was used: initial denaturation at 94°C for 2 min, then 20–40 cycles of denaturation at 94°C for 30 s, annealing at 50–60°C for 1 min, and extension for 1 min 45 s at 72°C. After the last cycle, the temperature was maintained at 72°C for 8 min. All PCR reactions were conducted in duplicate. Primers were subjected to the following three tests to ascertain their suitability as *P. colocasiae*-specific primers.

1. Annealing temperature: the maximum and optimum annealing temperatures per primer pair were empirically investigated starting with from 50 to 60°C.
2. PCR cycles: the specificity and efficiency of selected primer pairs in the PCR was tested from 20 to 40 cycles. Only those primer pairs yielding a single amplicon identical in size from 20 to 40 cycles were tested further.
3. Specificity and sensitivity: after successful amplification of the target DNA, the specificity of primer pairs was checked onto a range of bacterial, fungal, *Phytophthora* spp., and taro DNA samples. When no cross-reactivity was shown, the primer pairs were checked against *P. colocasiae*. To assess the sensitivity of the molecular detection using the primers, serial ten-fold dilutions of the *P. colocasiae* genomic DNA sample were used. Then, to test the negative effect of plant DNA on PCR, the ten-fold dilutions of *P. colocasiae* DNA were mixed with 600 ng/μL of plant DNA extracted from leaves of taro. In some cases, a nested PCR was developed to increase the sensitivity of the method. In this case, *Phytophthora*-specific primers, Phyto 2 and Phyto 3, were used in the first round and then 1 μL of the first round was used as template in the second round of amplification with *P. colocasiae*-specific primers, performed according to the PCR protocol described above.

Inoculation, sampling and PCR detection

For infection in taro tubers or leaves, *P. colocasiae* spores were generated by using the methodof Mishra et al. (2008b) and applied to wounds cut into tubers or leaf surfaces (cv. Telia) with a micropipette or paintbrush. Inoculated tubers or leaves were incubated in plastic bags (one tuber or leaf per bag), containing a wet paper towel to maintain high relative humidity, at 26°C in darkness until symptoms appeared (6–10 days). To determine when *P. colocasiae* could first be detected in infected tubers, sampling was performed at 12, 16, 24, 48, and 72 h post-inoculation by removing four small (ca. 10 mg fresh weight) tissue samples randomly from symptomless areas of tubers (eyes, or areas just adjacent to eyes) or leaves, and symptomatic areas from each tuber or leaf and processed by nested PCR. The experiment was performed three times, with four tubers or leaves inoculated and sampled in each experiment.

Additionally, field samples of taro tubers and leaves that either were asymptomatic, contained visible lesions, or were dead from infections caused by *P. colocasiae* were sampled from three field plots in Kerala. The lesions were cut in half to compare recovery after culture on isolation media to the PCR method. The tissue was surface disinfested in 0.05% sodium hypochlorite and plated on a *Phytophthora*-selective medium for isolation of the pathogen and a portion of the remaining lesion was used for nested-PCR assay. Twenty-five plants from each symptom category were sampled, and the PCR experiments were repeated twice.

Soil samples were also collected in the same farms where infected plants had been harvested and analyzed by designed primers.

Results

Primer design, specificity and sensitivity

DNA extracted from *P. colocasiae* isolates (02–08, 98–111, and PC-Tvm) was amplified with Phyto2 and Phyto 3 primers yielded a single 810-bp product in both the isolates. The PCR product was cloned with a pGEM-T® Easy vector and sequenced. DNA sequence analysis *P. colocasiae* isolates (98–111 and PC-Tvm) revealed 100% nucleotide sequence

homology with each other and 2–10% with different gene sequences of *Phytophthora* and fungal species present in the nucleotide database (data not shown). The candidate primers were designed from 100% sequence homology region of *P. colocasiae* isolates and from regions of the greatest sequence dissimilarity among other species and referred to as the PCSP primers. The designed *P. colocasiae*-specific forward primer PCSPF (5′-GTGAAGTGTTATGGGTAGAC-3′) and reverse primer PCSPR (5′-GCAATTTCCATATAGGCGG-3′), gave an approximately 750-bp product at standardized 53°C annealing temperature in all isolates of *P. colocasiae* (Fig. 1). To test the specificity of PCSP primers, purified DNA from one bacterial species, nine *Phytophthora* species, and six isolates from other fungal species, some pathogenic to taro, was amplified using PCSPF/PCSPR primer pair. No amplifications were recorded with the tested bacterial, *Pythium*, fungal, or *Phytophthora* species other than *P. colocasiae*, which indicated that no corresponding sites of the designed primer existed in the genomic DNA of the other tested organism (data not shown).

The *P. colocasiae*-specific primer pair produced a visible amplicon on an agarose gel after 25 cycles when starting with 200 ng of genomic DNA of *P. colocasiae*. In conventional PCR, the lowest amount of amplified DNA was 20 pg for the PCSPF/PCSPR primer pair (Fig. 2a). In nested PCR, after the first round amplification with the Phyto2 and Phyto 3 primers, the detection limit was 0.2 pg for each primer pair (Fig. 2b). The inclusion of plant DNA in the PCR reaction decreased the sensitivity limit two-fold in both conventional and nested PCR (data not shown).

Detection of *Phytophthora colocasiae* in plants and soils

Primers were tested for their ability to detect the *P. colocasiae* in healthy and infected taro tuber and leaf tissue. The designed primer pairs amplified *P. colocasiae* from water-soaked tissue at foliar lesion margins but not from yellowed marginal areas adjacent to lesions (data not shown). Amplification of *P. colocasiae* DNA was observed in artificially infected tubers of taro by the nested and conventional PCR at 16 h and 24 h post-inoculation, respectively. No amplification products were obtained after conventional and nested PCR for tuber samples that were healthy or for negative controls (data not shown).

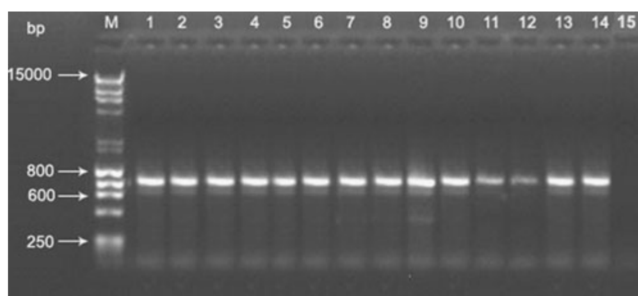

Fig. 1 Amplification of 750-bp product using the PCSP primer. *M* 1-kbp ladder, *lanes 1–14* isolates of *Phytophthora colocasiae* used in the study, *lane 15* no DNA template

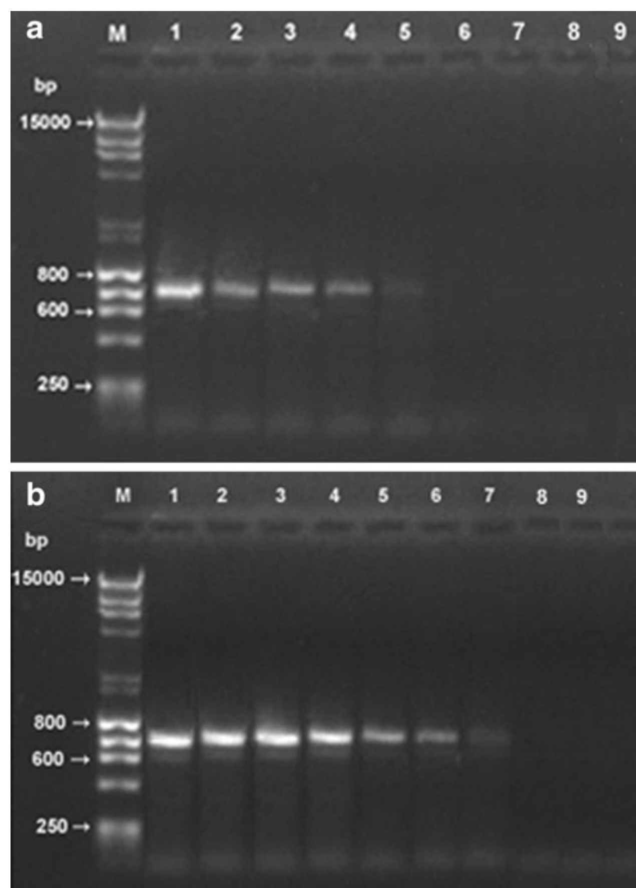

Fig. 2 Sensitivity of **a** conventional PCR or **b** nested PCR for detection of *Phytophthora colocasiae*. *M* 1-kbp marker, *lane 1* undiluted (200 ng/mL), *lane 2* 10^1 diluted, *lane 3* 10^2, *lane 4* 10^3, *lane 5* 10^4, *lane 6* 10^5, *lane 7* 10^6, *lane 8* 10^7, *lane 9* no DNA template

In the three replicate experiments of inoculated taro tubers by the nested PCR assay with both of the designed primers, the percentage of samples that were positive for *P. colocasiae* by PCR ranged from 75 to 80%, from 82 to 88%, and from 90 to 92% at 16, 24, and 48 h post-inoculation, respectively (data not shown). The visible lesion region yielded the highest percent detection by PCR (92–100% detection in the three experiments), while areas just adjacent to infection showed the next highest percent positive samples (92–94%). Healthy tuber tissue yielded negative PCR results in all the three experiments. Of the total of six tubers inoculated in the three replicated sampling experiments, all were tested positive by nested PCR at 48 h post-inoculation, before the occurrence of visible symptoms (data not shown). Since all six tubers eventually showed visible tuber rot lesions, this represents a frequency of 100% for detecting infected tubers

before lesions are visible. The above frequency is based on assay of seven 10-mg tissue samples per tuber. Samples taken from three symptomatic field-harvested tubers (designated A, B, and C) were subjected to the PCR assay with primer pairs. Tuber A, which showed evidence of infection with other fungal pathogens but no visible lesion symptoms, yielded only negative PCR results and *P. colocasiae* was not isolated on PDA medium. However, *P. colocasiae* was detected by PCR from tubers B and C (data not shown), which both contained areas of red brown discoloration typical of tuber rot. The presence of *P. colocasiae* in tubers B and C was confirmed by isolation of the fungus on PDA medium. Of infected taro plants with visible lesions that were positive by traditional isolation on media, 95% were also positive by PCR (data not shown). The PCR method also detected 49% of the infections in samples where the pathogen was not identified previously by traditional isolation on agar media.

Four subsamples of soil from each sample taken from three farms were assayed to test the presence of *P. colocasiae*; two out of three samples tested positive for *P. colocasiae*. In each one, the fungus was detected in the four sub-samples analyzed (data not shown).

Discussion

Prior to the development of DNA-based methods, identification of leaf blight in taro was solely based on visual observation and/or isolation on selective medium (Stamps et al. 1990). *Phytophthora colocasiae* dissemination depends on movement of infested soil and infected plant material; thus, an accurate and timely diagnosis of the presence of the pathogen is necessary to prevent huge losses and restrict the spread of the disease to uninfected areas. PCR has emerged as a powerful tool for the diagnosis of plant diseases because it is more sensitive, robust, rapid, and less labour-intensive than traditional diagnostic methods. We have developed a PCR-based assay of the diagnostic region of *P. colocasiae* which allows positive detection of this pathogen with greater speed and sensitivity, and that is rigorous based on visual observation or selective isolation. Primers designed to identify *P. colocasiae* did not amplify DNA from the other fungi and *Phytophthora* species. The PCR assay can be performed in 3–4 h, including sample processing, PCRs, gel electrophoresis, and staining. Visual identification may require placement of infected tissue on culture medium or in moist chambers to observe growth and/or sporulation characteristic of *P. colocasiae*, which may take 24 h or more. Combined with the use of the rapid NaOH method of DNA extraction from tuber/leaf tissue, the PCR primers we describe here offer a method that is useful in detecting, and moreover distinguishing among, species of *Phytophthora*. With the increased threat posed by new, more aggressive strains of *P. colocasiae* throughout India (Mishra et al. 2008c), the PCR assay we describe provides a novel tool to detect *P. colocasiae* in taro planting materials with greater sensitivity than was previously possible, and hence to reduce the potential for seed transmission of this destructive pathogen. The fact that *P. colocasiae* was detectable by PCR before the appearance of visible symptoms indicates that the assay we describe may prove useful in determining levels of latent infection in symptomless taro harvested from blighted fields and about to be placed into storage. Such information could assist growers in making decisions about the potential value and marketability of the harvested crop. Because the PCR assay is successful in detecting *P. colocasiae* in infected leaf as well as tuber tissue, it may also have applicability in studies of pathogen movement within the host plant, as a method for rapid disease diagnosis in the field, and as a tool for epidemiological studies.

The detection frequency based on four sampled areas per asymptomatic tuber in three replicate experiments, which later developed lesions, was 90–92% at 48 h post-inoculation. We believe that this frequency could be increased substantially by sampling more than four areas per tuber. Another sampling issue relates to the frequency of detection of *P. colocasiae* from visible lesions, which varied from 92 to 100% in our experiments. Several factors could be responsible for the lack of 100% detection, including possible variation in the ability to obtain PCR amplification from lesion areas differing in degree of tissue integrity (i.e., heavily infected brown or black areas versus firmer, less infected brown areas or lesion margins). The degree of rotting due to secondary organisms such as soft rot bacteria (the growth of which is enhanced by maintaining tubers at high humidity in plastic bags) may also affect the integrity of *P. colocasiae* DNA in sampled tissue, as could the occurrence of different levels of DNA degradation during grinding in NaOH prior to PCR. Lack of 100% recovery from lesions may be compensated for by sampling multiple areas of lesions, performing repeated tests, or by using PCR as an adjunct to direct visual identification.

The primers and the assay methods we have described will be useful for taro planting material testing programs, particularly since laboratory procedures for detection are needed as an adjunct to visual identification. Our sampling results have shown that the PCR assay can provide consistent detection of *P. colocasiae* from eyes and other areas of artificially infected tubers and may thus be applied to more routine planting material screening. This PCR assay may prove useful to the taro industry in the quest for a rapid, sensitive, and accurate method of detecting *P. colocasiae* in taro planting material. This will lead to better disease-management strategies as diseased plants and tubers can be identified more quickly before the spread of *P. colocasiae* can take place. In future studies, we will attempt to define

additional parameters that are important in field application of the PCR assay, including sampling methods and approaches for assaying tuber lots of different sizes. Although the use of a conventional PCR assay is the first step in obtaining information on pathogen detection and epidemiology, more accurate information can only be determined by quantitative measurements via the development real-time quantitative PCR assays, which could be the next step of this research.

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References

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM (2000) A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genet Biol 30:17–32

Drenth A, Sendall B (2004) Isolation of *Phytophthora* from infected plant tissue and soil and principles of species identification. In: Drenth A, Guest DI (eds) Diversity and management of *Phytophthora* in Southeast Asia. Australian Centre for International Agricultural Research, Canberra, pp 94–102

Erwin DC, Ribeiro OK (1996) *Phytophthora* diseases worldwide. American Phytopathological Society, St Paul

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hayden KJ, Rizzo D, Tse J, Garbelotto M (2004) Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay. Phytopathology 94:1075–1083

Jackson GVH, Gollifer DE, Pinegar JA, Newhook FJ (1979) The use of fungicides and polyethylene bags for control of post-harvest decay in stored taro corms in the Solomon Islands. Workshop on Small Scale Processing and Storage of Tropical Tuber Crops. University of Hawaii, Honolulu, pp 39–51

Lievens B, Thomma BPHJ (2005) Recent developments in pathogen detection arrays: implications for fungal plant pathogens and use in practice. Phytopathology 95:1374–1380

Mishra AK, Sharma K, Misra RS (2008a) Rapid and efficient method for the extraction of fungal and oomycetes genomic DNA. GenesGenomes Genomics 2:57–59

Mishra AK, Sharma K, Misra RS (2008b) Effect of Benzyl amino purine on the pathogen growth and disease development of taro leaf blight caused by *Phytophthora colocasiae*. J Plant Pathol 90:191–196

Mishra AK, Sharma K, Misra RS (2008c) Isozyme and PCR-based genotyping of *Phytophthora colocasiae* associated with taro leaf blight. Arch Phytopathol Plant Prot. doi:10.1080/03235400802476450

Misra RS, Sharma K, Mishra AK (2008) *Phytophthora* leaf blight of Taro (*Colocasia esculenta*) – a review. Asian Austral J Plants Sci Biotechnol 2:55–63

Raciborski M (1900) Parasitische Algen und Pilze, Java's (Java's Parasitic Algae and Fungi). I. Batavia. (Cited in Waterhouse 1970a under *P. colocasiae*)

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Stamps DJ, Waterhouse GM, Newhook FJ, Hall GS (1990) Revised tabular key to the species of *Phytophthora*. Mycology papers no. 162. C.A.B. International Mycol. Inst. Egham, England

Umabala KA, Rama R (1972) Leaf blight of *Colocasia* caused by *Phytophthora colocasiae*. Indian J Mycol Plant Pathol 2:182–188

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kupier M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wang H, Qi M, Cutler AJ (1993) A simple method of preparing plant samples for PCR. Nucleic Acids Res 21:4153–4154

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535