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Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China

Development of a PCR Assay for the Molecular Detection of *Phytophthora boehmeriae* in Infected Cotton

G. Shen, Y. C. Wang, W. L. Zhang and X. B. Zheng

Authors' address: Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China (correspondence to Y. C. Wang. E-mail: wangyc@njau.edu.cn)

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Abstract

Cotton blight, caused by the oomycete *Phytophthora boehmeriae*, is a serious disease of cotton in China. In wet weather conditions, *P. boehmeriae* is usually the primary pathogen, followed by many saprophytic fungi and pathogens such as *Pythium* spp., *Fusarium* spp., *Rhizoctonia* and others. As *P. boehmeriae* grows much slower than other pathogens, it is difficult to isolate and identify. A rapid and accurate method for its specific identification is necessary for the detection of blight in infected cotton tissue. The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) from three isolates of *P. boehmeriae* were amplified using the polymerase chain reaction (PCR) with the universal primers DC6 and ITS4. PCR products were cloned and sequenced. The sequences were aligned with those published of 50 other *Phytophthora* species, and a region specific to *P. boehmeriae* was used to construct the specific PCR primers PB1 and PB2. Over 106 isolates of 14 *Phytophthora* species and at least 20 other fungal species were used to check the specificity of the primers. PCR amplification with primers PB1 and PB2 resulted in the amplification of a product of approximately 750 bp only from isolates of *P. boehmeriae*. Using primers PB1 and PB2, detection sensitivity was approximately 10 fg DNA/μl. In inoculated plant material, *P. boehmeriae* could be detected in tissue 1 day after inoculation, prior to the appearance of symptoms. The PB primer-based PCR assay provides an accurate and sensitive method for detecting *P. boehmeriae* in cotton tissue.

Introduction

Phytophthora boehmeriae, first isolated from *Beohmeria niver* in Taiwan, was named by Sawada in 1927 and usually only infects *B. niver*, *Broussonetia papyriera* and *Gossypium*. *Phytophthora boehmeriae* has been reported in many areas, including mainland China, Japan, Australia and Argentina (He, 1992). However, there are no previous published reports of *P. boehmeriae* causing a serious disease.

Although blight is one of the most serious diseases of cotton in China, *P. boehmeriae* was only recognized as its cause in recent years. Prior to 1980, boll blight of cotton was infrequent, but during the 1980s severe boll blight epidemics were reported frequently in cotton production areas throughout China. This disease is one of the constraints to the expansion of cotton production in most areas of China, especially in central regions where wet weather conditions are ideal for disease development (He, 1992).

A rapid and accurate method for the specific detection of *P. boehmeriae* in plant material could simplify diagnosis of the disease. As several *Phytophthora* species have been reported to infect cotton (Hopkins, 1925; Patil-Kulkarni and Aswathaiah, 1962; Pinckard and Guidroz, 1973; Allen and West, 1986), disease diagnosis is difficult. In addition, *P. boehmeriae* generally infects cotton seedlings in complex with *Rhizoctonia solani*, *Colletotrichum gossypii* and/or *Fusarium* spp., and in cotton bolls in complex with *C. gossypii*, *Trichothecium roseum*, *Aspergillus* spp., *Fusarium* spp. and *Ascochyta gossypii* (He, 1992). Normally, *P. boehmeriae* infects cotton tissue first and is then followed by other pathogens. As the growth rate of *P. boehmeriae* is much slower than most other pathogenic fungi, its isolation and accurate identification are very difficult. Because most fungicides used to control pathogenic fungi are not effective against oomycetes, misidentification of the pathogen will lead to failure of disease management with higher fungicide usage and more severe contamination of the environment.

A rapid diagnostic assay will aid pathogen identification and lead to more effective management practices, such as directing proper fungicide application prior to the occurrence of serious disease. However, traditional methods of isolation and identification of *Phytophthora* are time-consuming, thus limiting management options. Polymerase chain reaction (PCR)

offers several advantages to more traditional methods of plant disease diagnosis: organisms need not be cultured prior to detection by PCR, the technique is sensitive and rapid (White et al., 1990; Ersek et al., 1994; Bonants et al., 1997; Kong et al., 2003).

The objective of this research was to develop PCR primers for specific amplification of *P. boehmeriae*, and to develop a rapid assay for detecting the pathogen in infected cotton tissues.

Materials and Methods

Source of isolates

Phytophthora boehmeriae isolates were collected from diseased cotton seedlings and bolls in China from 1989 to 2001. These isolates are maintained in the Department of Plant Pathology, Nanjing Agricultural University. Isolates of *Phytophthora* spp., *Pythium* and other fungi were collected for this work. All isolates are listed in Table 1.

DNA extraction

Mycelia of each *Phytophthora* and *Pythium* isolate were obtained by growth in tomato juice broth (100 g tomato juice/l sterile distilled water and 0.2 g $CaCO_3$) at 18–25°C (temperature isolate-dependent) for at least 5 days. Mycelia of other fungi were grown in potato dextrose broth (Fang, 1998). Mycelia were harvested by filtration and frozen at −20°C. DNA was extracted from mycelia according to a modification of the cetyltrimethylammonium bromide (CTAB)

Table 1
Isolates of *Phytophthora boehmeriae* and other fungi used to screen the polymerase chain reaction-specific primer (PB)

Species[a]	Isolation/origin		Number of isolates	PCR product[c]	
	Host/substrate	Source[b]		DC6/ITS4	PB1/PB2
P. boehmeriae	*Gossypium*	JS	21	+	+
	Gossypium	JX	5	+	+
	Gossypium	SD	2	+	+
	Beohmeria niver	JS	3	+	+
	Roussonetia papyriera	JS	2	+	+
P. nicotianae	*Nicotiana tabacum*	YN	6	+	−
	Citrus	JS	2	+	−
	Lycopersicum esculentum	JS	3	+	−
	Sophora chinensis	JS	1	+	−
P. cactorum	*Malus pumila*	W.H. Ko	2	+	−
P. drechsleri	*Cucumis sativus*	JS	1	+	−
P. palmivora	*Citrus*	JS	1	+	−
P. cinnamomi	*Cedrus deodara*	JS	1	+	−
P. capsici	*Capsicum annuum*	JS	7	+	−
P. tropicalis	*Leucospermum*	W.H. Ko	1	+	−
	Theobroma cacao	W.H. Ko	1	+	−
P. heveae	Unknown	HN	1	+	−
P. infestans	*Solanum tuberosum*	FJ	2	+	−
	Lycopersicon esculentum	JS	3	+	−
P. medicaginis	Unknown	ATCC	1	+	−
P. sojae	*Glycine max*	HLJ	3	+	−
	Glycine max	USA	2	+	−
P. colocasiae	Unknown	ATCC	1	+	−
P. cryptogea	*Gerbera jamesonii*	JS	3	+	−
Pythium ultimum	Irrigation water	JS	1	+	−
P. aphanidermatum	*Cucumis sativus*	ZJ	2	+	−
P. myriotylum	Irrigation water	JS	1	+	−
Pythium sp.	Soil	JS	2	+	−
Saprolegnia sp.	Irrigation water	JS	1	−	−
Chytridiomycetes sp.	Irrigation water	JS	1	+	−
Bremia lactucae	*Lactuca* L.	JS	1	+	−
Paranospora	*Gossypium*	JS	1	+	−
Fusarium solani	*Gossypium*	JS	3	−	−
Fusarium sp.	*Gossypium*	JS	2	−	−
Alternaria alternate	Soil	JS	1	−	−
Magnaporthe grisea	*Gossypium*	YN	3	−	−
Colletotrichum gossypii	*Oryza sativa*	JS	1	−	−
Rhizoctonia solani	*Gossypium*	JS	2	−	−
Trichothecium roseum	*Gossypium*	JS	1	−	−
Ascochyta gossypii	*Gossypium*	JS	1	−	−
Aspergillus spp.	*Gossypium*	JS	1	−	−
Verticillium dahliae	*Gossypium*	JS	3	−	−
V. albo-atrum	*Gossypium*	JS	1	−	−
Cryphonectria parasitica	*Castanea*	FJ	2	−	−

[a]All isolates of *P. boehmeriae* and other *Phytophthora* species were maintained in the collection of Nanjing Agricultural University. All other fungal genera were collected for this study.
[b]Isolates were isolated from: JS, Jiangsu Province; JX, Jiangxi Province; SD, handong Province; YN, Yunnan Province; ZJ, Zhejiang Province; FJ, Fujian Province; HLJ, Heilongjiang Province; W.H. Ko, Dr W.H. Ko from Hawaii University.
[c]Polymerase chain reaction (PCR) product: '+', clearly amplified PCR product; '−', no PCR product.

procedure. Frozen mycelia were placed in sterile 1.5 ml microcentrifuge tubes, 150 μl extraction buffer [0.35 M sorbitol, 0.1 M Tris, 0.005 M ethylenediaminetetraacetic acid (EDTA), pH 7.5, 0.02 M sodium bisulphite] was added, and tubes were vortexed. Nuclei lysis buffer (150 μl) containing 0.2 M Tris, 0.05 M EDTA, pH 7.5, 2.0 M NaCl, and 2% CTAB was added, followed by 60 μl of 5% sarkosyl (5 g *N*-lauryl sarcosine per 100 ml H_2O), and tubes were vortexed, then incubated at 65°C for 15–30 min. One volume of chloroform : isoamyl alcohol (24 : 1) was added to each tube, which were then centrifuged for 15 min at 13 000 × ***g*** at room temperature. The aqueous phase was transferred to a new tube and the chloroform extraction was repeated. DNA was precipitated at room temperature by the addition of 0.1 volumes of 3 M sodium acetate, pH 5.2, and two and half volumes of 100% ethanol. After centrifugation, the supernatant was discarded, the pellets were washed with 1 ml of 70% ethanol, and dried at room temperature. DNA was resuspended in TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Polymerase chain reaction

PCR was conducted in a reaction volume of 25 μl. Each reaction consisted of 1 μl of template DNA (approximately 20 ng), 2.5 μl 10X PCR buffer, 18.2 μl sterile distilled H_2O, 1 μl 2.0 mM dNTPs, 1 μl 10 mM $MgCl_2$, 1 μl each of 10 μM ITS4 and DC6 primers (Bonants et al., 1997) and 0.3 μl *Taq* polymerase (5 U/μl). All reactions were carried in a PE2400 PCR instrument. Thermal cycling parameters were: initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 62°C for 40 s, extension at 72°C for 1 min and a final extension at 72°C for 8 min. Negative controls (no template DNA) were used in every experiment to test for the presence of contamination in reagents. All reagents used for PCR amplification were purchased from Promega in Shanghai, China.

Amplified products from three isolates of *P. boehmeriae* were purified by the classic protocol described in Sambrook et al. (1989), and each then cloned in T-easy vector (Promega) according to the manufacturer's recommendations. The plasmid containing the PCR products were sent to Sanggon (Shanghai, China) for automated DNA sequencing on an ABI Prism System automated sequencer (Model 377, version 2.1.1, Perkin-Elmer Corp., Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA). Three clones of every isolate were sequenced. Sequences were aligned with published sequences from 50 other *Phytophthora* species (Cooke et al., 2000) downloaded from Genbank using CLUSTAL W in BIOEDIT, Version 4.8.2 (Hall, 1999). A region specific to *P. boehmeriae* was identified and used to design a pair of PCR primers (PB1 and PB2). These primers were then synthesized by Takara (Shanghai, China). PCR conditions used with primers PB1 and PB2 were identical to those described above for ITS4 and DC6.

Specificity and sensitivity of primers PB1 and PB2

Over106 isolates of 14 species of *Phytophthora* and 20 species of other fungi (Table 1) were used to check the specificity of primers PB1 and PB2. In order to check the sensitivity of the primer, the concentration of purified *P. boehmeriae* DNA was determined spectrophotometrically (Beckman DU640, Beckman Coulter Inc., Fullerton, CA, USA), and then a dilution series of DNA (10, 1.0, 0.1, 0.01 and 0.001 μl) was used for testing the sensitivity of the detection procedure. PCR conditions were as described above. Amplified products were electrophoresed on 0.8% agarose gel containing 0.5 μg/ml ethidium bromide with 1X TAE running buffer. A 100 bp DNA ladder (Takara) was included on each gel as a molecular size standard.

PCR amplification from diseased plant tissue

Phytophthora boehmeriae was grown for 5 days on tomato juice agar medium at 25°C in the dark, mycelium plugs were transferred to tomato juice broth, and grown for 2 days at the same temperature. The broth was discarded and 10 ml sterile distilled water was added to the dish, and the mycelium was grown for 2 more days under constant light to induce sporangia production. The sporangial suspension was chilled at 4°C for 20 min to release zoospores (Zheng, 1995). The zoospore concentration was calculated microscopically, and then 50 μl of the zoospores suspension (containing approximately 200 zoospores) was drawn up into absorbent cotton, and use to inoculate the roots of cotton seedlings, which were grown for approximately 2 weeks in a growth chamber, and were at the two true leaf stage. After 0.5, 1 and 2 days, the inoculated root tissue was washed with double-distilled H_2O, then excised and subjected to a NaOH lysis as a preparation for PCR according to the method of Wang et al. (1993). Healthy cotton root tissue was used as a template for checking the sensitivity of the specific primers in infected plant tissues. The NaOH lysis procedure consisted of grinding 10 mg of plant tissue in 0.5 N NaOH (10 μl/mg), then immediately transferring 5 μl of this extract to a sterile 1.5 ml tube containing 495 μl 100 mM Tris, pH 8.0. An aliquot (1 μl) of this extract was used as the DNA template for PCR under conditions identical to those described above. PCRs were repeated at least three times. In addition, lesions obtained from diseased cotton plants collected in the field from Jiangsu province in 2003 were excised and subjected to a NaOH lysis for subsequent PCR as described above. Healthy root and boll pieces from cotton were included in the NaOH extractions and subsequent PCRs as negative controls. In order to check for inhibitory compounds in the plant tissue DNA extracts, the primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the plant internal transcribed spacer (ITS) region.

In order to check the specificity of the PCR assay in plant tissues 1 day after inoculation with *P. boehmeriae*, the mycelial plugs of *F. solani* and *R. solani* were

used to inoculate the cotton root, and *C. gossypii* and *F. solani* used to inoculate the cotton boll. After 5 days, the infected cotton tissue developed obvious symptoms. Ten days later, the lesion was excised and subjected to NaOH lysis and PCR assays. Inoculated cotton tissue without *P. boehmeriae* was used as a negative control. All PCR assays were repeated three times.

Results

Sequence analysis

Sequences of the ITS regions of three *P. boehmeriae* isolates from the different hosts were 100% identical. These sequences were aligned with previously published sequences from a similar region of other 50 *Phytophthora* spp., including *P. parasitica*, *P. palmivora*, *P. drechsleri*, *P. cactorum* and *P. nicotianae* var. *parasitica*. The variable sequences in ITS1 and ITS2 regions were identified and a pair of potential *P. boehmeriae*-specific primers PB1 (5′-CGGCTTTCGGGCTGCTGC-3′) and PB2 (5′-ATACCCGAAGGCAAAGCGC-3′) were designed and synthesized. The complete ITS1 and ITS2 sequences from *P. boehmeriae* isolate Boe1 were deposited in GenBank (accession numbers AF204921, and AF204922, respectively).

Specificity

Over 106 isolates representing 14 species of *Phytophthora* and 20 other species of fungi were examined in PCR with oomycete universal primers DC6 and ITS4 and *P. boehmeriae*-specific primers PB1and PB2. With the exception of *Saprolegnia*, all of the oomycete isolates including *Phytophthora, Pythium, Parenospora* and *Chytridiomycetes* amplified a product of approximately 1.3 kb with primers DC6 and IT4 (Table 1). However, only the 33 isolates of *P. boehmeriae* yielded a product of approximately 750 bp (Table 1 and Fig. 1) with primers PB1 and PB2. Isolates of all other *Phytophthora* species and other genera tested yielded no amplification product with primers PB1 and PB2.

Sensitivity

The extracted purified DNA of *P. boehmeriae* was used to check the sensitivity of PCR using primers PB1 and PB2 (Fig. 2a). PCR with PB primers PB1 and PB2 yielded positive results at five concentrations from 10 to 0.01 pg DNA, but no positive signal at 0.001 pg DNA as template.

Using the described PCR assay, *P. boehmeriae* was detected in the roots of the cotton seedlings 1 and 2 days, but not 12 h, after inoculation (Fig. 2b).

PCR amplification from disease plant tissue

After a simple template preparation from cotton blighted tissue by NaOH and PCR amplification using primers PB1 and PB2, a single PCR product of approximately750 bp was detected in the infected cotton samples collected in 2003 (Fig. 3, lane 4). The presence of *P. boehmeriae* in the diseased plants was

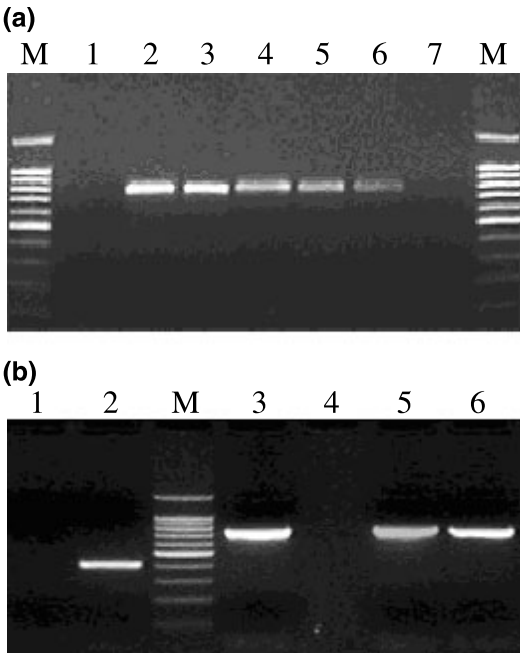

Fig. 2 Sensitivity of molecular detection using the *Phytophthora boehmeriae*-specific primers PB1 and PB2 designed in this study. (a) The purified DNA was used as template; no template DNA (lane 1); lanes 2–7 were purified genomic DNA from 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, respectively. (b) DNA from the pathogen-infected cotton tissue was used as template; healthy cotton tissue without pathogen infection (lane 1), healthy cotton tissue, but plant ITS2 universal primers used (lane 2); 100 fg of purified DNA of *P. boehmeriae* (lane 3), DNA from cotton seedling tissue, 12 h, 1 and 2 days postinoculation with *P. boehmeriae* (lanes 4, 5 and 6, respectively), M: 100 bp DNA ladder

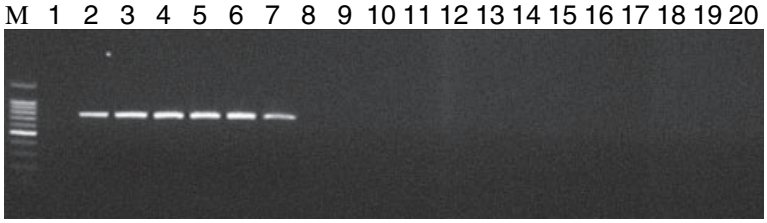

Fig. 1 Agarose gel electrophoresis of polymerase chain reaction (PCR) products using the *Phytophthora boehmeriae*-specific primers PB1 and PB2 designed in this study. PCR amplification products of 19 isolates of 11 species of *Phytophthora* and three species of *Pythium*. Isolates included: *P. boehmeriae* (lanes 2–7), *P. drechsleri*, *P. cactorum*, *P. nicotianae*, *P. palmivora*, *P. tropicalis*, *P. capsici*, *P. cinnamomi*, *P. infestans*, *P. sojae*, *P. cryptogea* and *P. ultimum*, *P. aphanidermatum* and *Pythium* sp. (lanes 8–20), respectively. Lane 1, no template DNA; M, 100 bp DNA ladder

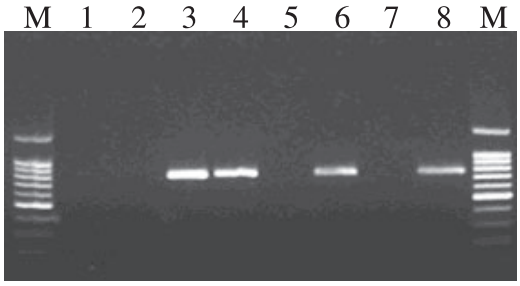

Fig. 3 PB primer amplification of *Phytophthora boehmeriae* from cotton tissue with complex infection. Lane 1: no template DNA; lane 2: healthy cotton tissue; lane 3: 100 fg of purified DNA of *P. boehmeriae*; lane 4: *P. boehmeriae*-infected cotton tissue only; lane 5: *Fusarium solani*- and *Rhizoctonia solani*-infected cotton seedling; lane 6: *P. boehmeriae*, *F. solani* and *R. solani* inoculated cotton seedling; lane 7: boll inoculation of *Colletotrichum gossypii* and *F. solani* only; lane 8: *P. boehmeriae*, *C. gossypii* and *F. solani* were inoculated to cotton boll; M: 100 bp DNA ladder

confirmed by isolating the fungus into pure culture. In an artificial inoculation experiment, both root inoculation with *P. boehmeriae, F. solani* and *R. solani* (Fig. 3, lane 6), and boll inoculation with *P. boehmeriae, C. gossypii* and *F. solani* (Fig. 3, lane 8) yielded only the predicted PCR product of approximately 750 bp. However, no PCR products were obtained from plants without *P. boehmeriae* (Fig. 3, lanes 5 and 7). The NaOH lysis technique was rapid and eliminated the necessity to perform laborious DNA extractions prior to PCR amplification.

Discussion

The objective of this work was to develop PCR primers for specific amplification of *P. boehmeriae* in diseased cotton tissue. Amplification with primers PB1 and PB2 resulted in a product of approximately 750 bp with all 33 isolates of *P. boehmeriae*. No amplification products were produced by the DNA of other 13 tested *Phytophthora* species or 31 isolates of 20 other species of fungi, including these which infect cotton seedlings and bolls, such as *R. solani*, *C. gossypii*, *F. solani*, *T. roseum*, *Aspergillus* spp., and *A. gossypi*.

Specificity is important for detecting of *P. boehmeriae* in disease plant tissue. Five other species of *Phytophthora* have been reported to cause cotton blight. However, only *P. boehmeriae* and *P. drechsleri* have been reported in China (He, 1992). During the last 10 years, we have not isolated any other *Phytophthora* species except *P. boehmeriae* from cotton blight tissue. Isolates of the five species known to infect cotton (*P. boehmeriae*, *P. palmivora*, *P. drechsleri*, *P. cactorum* and *P. nicotianae*) were examined in this study, but only *P. boehmeriae* isolates yielded amplification products with primers PB1 and PB2. With this set of specific primers, no amplification was obtained from any other *Phytophthora* species or other fungi tested.

The PCR assay was also very sensitive for detecting *P. boehmeriae* in artificially inoculated cotton seedlings. *Phytophthora boehmeriae* is a homothallic species, which can yield high numbers of oospores in infected cotton tissue. Soil-borne oospores, which can survive for at least 1 year in soil, are the primary infection sources (He, 1992; Zheng, 1995). In further work, attempts will be made to develop a DNA extraction method from soil-borne oospores which, coupled with the PCR assay described here, will provide a very sensitive detection technique and facilitate disease control. The molecular detection sensitivity of *P. nicotianae* is between 80 and 800 fg DNA/μl (Kong et al., 2003). However, in this work, the PB primer provided a more sensitive diagnostic technique for detection of *P. boehmeriae*; using purified DNA, only 10 fg DNA was needed to detect the pathogen. The greater sensitivity may be due to the high copy number of ITS target sequences in the genome of *Phytophthora* (Cooke et al., 2000).

Molecular tools including isozyme analysis, restriction fragment length polymorphisms in nuclear and mitochondrial DNA, random amplified polymorphic DNA PCR, serological assays, DNA probes, PCR of ITS regions and nuclear small and large subunit rRNA, and PCR of some conserved genes such as elicitins have been developed to detect and discriminate *Phytophthora* species (Goodwin et al., 1989; Panabieres et al., 1989; Förster et al., 1990; Oudemans and Coffey, 1991; Lee and Taylor, 1992; Lee et al., 1993; Möller et al., 1993; Ersek et al., 1994; Ristaino et al., 1995; Tooley et al., 1997; Fraser et al., 1999; Kong et al., 2003). Most of these techniques involve isolating the pathogen into pure culture followed by complex extraction procedures to isolate DNA. Recently, Tooley et al. (1997) and Trout et al. (1997) reported the development of PCR primers to detect *P. infestans* from infected potato and Kong et al. (2003) developed PCR primers to detect *P. nicotianae* in irrigation water. To our knowledge, there are no reports on the molecular diagnosis of *P. boehmeriae*, which is difficult to identify when sporangia or other characteristic morphological structures are absent and when it may be confused with other *Phytophthora* species, such as *P. cactorum*. *Phytophthora* spp. are often difficult to isolate into pure culture, and traditional culture work is time-consuming. The techniques described here eliminate the need for isolation. We have adopted a quick lysis assay for use with infected plant material that eliminates the need for complex DNA extractions prior to PCR (Wang et al., 1993; Trout et al., 1997). These techniques, coupled with traditional disease diagnostics, should provide rapid, accurate diagnosis of blight-infected plants, leading to more effective disease management practices and minimal losses from disease.

Development of this PCR assay has significant practical applications. Its use can provide rapid and accurate detection of *P. boehmeriae* in diseased cotton tissue, even before the appearance of symptoms (i.e. only 1 day after infection). This will be very useful for detecting *P. boehmeriae* in the field and in direct the practical management. Complex infection of *P. boehmeriae* with other fungi such as *F. solani*,

R. solani and *C. gossypii* complicates disease diagnosis, but in such cases, the PCR assay provides a powerful tool for disease management.

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