A Specific and Sensitive Method for the Detection of *Colletotrichum lindemuthianum* in Dry Bean Tissue

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ABSTRACT

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To facilitate early diagnosis and improve control of bean anthracnose, a rapid, specific, and sensitive polymerase chain reaction (PCR)-based method was developed to detect the causal agent, *Colletotrichum lindemuthianum*, in bean (*Phaseolus vulgaris*) seed. Based on sequence data of the rDNA region consisting of the 5.8S gene and internal transcribed spacers (ITS) 1 and 2 of four *C. lindemuthianum* races and 17 *Colletotrichum* species downloaded from GenBank, five forward primers were designed and evaluated for their specificity. Among them, one forward primer was selected for use in combination with ITS4 to specifically detect *C. lindemuthianum*. A 461-bp specific band was amplified from the genomic DNA template of 16 representative isolates of *C. lindemuthianum*, but not from 58 representative isolates of 17 other *Colletotrichum* species or 10 bean pathogens. Moreover, to enhance the sensitivity of detection, nested PCR was applied, which allowed the detection of as little as 10 fg of *C. lindemuthianum* genomic DNA and 1% infected seed powder, which was mixed with 99% healthy seed powder. The diagnostic analysis can be completed within 24 h, compared with about 2 weeks required for culturing. Furthermore, this method can be performed and interpreted by personnel with no specialized taxonomic expertise.

Additional keywords: PCR-based detection

Bean anthracnose, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib., is a serious seed-borne disease of common beans (*Phaseolus vulgaris* L.). This pathogen is distributed worldwide and causes devastating losses in fields planted with infected seed (29), especially when climatic conditions favor disease development (12). Anthracnose has caused serious reductions in the yield of beans in many parts of the world, resulting in yield losses as high as 95% (21). Seed infection by *C. lindemuthianum*

can also reduce seed quality and result in the introduction of the disease into new areas or new races into new geographic regions (29). Anthracnose control is complicated by the ability of *C. lindemuthianum* to survive for up to 22 months in plant debris in northern latitudes (5).

During the first 50 years or so of the last century, traditional *Colletotrichum* systematics relied heavily upon host-related criteria, and up to 750 species belonging to the *Colletotrichum* genus have been described based primarily on host specificity. Subsequently, von Arx (30) reduced the genus to 11 species with 12 forms based on morphological traits rather than the host-related criteria. Species numbers have since crept upward with more detailed studies on morphology, cultural characteristics, and pathogenicity, so that currently 38 species have been accepted (28). However, crop breeders and plant pathologist still prefer to use the original species names that are based on the host-related criteria due to their importance and specificity in causing plant diseases. The different taxonomic approaches have resulted in

confusion regarding species identification within the genus *Colletotrichum*.

The planting of pathogen-free seed is a key recommendation for the control of bean anthracnose (25). However, this requires a highly sensitive assay system to determine whether or not seed is free of the pathogen. Current assays to detect and identify *C. lindemuthianum* in bean tissues include plating on microbial media, which is sometimes followed by the spray inoculation of seedlings with a spore suspension. Although valuable, the plating process can require up to 14 days to complete and is not completely reliable. Polymerase chain reaction (PCR)-based techniques have several potential advantages over conventional diagnostic methods in that they are more accurate, faster, and can be used by personnel with little experience in mycology or plant pathology (1,15,20). By employing specific PCR primers, it is possible to selectively amplify DNA of the pathogen from infected tissues without the need for isolation of the pathogen. The specificity of PCR is derived from the selection of the synthetic oligonucleotide primers. Depending on the choice of primers, PCR can facilitate the detection of a single pathogen or many members of a group of related pathogens (15). Ultimately, PCR procedures will be available for the detection of all seedborne pathogens (32).

In fungi, there are three common DNA sequences used for diagnostic development. The first is the nuclear rDNA region, which usually consists of three genes—the large subunit gene, the small subunit gene, and the 5.8S gene—separated by internal transcribed spacer (ITS) regions, which are repeated many times in a unit (20). The second is the β -tubulin gene (10,16). The third is an ascomycete mating-type gene (8,9). Among them, the ITS region is the most widely sequenced DNA region in fungi. It typically has been most useful for molecular systematics at the species level and even within species (e.g., to identify geographic races) (34). Some primers based on ITS sequence information have

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been designed to specifically detect related fungal pathogens (11,13,17,23,35,37). In common bean, rapid, highly sensitive PCR-based detection systems have been developed for viral diseases such as *Bean yellow mosaic virus* (31) and bacterial diseases such as halo blight and common bacterial blight (2). For bean anthracnose, three race-specific random amplified polymorphic DNA (RAPD) markers have been developed to identify races 64, 65, and 73 from fungal DNA (22).

Touchdown PCR was developed by Don et al. (6) and used to circumvent spurious priming during DNA amplification. This method can avoid amplifying misprimed products and conveniently bypasses spurious amplifications without the need for lengthy optimization procedures. Even in cases where an appropriate discriminatory temperature has been empirically determined, the touchdown approach can also help avoid secondary problems, such as an inconsistency of well temperatures within or between thermal cyclers (6). Moreover, nested PCR has been identified as an effective technique to enhance detection sensitivity (20,36). For this procedure, two PCRs are carried out with the first reaction increasing the amount of template for the second reaction (33). In some situations, nested PCR is necessary to achieve consistently high sensitivity and reduce the incidence of false negatives (18).

Although the timely application of foliar fungicides provides an effective means for controlling *C. lindemuthianum* (4), using disease-free seed is also an effective and practical strategy for control of bean anthracnose. Detection of pathogen contamination requires a quick and effective diagnostic test assay that can be used on seed and plant samples. Currently, no research on the development of a PCR-based method for anthracnose detection in common bean has been reported. The purpose of this study was to develop a reliable, rapid, species-specific, sensitive molecular assay to detect *C. lindemuthianum* in bean tissues.

MATERIALS AND METHODS

Sources of isolates and production of mycelial mats. The original sources of all microbial isolates used in this study are listed in Table 1. Each fungal culture was revived from a stock culture and grown on potato dextrose agar (PDA) (Difco, Sparks, MD) and/or Mathur's medium (19) agar (MMA) at 21°C. Agar plugs taken from the actively growing margins of colonies were transferred to flasks containing 50 ml of potato dextrose broth (PDB). The flasks were incubated in shake culture at 100 rpm at 21°C in the dark for 3 to 7 days. The mycelial mats were removed, dried on filter paper for immediate DNA extraction, or lyophilized with liquid nitrogen and stored at -80°C for later use.

DNA extraction. Fungal genomic DNA was extracted from mycelial mats using

either a modified CTAB (cetyl trimethyl ammonium bromide) procedure (7) or a DNeasy Plant Mini Kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol. For the CTAB method, approximately 50 mg of fresh, filter paper-dried mycelium was transferred to a 1.5-ml microcentrifuge tube containing 588 µl of 2× CTAB buffer preheated at 65°C (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 1% β-mercaptoethanol, 1% PVP-40, pH 8.0) and 12 µl of 10 mg ml⁻¹ Proteinase K (Sigma-Aldrich, Steinheim, Germany). The mycelial tissue was ground with a plastic minipebble (Fisher Scientific, Ottawa, ON) for 3 to 5 min and incubated at 65°C in a water bath for 1 h with occasional gentle swirling. The mix was directly subjected to a chloroform:isoamyl alcohol (24:1) extraction step. The DNA pellet was dissolved in 100 µl of 10 mM Tris buffer, treated with RNase A (Sigma-Aldrich, Steinheim, Germany) (10 µg ml⁻¹) at 37°C for 1 h.

Total DNA from healthy and infected bean seed, and from infected plant leaves, were separately extracted using either the modified CTAB method or DNeasy Plant Mini Kit according to the manufacturer's protocol. The seeds were surface-sterilized in 70% ethanol for 30 s and washed three times with sterilized distilled water prior to DNA isolation. For the bacterial pathogens of common bean, total genomic DNA was extracted from cultures growing on PDA plates with a DNeasy Plant Mini Kit. The concentration of DNA stocks was estimated using a DU 640B spectrophotometer (Beckman, Fullerton, CA) set at 260 nm. DNA quality was evaluated according to the value of $A_{260\text{nm}}/A_{280\text{nm}}$ (24).

Primer design for *C. lindemuthianum*. Twenty-one DNA referential ITS sequences of four *C. lindemuthianum* races and 17 other *Colletotrichum* species were downloaded from GenBank through the National Center for Biotechnology Information (NCBI), National Library of Medicine, National Institutes of Health website, and edited and aligned with BioEdit version 7.0 software (14). Only one species-specific single nucleotide polymorphism (SNP) site located in the ITS1 region distinguished *C. lindemuthianum* from other *Colletotrichum* species. The sequence diversity of the 5.8S and ITS2 was too small to design any species-specific reverse primers. Therefore, based on the relative higher diversity in the ITS1 region among sequences of four *C. lindemuthianum* and 17 other *Colletotrichum* species, five forward primers, CIF1, CIF2, CIF3, CIF4, and CIF5, were manually designed for *C. lindemuthianum*. The possibility of hairpin formation and other primer parameters were evaluated with Primer Premier 5.0 software (26). The above primers were synthesized (Invitrogen Corp., Carlsbad, CA).

PCR amplification. The target band was amplified with a touchdown PCR program (6). Reaction volumes were 25 µl and consisted of 1× PCR buffer, 1.5 mM MgCl₂, 125 µM each of dNTP, 250 nM of each forward and reverse primer, 0.5 units of AmpliTaq DNA polymerase (ABI, Foster City, CA), and 25 to 60 ng of sample DNA. PCR was performed in a PTC-100 thermocycler (MJ Research Inc., Watertown, MA). The PCR program was optimized and consisted of initial denaturation at 95°C for 5 min, 12 cycles of 30 s at 94°C, 30 s from 62°C to a "touchdown" (the annealing temperature was lowered 1°C in each subsequent cycle) at 50°C for primer annealing, 1 min at 72°C for primer extension, followed by 30 cycles with a 50°C annealing temperature, and ended with 1 cycle of 5 min at 72°C.

For nested PCR, the universal primer set ITS5/ITS4 (34) was used as the outer primers in the first round of PCR, 1-µl aliquot of 10 times diluted first-round PCR product was used as the template in the second PCR using the selected forward primer CIF4 and ITS4 with the same touchdown PCR program. The PCR products were separated on a 1.4% agarose gel in 1× TBE buffer at 100 V for 1 h. The gels were stained with ethidium bromide and visualized under UV light using an AlphaImager gel documentation system (Alpha Innotech, San Leandro, CA).

Test of primer specificity. Combined with the reverse primer ITS4, five forward primers CIF1 to CIF5 were separately tested for species-specificity to *C. lindemuthianum* using genomic DNA of seven *C. lindemuthianum* races and 16 representatives of other *Colletotrichum* species. The selected species-specific primer set was evaluated further with DNA from an additional 29 *Colletotrichum* isolates, 18 isolates of other bean pathogens, and the anthracnose-resistant bean differential line, G2333 (Table 1).

Detection of *C. lindemuthianum* in bean seed and plant leaves. The selected primer set CIF4 and ITS4 was used to detect *C. lindemuthianum* from bean seed and infected plant leaves using nested PCR. Seeds of the pinto bean cultivar AC Pintoba were used. Sixteen seeds with typical symptoms and 19 seeds with a healthy appearance were surface-sterilized in 70% ethanol for 30 s and washed three times in sterilized distilled water. The seeds were dried on sterilized filter paper, and batches of healthy and diseased seed were separately ground into a fine powder using a new coffee grinder (Proctor-Silex, Hamilton, CA) for later DNA extraction. Different percentages of the *C. lindemuthianum*-infected seed powder were serially mixed with healthy seed powder to test the minimum level of detection using the species-specific primer set. A total of 0.1 g of ground seed powder was used for each sample. The infected and healthy seed

Table 1. Species, isolates, host, and origin of fungal and bacterial isolates and plant samples used, and results of the polymerase chain reaction (PCR) amplification using the CIF4 and ITS4 primer set

Code	Species	Isolates	Host	Origin	PCR product ^a
1	<i>Colletotrichum lindemuthianum</i>	Race 2	<i>Phaseolus vulgaris</i>	Mich. St. Univ.	+
2	<i>Colletotrichum lindemuthianum</i>	Race 17	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+
3	<i>Colletotrichum lindemuthianum</i>	Race 23	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+
4	<i>Colletotrichum lindemuthianum</i>	Race 31	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+
5	<i>Colletotrichum lindemuthianum</i>	Race 73	<i>Phaseolus vulgaris</i>	AAFC-Morden	+
6	<i>Colletotrichum lindemuthianum</i>	Race 89	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+
7	<i>Colletotrichum lindemuthianum</i>	Race 1096	<i>Phaseolus vulgaris</i>	AAFC-Morden	+
8	<i>Colletotrichum lindemuthianum</i>	DAOM 225470	<i>Phaseolus vulgaris</i>	CCFC ^b	+
9	<i>Colletotrichum lindemuthianum</i>	DAOM 225471	<i>Phaseolus vulgaris</i>	CCFC	+
10	<i>Colletotrichum lindemuthianum</i>	DAOM 225472	<i>Phaseolus vulgaris</i>	CCFC	+
11	<i>Colletotrichum lindemuthianum</i>	DAOM 225473	<i>Phaseolus vulgaris</i>	CCFC	+
12	<i>Colletotrichum lindemuthianum</i>	DAOM 225581	<i>Phaseolus vulgaris</i>	CCFC	+
13	<i>Colletotrichum lindemuthianum</i>	DAOM 225739	<i>Phaseolus vulgaris</i>	CCFC	+
14	<i>Colletotrichum lindemuthianum</i>	DAOM 225740	<i>Phaseolus vulgaris</i>	CCFC	+
15	<i>Colletotrichum lindemuthianum</i>	DAOM 225741	<i>Phaseolus vulgaris</i>	CCFC	+
16	<i>Colletotrichum lindemuthianum</i>	DAOM 225742	<i>Phaseolus vulgaris</i>	CCFC	+
17	<i>Colletotrichum acutatum</i>	DAOM 185681	Unknown	CCFC	–
18	<i>Colletotrichum acutatum</i>	DAOM 212360A	Unknown	CCFC	–
19	<i>Colletotrichum acutatum</i>	DAOM 214715	Unknown	CCFC	–
20	<i>Colletotrichum acutatum</i>	DAOM 214992	Unknown	CCFC	–
21	<i>Colletotrichum acutatum</i>	DAOM 215166	Unknown	CCFC	–
22	<i>Colletotrichum brassicae</i>	DAOM 116226	Unknown	CCFC	–
23	<i>Colletotrichum capsici</i>	DAOM 212661	Unknown	CCFC	–
24	<i>Colletotrichum circinans</i>	DAOM 151616	Unknown	CCFC	–
25	<i>Colletotrichum coccodes</i>	DAOM 60413	Unknown	CCFC	–
26	<i>Colletotrichum coccodes</i>	DAOM 145740	Unknown	CCFC	–
27	<i>Colletotrichum coccodes</i>	DAOM 182572	Unknown	CCFC	–
28	<i>Colletotrichum coccodes</i>	DAOM 214592	Unknown	CCFC	–
29	<i>Colletotrichum coccodes</i>	DAOM 216029	Unknown	CCFC	–
30	<i>Colletotrichum dematium</i>	DAOM 96293	Unknown	CCFC	–
31	<i>Colletotrichum dematium</i>	DAOM 147549	Unknown	CCFC	–
32	<i>Colletotrichum dematium</i>	DAOM 188792	Unknown	CCFC	–
33	<i>Colletotrichum dematium</i>	DAOM 212662	Unknown	CCFC	–
34	<i>Colletotrichum destructivum</i>	DAOM 179749	Unknown	CCFC	–
35	<i>Colletotrichum destructivum</i>	DAOM 196849	Unknown	CCFC	–
36	<i>Colletotrichum destructivum</i>	DAOM 216020	Unknown	CCFC	–
37	<i>Colletotrichum destructivum</i>	DAOM 225584	Unknown	CCFC	–
38	<i>Colletotrichum fuscum</i>	DAOM 216112	Unknown	CCFC	–
39	<i>Colletotrichum gloeosporioides</i>	DAOM 183087	Unknown	CCFC	–
40	<i>Colletotrichum gloeosporioides</i>	DAOM 214710	Unknown	CCFC	–
41	<i>Colletotrichum gloeosporioides</i>	DAOM 225300	Unknown	CCFC	–
42	<i>Colletotrichum graminicola</i>	DAOM 231462	Unknown	CCFC	–
43	<i>Colletotrichum graminicola</i>	DAOM 232153	Unknown	CCFC	–
44	<i>Colletotrichum higginsianum</i>	DAOM 225478	Unknown	CCFC	–
45	<i>Colletotrichum lini</i>	DAOM 183091	Unknown	CCFC	–
46	<i>Colletotrichum musae</i>	DAOM 182828	Unknown	CCFC	–
47	<i>Colletotrichum pisi</i>	DAOM 196850	Unknown	CCFC	–
48	<i>Colletotrichum subliniolum</i>	DAOM 212374	Unknown	CCFC	–
49	<i>Colletotrichum truncatum</i>	DAOM 215174	Unknown	CCFC	–
50	<i>Colletotrichum truncatum</i>	DAOM 233303	Unknown	CCFC	–
51	<i>Colletotrichum truncatum</i>	DAOM 233308	Unknown	CCFC	–
52	<i>Colletotrichum truncatum</i>	DAOM 233314	Unknown	CCFC	–
53	<i>Uromyces phaseoli</i>	Mb05-70	<i>Phaseolus vulgaris</i>	AAFC-Morden	–
54	<i>Sclerotinia sclerotiorum</i>	...	<i>Phaseolus vulgaris</i>	AAFC-Morden	–
55	<i>Fusarium solani</i> f. sp. <i>phaseoli</i>	...	<i>Phaseolus vulgaris</i>	AAFC-Morden	–
56	<i>Fusarium solani</i> f. sp. <i>pisi</i>	98Fus016-1	<i>Pisum sativum</i>	AAFC-Morden	–
57	<i>Rhizoctonia solani</i>	...	<i>Phaseolus vulgaris</i>	AAFC-Morden	–
58	<i>Pythium</i> spp.	P-p3	<i>Pisum sativum</i>	AAFRD-Lacombe	–
59	<i>Pythium</i> spp.	P-p9	<i>Pisum sativum</i>	AAFRD-Lacombe	–
60	<i>Pythium</i> spp.	P-p31	<i>Pisum sativum</i>	AAFRD-Lacombe	–
61	<i>Pythium</i> spp.	P-P88-P3	<i>Pisum sativum</i>	AAFRD-Lacombe	–
62	<i>Botrytis cinerea</i>	B-3	<i>Lupinus angustifolius</i>	Devon	–
63	<i>Botrytis cinerea</i>	B-12	<i>Lupinus angustifolius</i>	Devon	–
64	<i>Botrytis cinerea</i>	B-89	<i>Lupinus angustifolius</i>	Ellerslie	–
65	<i>Botrytis cinerea</i>	B-16	<i>Lupinus angustifolius</i>	Penhold	–
66	<i>Botrytis cinerea</i>	B-4c	<i>Lupinus angustifolius</i>	Barthead	–
67	<i>Xanthomonas campestris</i> pv. <i>phaseoli</i>	BXP-2	<i>Phaseolus vulgaris</i>	AAFC-Morden	–
68	<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	HB06-2	<i>Phaseolus vulgaris</i>	AAFC-Morden	–
69	<i>Corynebacterium flaccumfaciens</i>	OSB-3	<i>Phaseolus vulgaris</i>	AAFC-Lethbridge	–
70	<i>Phaeoisariopsis griseola</i>	MYA-2353	<i>Phaseolus vulgaris</i>	Univ. of Guelph	–
71	<i>Phaseolus vulgaris</i> (G2333)	...	...	AAFC-Morden	–
72	DNA grade water	...	...	...	–

^a + = PCR product was observed after electrophoresis, and – = no PCR product was observed after electrophoresis.

^b Canadian Collection of Fungal Cultures.

powder were mixed sequentially starting with the lowest dilution. The rates of infected seed powder included 100, 80, 60, 40, 20, 10, 8, 6, 4, 2, 1, and 0% (wt/wt). Additionally, genomic DNA extracted from the seeds of the resistant differential line, G2333, and the reaction mixture without DNA were used as negative controls. DNA from a pure culture of *C. lindemuthianum* race 73 was used as the positive control. Total DNA from infected leaves was used to detect *C. lindemuthianum* employing the same PCR procedure.

Moreover, a 10-fold dilution series of *C. lindemuthianum* genomic DNA was used to test the minimum amount of DNA template required for producing a detectable target band with the primer set CIF4 and ITS4 directly by nested PCR.

RESULTS

Sequence analysis of the ITS region of *Colletotrichum* species and primer design. ITS fragments were sequenced from seven identified *C. lindemuthianum* races in this study, and four other *C. lindemuthianum* ITS sequences were downloaded from the GenBank. The ITS sequences of six races (race 17, 23, 31, 73, 89, and 1096) and isolate MAFF 305390 (GenBank accession no. AB087222) were identical. The sequences of the ITS region of race 2, strain BBA 65483 (GenBank accession no. AJ301958), strain CBS 151.28 (GenBank accession no. AJ301946), and strain CBS 132.57 (GenBank accession no. AJ301947) also were identical. There were only three base substitutions between these two ITS sequences. In an aligned ITS se-

quence matrix consisting of four *C. lindemuthianum* strains and 17 other *Colletotrichum* species, sequence identity ranged from 86.9 to 99.2%. Based on sequence differences in a 21-sequence data matrix, five forward primers, CIF1, CIF2, CIF3, CIF4, and CIF5, were designed and tested for their specificity in detecting *C. lindemuthianum* (Table 2).

Specific amplification of *C. lindemuthianum*. With genomic DNA extracted from pure microbial cultures, the touchdown PCR program was used to select a species-specific primer set among the five designed forward primers CIF1, CIF2, CIF3, CIF4, and CIF5. Seven identified *C. lindemuthianum* races (races 2, 17, 23, 31, 73, 89, and 1096) and 16 representatives of other *Colletotrichum* species were tested to determine the species-specificity of the five primer sets. All primer sets could direct successful amplification from the seven *C. lindemuthianum* races. However, CIF1, CIF2, CIF3, and CIF5 forward primers, combined with ITS4, also produced target fragments from some of the other *Colletotrichum* species. The primer pair CIF4/ITS4 produced a 461-bp amplified fragment from the seven *C. lindemuthianum* races, and none from the other 16 *Colletotrichum* species (Table 2). A total of 74 genomic DNA samples derived from different microbial isolates were used to evaluate specificity of PCR amplification with primer set CIF4/ITS4 (Table 1). These microbial isolates included the other 16 *Colletotrichum* species, causal pathogens of seven fungal diseases and three bacterial diseases of common bean. The primer set CIF4/ITS4 directed a 461-bp DNA frag-

ment amplification from only the 16 identified *C. lindemuthianum* isolates, but not from the other *Colletotrichum* species or bean pathogens. Therefore, this primer pair was further used to detect *C. lindemuthianum* in infected bean tissues.

Evaluation of detection sensitivity. A 10-fold dilution series of *C. lindemuthianum* race 2 genomic DNA was used to evaluate the detection sensitivity of the selected primer set CIF4 and ITS4. The primer set could direct visible amplified fragments from as little as 10 pg of DNA using single-step PCR (Y. Chen, unpublished data). In nested PCR, the universal primer set ITS5/ITS4 was used in the first-round and the CIF4/ITS4 primer set was used in the second-round PCR reaction. One microliter of 10-times diluted first-round PCR products was used as template for the second-round PCR, and as little as 10 fg of *C. lindemuthianum* genomic DNA could be detected (Fig. 1). The nested PCR was approximately 1,000 times more sensitive than the single-step PCR assay.

Using single-step PCR, the PCR results were unstable and the sensitivity was low for genomic DNA from infected leaves and different mixtures of infected and healthy bean seed powder (Y. Chen, unpublished data). Therefore, the nested PCR method was employed to directly detect *C. lindemuthianum* in infected bean tissues. A 461-bp DNA fragment was produced from genomic DNA of individual infected leaves with nested PCR (Fig. 2). Visible PCR products in ethidium bromide-stained gels after electrophoresis could be produced from the template DNA extracted from mixed seed samples, which included 100

Table 2. Species, isolates, host, origin of fungal isolates used, and results of the polymerase chain reaction (PCR) amplification using CIF1 to CIF5 and ITS4 primer sets

Code	Species	Isolates	Host	Origin	PCR product ^a				
					CIF1	CIF2	CIF3	CIF4	CIF5
1	<i>Colletotrichum lindemuthianum</i>	Race 2	<i>Phaseolus vulgaris</i>	Mich. St. Univ.	+	+	+	+	+
2	<i>Colletotrichum lindemuthianum</i>	Race 17	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+	+	+	+	+
3	<i>Colletotrichum lindemuthianum</i>	Race 23	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+	+	+	+	+
4	<i>Colletotrichum lindemuthianum</i>	Race 31	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+	+	+	+	+
5	<i>Colletotrichum lindemuthianum</i>	Race 73	<i>Phaseolus vulgaris</i>	AAFC-Morden	+	+	+	+	+
6	<i>Colletotrichum lindemuthianum</i>	Race 89	<i>Phaseolus vulgaris</i>	AAFC-Harrow	+	+	+	+	+
7	<i>Colletotrichum lindemuthianum</i>	Race 1096	<i>Phaseolus vulgaris</i>	AAFC-Morden	+	+	+	+	+
8	<i>Colletotrichum acutatum</i>	DAOM 214715	Unknown	CCFC	+	+	—	—	+
9	<i>Colletotrichum brassicae</i>	DAOM 116226	Unknown	CCFC	+	+	—	—	+
10	<i>Colletotrichum capsici</i>	DAOM 212661	Unknown	CCFC	—	+	+	—	+
11	<i>Colletotrichum circinans</i>	DAOM 151616	Unknown	CCFC	+	—	+	—	+
12	<i>Colletotrichum coccodes</i>	DAOM 60413	Unknown	CCFC	+	+	+	—	+
13	<i>Colletotrichum dematium</i>	DAOM 188792	Unknown	CCFC	+	+	—	—	+
14	<i>Colletotrichum destructivum</i>	DAOM 179749	Unknown	CCFC	+	+	+	—	+
15	<i>Colletotrichum fuscum</i>	DAOM 216112	Unknown	CCFC	—	+	—	—	+
16	<i>Colletotrichum gloeosporioides</i>	DAOM 183087	Unknown	CCFC	—	+	+	—	+
17	<i>Colletotrichum graminicola</i>	DAOM 231462	Unknown	CCFC	+	+	+	—	+
18	<i>Colletotrichum higginsianum</i>	DAOM 225478	Unknown	CCFC	+	+	+	—	+
19	<i>Colletotrichum lini</i>	DAOM 183091	Unknown	CCFC	+	—	—	—	+
20	<i>Colletotrichum musae</i>	DAOM 182828	Unknown	CCFC	+	—	—	—	+
21	<i>Colletotrichum pisi</i>	DAOM 196850	Unknown	CCFC	—	—	—	—	+
22	<i>Colletotrichum subliniolum</i>	DAOM 212374	Unknown	CCFC	+	+	+	—	+
23	<i>Colletotrichum truncatum</i>	DAOM 215174	Unknown	CCFC	+	+	—	—	+

^a Primers designed for PCR were CIF1 (5'-CCCTTTGTGAACATACCAAACCG-3'); CIF2 (5'-TGTGAACATACCAAACCGTTGC-3'); CIF3 (5'-TCCCCCTGCCCCGCTCGCG-3'); CIF4 (5'-TCCCCCTGCCCCGCTCG-3'); and CIF5 (5'-CGCCGGAGGAAAACCCAAC-3'). + = PCR product was observed after electrophoresis, and — = no PCR product was observed after electrophoresis.

to 1% infected seed powder (Fig. 3). The negative controls did not produce any amplicons, whereas the positive control showed a strong DNA band of the expected size.

DISCUSSION

In this study, a PCR-based assay was developed to specifically detect *C. lindemuthianum* from pure fungal DNA and mixed DNA from infected bean tissues such as seeds and leaves. The forward primer CIF4 was selected to be combined with the universal reverse primer ITS4 to detect *C. lindemuthianum* DNA from pure fungal DNA and fungal-plant background mixed DNA. The ITS region has proven to be useful for molecular systematics at the species level and within species. Sreenivasaprasad et al. (27) reported that in *Colletotrichum* species the ITS1 region has a greater degree of intra- and inter-specific divergency (50.3% variable sites) compared with the ITS2 region (12.4% variable sites). The maximum intraspecific divergence was recorded with *C. acutatum* (5.8%). *Colletotrichum capsici* showed the greatest level of interspecific divergence (8.9 to 23.3%). Genetic distances calculated from the ITS sequence ranged from 0.2 to 1.8% among 14 isolates of *C. lindemuthianum* that were collected in North, Central, and South America and the Netherlands (3). However, *C. lindemuthianum* is a species primarily based on a host-pathogen criterion, not a real phylogenetic species (28,30). In this study, the average ITS sequence divergence between *C. lindemuthianum* and other *Colletotrichum* species was 11.5%, and no species-specific sequence information could be found in the 5.8S and ITS2 regions of the *C. lindemuthianum* sequence. It was difficult to find an opportunity to design possible specific reverse primers in the 5.8S or ITS2 region. Similarly, using the specific forward primer CgInt and the universal reverse primer ITS4, a PCR-based assay had been developed to detect *C. gloeosporioides* Penz. from infected tomato fruits (23).

The annealing temperature of the touchdown PCR used in this study was decreased 1°C per cycle from 62°C to a touchdown temperature of 50°C. Any imbalance between correct and spurious annealing was automatically redressed, which eliminated the time required to determine the best annealing temperature for every primer set during the PCR program optimization procedure. In this study, five designed primers with different T_m (melting temperature) values directed successful amplification with the same touchdown PCR program. Touchdown PCR has several advantages in comparison to a PCR program with fixed annealing temperature, since it can be applied in several situations, such as running in different thermal cyclers, with different PCR kits or different

operators. For plant pathogen detection, touchdown PCR can permit PCR-based testing of several plant pathogens in one thermal cycler simultaneously. Furthermore, it has the potential to allow the use of different primer sets in PCR detection for several related plant pathogens in one tube.

Apart from specificity, the sensitivity of detection also is important for early disease diagnosis. The sensitivity of a PCR assay depends on several factors, including

the quality of the prepared genomic DNA, primer composition, structure and homology of the target molecule, concentration of magnesium, other components of the PCR reaction buffer, and the PCR program. In this study, nested PCR was employed to improve the detection sensitivity of the pathogen in the host tissues. The first-round PCR with universal primer set ITS5/ITS4 enriched the ITS fragments both from plant and fungal genomic DNA. A designed specific forward primer com-

Fig. 1. Evaluation of detection sensitivity using nested polymerase chain reaction (PCR) with fungal genomic DNA extracted from a pure *Colletotrichum lindemuthianum* culture in a 10-fold dilution series. Lanes: M = 100-bp DNA ladder (Invitrogen), 1 = 10 ng μl^{-1} , 2 = 1 ng μl^{-1} , 3 = 100 pg μl^{-1} , 4 = 10 pg μl^{-1} , 5 = 1 pg μl^{-1} , 6 = 100 fg μl^{-1} , 7 = 10 fg μl^{-1} , 8 = 1 fg μl^{-1} , 9 = negative control (water replaced genomic DNA as the template).

Fig. 2. Detection of *Colletotrichum lindemuthianum* from infected bean leaves using nested polymerase chain reaction (PCR). Lanes: M = 100-bp DNA ladder (Invitrogen), 1 to 3 = *C. lindemuthianum* infected leaves, 4 = anthracnose-resistant bean genotype G2333, 5 = negative control (water replaced genomic DNA as the template), 6 = positive control (*C. lindemuthianum* DNA).

Fig. 3. Amplification of *Colletotrichum lindemuthianum* DNA from mixed infected and healthy bean seeds powder at different concentrations using nested polymerase chain reaction (PCR). Lanes: M = 100-bp DNA ladder (Invitrogen), 1 = 100%, 2 = 80%, 3 = 60%, 4 = 40%, 5 = 20%, 6 = 10%, 7 = 8%, 8 = 6%, 9 = 4%, 10 = 2%, 11 = 1%, 12 = 0%, 13 = anthracnose-resistant bean genotype G2333, 14 = negative control (water replaced genomic DNA as the template), 15 = positive control (*C. lindemuthianum* DNA).

bined with universal reverse primer was then used to selectively amplify the target pathogen DNA. As little as 10 fg of *C. lindemuthianum* DNA was detected by nested PCR in this study, approximately the equivalent to a single fungal genome (36). Thus, the nested PCR system satisfies both the specificity and sensitivity requirements for diagnostic applications.

The PCR assay system developed in the current study should assist the dry bean seed industry in the production and distribution of anthracnose-free seed. In the past, some difficulties have been experienced with identification of infected bean plants based on symptomology. The use of this new PCR-based assay system will improve anthracnose detection and will reduce unnecessary fungicide application. This improvement in the speed of detection of the anthracnose fungus will promote more timely application of control measures when they are required.

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