Identification of Races of *Colletotrichum lindemuthianum* with the Aid of PCR-Based Molecular Markers

A. G. G. Mesquita, Universidade Federal do Acre, 69915-900, Rio Branco, AC, Brazil; T. J. Paula, Jr., EPA-MIG/BIOAGRO; M. A. Moreira, Dept. de Bioquímica e Biologia Molecular/BIOAGRO; and E. G. de Barros, Dept. de Biologia Geral/BIOAGRO - Universidade Federal de Viçosa, 36571-000, Viçosa, MG, Brazil

ABSTRACT

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Inoculation of a common bean differential series is the usual method for identification of races of *Colletotrichum lindemuthianum*. This procedure is extremely useful for phytopathological as well as breeding purposes, but it requires strict control of the number of spores and incubation conditions. Furthermore, this method may result in misclassifications of isolates because of the subjectivity of symptom evaluation. We propose the use of DNA-based molecular markers as an auxiliary tool to aid the classification of races of *C. lindemuthianum*. Specific DNA bands were identified for races 73, 65, and 64 by polymerase chain reaction (PCR) amplification of bulked DNA samples from isolates of these three races with random primers. The presence of these bands was checked on four isolates previously classified by inoculation on a differential series as belonging to races 23, 72, 79, and 585. The molecular procedure showed that two of these isolates had been misclassified, confirming the high potential of the proposed procedure to aid the identification of races of *C. lindemuthianum*. Amplification products obtained with 44 different primers also allowed the determination of the genetic distances among isolates from races 73, 65, and 64. These data were used to cluster the isolates into three groups that coincide with the ones obtained by inoculation.

Fungal, viral, and bacterial diseases represent a permanent challenge to the common bean (*Phaseolus vulgaris* L.) growers. Angular leaf spot, caused by *Phaeoisariopsis griseola*, rust, caused by *Uromyces appendiculatus*, white mold, caused by *Sclerotinia sclerotiorum*, and anthracnose, caused by *Colletotrichum lindemuthianum*, are among the main fungal diseases affecting the common bean.

C. lindemuthianum attacks susceptible varieties grown under moderate to cold temperatures and high relative humidity (12). Pathogenic variability in *C. lindemuthianum* was first reported in 1911 (3). Since then, several races of this fungus were reported in the literature (15). Initially, only three differential common bean cultivars were used, allowing the identification of eight races of *C. lindemuthianum*. It soon became obvious that the differential series was not large enough to allow the classification of the increasing number of fungus races. In 1988, researchers at CIAT defined a group of 12 common bean differentials to be used internationally and to

facilitate the exchange of information and of resistant germ plasm. At the same time, a binary system of race classification was proposed (4).

Despite this concerted effort to establish a uniform classification system for *C. lindemuthianum* races, several uncertainties still exist in the present classification procedure. For instance, the environment, the number of spores, and incubation conditions used may vary considerably from one laboratory to the other. In addition, the subjectivity during symptom evaluation may lead to misclassifications and to disagreements on classification of the same race by different research groups (20).

DNA-based molecular markers have increased the potential to detect and measure variability among individuals. These markers are almost unlimited in number and are not affected by the environment (22). The distinct patterns obtained for each individual can be taken as specific fingerprints that describe and identify them. Several authors have proposed the use of molecular markers as a fast and safe alternative to differentiate and characterize fungal isolates (2,5-7,9). We propose the use of random amplified polymorphic DNA (RAPD) markers as an auxiliary tool for the classification of races of *C. lindemuthianum*. Toward this goal, we have isolated a set of DNA bands that can specifically identify races 73, 65, and 64 of *C. lindemuthianum*.

MATERIALS AND METHODS

Genetic material. The genotypes of *C. lindemuthianum* used in this work were 11 isolates from race 73, 6 isolates from race 65, and 10 isolates from race 64 (Table 1). Isolates previously classified as belonging to races 23, 72, 79, and 585 were also used. The original cultures were kindly provided by Carlos Rava and Aloisio Sartorato (CNPq/EMBRAPA, Goiânia, GO, Brazil). Monospore cultures were maintained at 4°C in culture tubes containing potato dextrose agar (PDA) medium. Cultures of the 27 isolates from races 73, 65, and 64 were deposited at the Tropical Culture Collection of the Fundação André Tosello, in Campinas, São Paulo, Brazil (E-mail: cristina@bdt.org.br; phone: +55 019 242-7022; fax: +55 019 242-7827).

Identification of races. Race identification was based on procedures defined elsewhere (1,13). For each isolate, 10 seeds from each of the 12 differential common bean varieties (11) were sown on a plastic tray containing sterile soil. After 12 days, the plants were sprayed with 1.2×10^6 conidia per ml with the aid of a DeVilbiss no. 15. The plants were then transferred to a mist chamber ($20 \pm 2^\circ\text{C}$, 95% relative humidity) where they stayed for 7 days. Disease symptoms were evaluated according to an infection scale (16) in which 1 = plants with no visible symptoms; 2 = few isolated small lesions on midveins in the lower leaf surface; 3 = a higher frequency of small lesions on midveins in the lower leaf surface; 4 = lesions in the midvein and occasionally in secondary leaf veins; 5 = many small lesions scattered on mid- and secondary veins; 6 = many small lesions as described in grade 5 in the lower and upper leaf surface and in the stems and petioles; 7 = large lesions scattered over the leaf blade and many lesions in the stems and petioles; 8 = many large, coalesced lesions accompanied by tissue breakdown and chlorotic or abscised leaflets, reduced plant growth, and many lesions in stems and petioles; and 9 = severely diseased or dead. Resistant (R) phenotype was assigned to plants with no or limited symptoms (scores 1 to 3); whereas plants graded 4 or greater were considered to be susceptible (S).

Production of mycelial mass. Mycelium from each of the 27 isolates was plated on petri dishes containing PDA medium and incubated at 22°C for 12 days. Agar plugs taken from the actively grow-

Corresponding author: Everaldo G. de Barros
E-mail: ebarros@mail.ufv.br

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ing margins of the colonies were transferred to Erlenmeyer flasks, each containing 50 ml of potato dextrose broth. The flasks were then incubated under constant agitation at 108 rpm at 24°C in the dark for 8 days. The mycelial mass was filtered through cheesecloth, washed with 0.05 M EDTA, and kept at -80°C for DNA extraction.

DNA extraction and amplification.

DNA extraction was according to Raeder and Broda (14) with the following modification: only chloroform, no phenol, was used in the deproteinization step. DNA samples were quantitated spectrophotometrically at 260 nm (18). The samples were diluted to a final concentration of 10 ng/μl with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and mixed in equal amounts to form three DNA bulks, one for each race. DNA amplification was according to Vilarinhos et al. (20). Initially, the DNA bulks were amplified with 398 primers. Forty-four of them (Table 2) were informative, i.e., they revealed polymorphisms among the three bulks. These primers were used to analyze the individual components of each bulk and to determine pairwise genetic distances among them.

Data analysis. The DNA bands obtained for each bulk or individual were scored based on their presence (1) or absence (0). Only the most intense bands were considered. Pairwise genetic distances were expressed as the complement of Nei and Li's *F* statistic (8). Cluster analysis was done by the nearest neighbor method. All calculations were done with the aid of the program SPSS for Windows, version 5 (21).

RESULTS

To search for primers that could amplify specific bands for races 73, 65, and 64 of *C. lindemuthianum*, 398 arbitrary primers were tested. Forty-four of them (Table 2) revealed polymorphisms among bulked DNA samples from the three races. These primers were used to amplify individual DNA samples from the 27 isolates, and several of these primers revealed bands that were race specific. Figure 1 shows the amplification patterns obtained with primers that were specific for races 73 (OPAT-09), 65 (OPAT-18), and 64 (OPAR-09).

To confirm the usefulness of this approach for the identification of specific races of *C. lindemuthianum*, primer OPAP-08, which is specific for race 73, was used to amplify DNA from isolates previously classified as belonging to races 23, 72, 79, and 585 (Fig. 2). Isolates from races 23 and 79 had distinct amplification patterns from that of race 73. However, the other two isolates presented a DNA amplification pattern similar to that of race 73 and a DNA band characteristic of that race. This was confirmed with another primer (OPAP-16) which is also specific for race 73 (data not shown). For this reason, both

isolates were reinoculated on the differential series. The isolate previously classified as belonging to race 72 gave a compatible reaction with cultivar Michelite. Consequently, it was originally misclassified and

in fact belonged to race 73. The isolate belonging to race 585 gave an incompatible reaction with cultivar TU. Consequently, this isolate also belonged to race 73.

Table 1. Isolates from races 73, 65, 64, 23, 72, 79, and 585 of *Colletotrichum lindemuthianum*, geographic origins, and common bean varieties from which the isolates were obtained

Race	Isolate	Entry number ^a	Common bean variety ^{b,c}	Geographic origin (Brazilian state) ^b
73	1	CCT 6123	RH 5-27	Goiás
	2	CCT 6124	RH 5-08	Goiás
	3	CCT 6125	Capixaba Precoco	Espírito Santo
	4	CCT 6126	Capixaba Precoco	Espírito Santo
	5	CCT 6127	Capixaba Precoco	Espírito Santo
	6	CCT 6128	Capixaba Precoco	Espírito Santo
	7	CCT 6129	Capixaba Precoco	Espírito Santo
	8	CCT 6130	LM 30630	Goiás
	9	CCT 6131	Capixaba Precoco	Espírito Santo
	10	CCT 6132	Rio Tibagi	—
	11	CCT 6120	RH 5-08	Goiás
65	12	CCT 6115	—	Paraná
	13	CCT 6116	—	Paraíba
	14	CCT 6117	Bagajó	Bahia
	15	CCT 6118	Bagajó	Bahia
	16	CCT 6119	Pitoco	Bahia
	17	CCT 6122	FT 120	—
	18	CCT 6121	Capixaba Precoco	Espírito Santo
64	19	CCT 6106	Iguaçu	Rio Grande do Sul
	20	CCT 6107	Carioca	Paraná
	21	CCT 6108	Capixaba Precoco	Espírito Santo
	22	CCT 6109	Capixaba Precoco	Espírito Santo
	23	CCT 6110	Capixaba Precoco	Espírito Santo
	24	CCT 6111	Capixaba Precoco	Espírito Santo
	25	CCT 6112	Capixaba Precoco	Espírito Santo
	26	CCT 6113	Capixaba Precoco	Espírito Santo
	27	CCT 6114	Capixaba Precoco	Espírito Santo
23			Mulatinho	Goiás
72			Capixaba Precoco	Espírito Santo
79			Capixaba Precoco	Espírito Santo
585			Roxinho	Espírito Santo

^a Entry number at the Tropical Culture Collection of the Fundação André Tosello.

^b — = variety or geographic origin unknown.

^c Cultivar Bagajó is Andean; all others are Mesoamerican.

Table 2. List of primers that revealed polymorphisms among races 73, 65, and 64 of *Colletotrichum lindemuthianum*

Primers	Nucleotide sequence (5' → 3')	Primers	Nucleotide sequence (5' → 3')
OPAA-02	GAGACCAGAC	OPAR-06	GTCTACGGCA
OPAA-11	ACCCGACCTG	OPAR-09	TGAGCACGAG
OPAA-18	TGGTCCAGCC	OPAR-15	GGACAACGAG
OPAC-18	TGCGGGGAGA	OPAR-16	CTCTGCGCGT
OPAD-05	ACCGCATGGG	OPAT-09	CACCCCTGAG
OPAG-01	CTACGGCTTC	OPAT-12	GGGTGTGTAG
OPAG-04	GGAGCGTACT	OPAT-15	GGATGCCACT
OPAG-20	TGCGCTCCTC	OPAT-18	GATGCCAGAC
OPAH-01	TCCGCAACCA	OPAV-10	GGACCTGTGT
OPAH-09	ACAACCGAGG	OPAV-11	CTCAGACAGAG
OPAH-11	CTTCCGAGT	OPAV-12	ACCCCCCACT
OPAM-01	GTTGGTGGCT	OPAV-15	AGATCCCGCC
OPAM-02	ACAACGCCTC	OPAV-18	TGGTGGCGTT
OPAM-04	GGCGGTGTGC	OPAW-01	CTCAGTGTGC
OPAM-07	CCGTGACTCA	OPAW-07	CTGGACGTCA
OPAO-02	ACGTAGCGTC	OPAW-08	GACTGCCTCT
OPAO-08	CCTCCAGTGT	OPAW-11	CTGATGCGTG
OPAP-07	GTCCATGCCA	OPAW-14	CTGCTGAGCA
OPAP-09	GTGGTCCGCA	OPAW-16	CAGCCTACCA
OPAP-16	CCAAGCTGCC	OPAX-14	ACAGGTGCTG
OPAQ-12	AGTAGGGCAC	OPN-09	TGCCGGCTTG
OPAQ-20	TCGCCAGTC	OPN-16	AAGCGACCTG

Fig. 1. Electrophoretic analyses of DNA amplification patterns specific for races 73 (A), 65 (B), and 64 (C) of *Colletotrichum lindemuthianum*. Lanes 1 through 27 correspond to the identification of the isolates in Table 1. Stars indicate DNA bands specific for races 73, 65, or 64.

Fig. 2. Electrophoretic analyses of DNA amplification patterns of isolates from races 73, 23, 72, 79, and 585 of *Colletotrichum lindemuthianum*. Stars indicate a DNA band specific for race 73. The first four lanes correspond to isolates 1, 2, 3, and 4 from race 73 (Table 1). The other lanes are labeled with the number of the respective races.

The 44 primers generated a total of 182 DNA bands, which allowed the determination of the genetic distances among all 27 isolates (Fig. 3). The genetic distances varied between 4% (isolate pairs 21/25, 22/25, and 25/27, all from race 64) and 72% (isolate pairs 4/26 and 4/27 from races 73 [isolate 4] and 64 [isolates 26 and 27]). The average distance among all isolates was 39.02%; the average distances within races 73, 65, and 64 were 22.6%, 24.7%, and 12.24%, respectively.

Cluster analyses based on the genetic distances divided the isolates into three

distinct groups, which coincided with their classification based on inoculation on a differential series (Fig. 3).

DISCUSSION

RAPD markers have been used with success for the intraspecific characterization of several plant pathogens (5,6,20,23). Attempts have also been made to distinguish between aggressive and nonaggressive forms of *Phoma lingam* (19), and even to identify specific races of *Fusarium oxysporum* in cotton (2). In this work, we developed a strategy to identify race-specific DNA bands for *C. lindemuthianum*. This

molecular approach can be extremely useful for proper identification of races of this important pathogen. DNA bulks containing samples of at least six isolates from each of three races of *C. lindemuthianum* (races 73, 65, and 64) were PCR amplified with random primers. Some of these primers revealed bands that were specific for each bulk. These data were confirmed by amplification of DNA samples from each individual isolate (Fig. 1). To confirm the usefulness of the identification procedure based on DNA markers, primers specific for race 73 were tested with isolates previously classified by inoculation on the differential series as races 23, 72, 79, and 585. Two of these isolates (races 72 and 585) had been apparently misclassified, as their amplification patterns were similar to those of race 73 (Fig. 2). Indeed, reinoculation of these isolates on the differential series confirmed that they in fact belonged to race 73. At the moment, isolates from the most prevalent races in the state of Minas Gerais, Brazil, such as 89 and 81, are being collected to allow the identification of specific bands to other races of *C. lindemuthianum*. In addition, DNA samples from the 25 races identified in Brazil (17) will be analyzed to confirm if the bands we identified for races 73, 65, and 64 are indeed specific. These DNA bands will be sequenced to allow the construction of longer and more specific primers, which will provide a more reliable and reproducible amplification (10). Our findings confirm the usefulness of the molecular procedure for the identification of races of *C. lindemuthianum*, and also possibly as a diagnostic tool.

The genetic distances among the isolates and the cluster analysis based on the molecular data (Fig. 3) are in accordance with their classification based on inoculation of differential cultivars. Although the number of isolates analyzed in this work is too small and their geographic origin is too limited to make a definitive evolutionary statement, the clustering pattern observed suggests that the evolution of these three races followed a pathway in which gain or loss of a virulence genotype was followed by divergence of the isolates within each new race. It would be extremely interesting to analyze the divergence of isolates from the same race collected in different parts of the world.

Our findings open up the possibility for a rational use of molecular markers to aid the identification of races of *C. lindemuthianum* and suggest that these markers could be used as reliable diagnostic tools.

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Fig. 3. Cluster analyses of 27 isolates of *Colletotrichum lindemuthianum* based on pairwise genetic distances obtained from random amplified polymorphic DNA (RAPD) data. Identification of isolates in Table 1.

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