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Genetic Divergence Among and Within *Colletotrichum lindemuthianum* Races Assessed by RAPD

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ABSTRACT

Genetic divergence within and among races of *Colletotrichum lindemuthianum* was determined using RAPD markers. In addition to the different races of the fungus three isolates of the sexual stage of *Colletotrichum lindemuthianum* (*Glomerella cingulata* f.sp. *phaseoli*) were included in this study. The band patterns generated using 11 primers produced 133 polymorphic bands. The polymorphic bands were used to determine genetic divergence among and within the pathogen races. The isolates analyzed were divided into six groups with 0.75 relative similarity. Group VI, formed by three isolates of the sexual phase of *Colletotrichum lindemuthianum,* was the most divergent. Races previously determined using differential cultivars did not correlate with the results obtained using RAPD markers.

Additional Keywords: *Phaseolus vulgaris*, common bean, DNA, molecular characterization, variability.

RESUMO

Divergência genética entre e dentro de raças de *Colletotrichum lindemuthianum* utilizando RAPD.

A divergência genética entre raças de *Colletotrichum lindemuthianum* foi obtida utilizando marcadores RAPD. Além das diferentes raças deste fungo, foram incluídos neste estudo três isolados da fase sexuada de *Colletotrichum lindemuthianum* (*Glomerella cingulata* f.sp. *phaseoli*). A informação do padrão de bandas geradas utilizando 11 primers, permitiu a identificação de 133 bandas polimórficas. As bandas polimórficas foram utilizadas para determinar a divergência genética entre e dentro de raças do patógeno. Os isolados analisados foram distribuídos em seis grupos com similaridade relativa de 0,75. O grupo VI, formado pelos três isolados da fase sexual do *Colletotrichum lindemuthianum,* foi o mais divergente. As raças agrupadas previamente pelo uso de cultivares diferenciadoras não apresentou qualquer relação com a classificação obtida pelos marcadores RAPD.

Palavras-chave adicionais: *Phaseolus vulgaris*, feijão comum, DNA, caracterização molecular, variabilidade.

INTRODUCTION

Colletotrichum lindemuthianum (Sacc. & Magnus) Scribn., causal agent of anthracnose in common bean (*Phaseolus vulgaris* L.), is widely distributed in bean-producing regions of Minas Gerais State and in other locations in Brazil. Association of the pathogen with various crops and the different environmental conditions found from region to region may lead to the high pathogenic variability which has been observed in this fungus. This diversity may be mostly the result of the pathogen-host co-evolution (Alzate-Marin *et al.*, 1999). A number of races may be found in each producing region, which may hinder the development of cultivar resistance to *C. lindemuthianum* (Rava *et al.*, 1994).

Genetic and biochemical markers can provide new means to study genetic variability in phytopathogenic fungi (Maclean *et al.*, 1993). RAPD markers permit the direct analysis of genome variations without environmental influence. Thus the polymorphisms found among *C. lindemuthianum* isolates detected by RAPD can facilitate studies on genetic diversity within and among races determined by a differential cultivar system (Alzate-Marin *et al.*, 1999). In previous studies using RAPD markers, band patterns generated by 44 primers enabled the determination of the genetic distance between isolates of races 64, 65, and 73. These data grouped these isolates in three groups which were coincident with the results obtained by inoculating the differential cultivars (Mesquita *et al.*, 1998). Other RAPD studies have confirmed the high variability of *C. lindemuthianum* but these data did not correlate with those obtained by inoculation of differential cultivars (Vilarinhos *et al.*, 1995; Alzate Marin *et al.*, 1997; 2001; Thomazella *et al.*, 2002).

Gonzalez *et al.* (1998) used the RAPD technique and detected a slight tendency to group isolates in relation to the geographic origin and did not observe direct correlation between

genotype and races identified by inoculation in the differential cultivars. Similar results were obtained by Sicard *et al.* (1997). However, in another study, the relationship between geographic origin and clustering by this technique was not observed (Fabre *et al.*, 1995). Thus, the present study was carried out to determine if genetic variation found in *C. lindemuthianum,* including isolates of the sexual stage of the pathogen, *Glomerella cingulata* f.sp. *phaseoli* Kimati, can be correlated with race using RAPD markers.

MATERIALS AND METHODS

The study was carried out in the Laboratory of Plant Disease Resistance, in a greenhouse, and in the Molecular Genetics laboratory located in the experimental area of the Department of Biology and the Epidemiology and Management Laboratory of the Plant Pathology Department, both at the Federal University of Lavras (UFLA).

Colletotrichum lindemuthianum race isolates

Isolates of *C. lindemuthianum* obtained from naturally infected common bean cultivars were used in this study. Small pieces of infected plant tissue were surface-sterilized and placed on Petri dishes containing M3 culture medium, and each isolate was purified by single spore isolation technique.

Thirty-one isolates were collected from three regions from Minas Gerais State, Brazil: Coromandel and Patos de Minas (Alto Paranaíba Region); Lavras, Luminárias and Lambari (Sul de Minas Region); and Viçosa (Zona da Mata Region). Four were collected from other States of Brazil: one from Cristalina, Goiás State; one from Pindamonhangaba, São Paulo State; and two from Lapa, Paraná State. Three isolates previously identified and stored in the laboratory were also used, one of race 55 from Embrapa CNPAF (Santo Antonio de Goiás-GO) and two belonging to races 89 and 2047 from the Resistance Laboratory of the Department of Biology at UFLA. Three isolates of the sexual stage of *C. lindemuthianum* (*Glomerella cingulata* f.sp. *phaseoli*) obtained from a single ascospore in plates with colonies of the *C. lindemuthianum* race 73 were also included in this experiment. Therefore, 41 isolates were used in this study (Table 1).

The isolates were races determined by inoculation in the set of 12 differential cultivars proposed by the Latin American Meeting for Anthracnose in the common bean plant, held at the International Center for Tropical Agriculture (Cali, Colombia) (Ciat, 1990). The identified races were assigned a value based on the binary nomenclature system (Habgood, 1970).

DNA extraction and RAPD marker generation

Mycelia for DNA extraction were obtained by inoculating liquid M3 culture medium (Junqueira *et al.*, 1984) and incubating at 20ºC with agitation at 110 RPM rotation until abundant, pale-colored mycelia were obtained (between 5 to 7 days). The mycelia were freeze-dried for 24 hours and stored in a cool, dry

TABLE 1 - Geographic origin and races of isolates of *Colletotrichum lindemuthianum* used in the RAPD

Isolate	Region of the origin	RACE/Nº	Isolate	Region of the origin	RACE/N
1	Sul de Minas	8*1	21	Zona da Mata	81*3
2	EMBRAPA/CNPAF [1]	55*1	22	Zona da Mata	81*4
3	Lapa/PR	64*1	23	Alto Paranaíba	81*5
4	Sul de Minas	64*2	24	Sul de Minas	81*6
5	Zona da Mata	65*1	25	Sul de Minas	81*7
6	Zona da Mata	65*2	26	Zona da Mata	83*1
7	Zona da Mata	65*3	27	Zona da Mata	87*1
8	Zona da Mata	65*4	28	Zona da Mata	87*2
9	Sul de Minas	65*5	29	Alto Paranaíba	87*3
10	Sul de Minas	65*6	30	Sul de Minas	89*1
11	Sul de Minas	65*7	31	DBI/UFLA	89*2
12	Alto Paranaíba	65*8	32	Zona da Mata	337*1
13	Zona da Mata	65*9	33	Zona da Mata	337*2
14	Sul de Minas	65*10	34	Zona da Mata	337*3
15	Sul de Minas	65*11	35	Zona da Mata	337*4
16	Pindamonhangaba/SP	73*1	36	Cristalina/GO	593*1
17	Sul de Minas	73*2	37	DBI/UFLA[2]	2047*1
18	Sul de Minas	73*3	38	Lavras [3]	73*1
19	Alto Paranaíba	81*1	39	Lavras [3]	73*2
20	Sul de Minas	81*2	40	Lavras [3]	73*3
			41	Lapa/PR	0*1

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[3]*Glomerella cingulata* f.sp. *phaseoli.*

environment for later DNA extraction. DNA was extracted according to methodology by Roaeder and Broda (1987) with modifications.

Twenty-one primers from Operon Technologies were used to analyze the genetic variability of the *C. lindemuthianum* isolates. Some primers were designated at random and others were based on related articles, for example: OPAT09 and OPAT18 primers were used by Mesquita *et al.* (1998), and Alzate-Marin *et al.*, (2001) (Table 2).

Each RAPD reaction contained 30ng of DNA, 100 µM of each dNTP; 04 µM primer; 0.6U Taq DNA polymerase; 2.5mM $MgCl_2$; 20mM KCl; 50mM Tris pH 8.3; 250 µg bovine serum albumin/mL; 1% de ficoll 400; 1mM tartrazine and water up to final volume of 16 µL (Hagiwara *et al.,* 2001). Amplification reaction was conducted in a Gradient 5331 Eppendorf Master Cycler thermocycler, programmed for 39 cycles. In the first two cycles, the denaturation was conducted at 94 ºC for 2 minutes, annealing at 37º for 15 seconds, and elongation at 72 ºC for 60 seconds followed by an additional 37 cycles, which differed for denaturation by 15 seconds. A final extension was done for 2 minutes at 72ºC. The RAPD fragments were analyzed by agarose gel electrophoresis at a concentration of 1% agarose in 1X TBE buffer. The bands were visualized under ultra-violet light and photographed with Polaroid 667 film.

Analysis of RAPD markers

Polymorphic bands were confirmed in two replications. They were scored for each individual based on presence or absence. The genetic similarity was estimated by the Nei and Li procedure by the expression $sg_{ij}=2a/(2a+b+c)$, where a= presence of a determined band in the individuals i and j; b= presence of the band in i and absence in j; c= absence of the band in i and presence in j. Cluster analysis was performed using the mean of similarities method (UPGMA) using the NTSYS-PC 2.0 program (Rohlf, 1992).

RESULTS AND DISCUSSION

Eleven of the twenty-one primers used produced band patterns in each of the two replicate reactions (Table 2) and were used to analyze the 41 *C. lindemuthianum* isolates. One hundred and thirty-three polymorphic bands could be identified across all isolates and these were analyzed to determine genetic similarity among isolates and construct the dendogram (Figure 1). The isolates analyzed in this study were divided into six groups with 0.75 relative similarity. Group I was formed by an isolate of the races 8, 64, 83, 593, 55, 0, two isolates from races 73 and 89, three isolates from races 87 and 337, seven isolates from race 81 and seven isolates of race 65. Group II was formed by one isolate of race 2047, group III was formed by one isolate of race 65, group IV by one isolate of races 64, 73 and 337 and two isolates from race 65. Group V was formed by one isolate from race 65 and group VI was formed by three isolates of the sexual stage *G. cingulata* f.sp. *phaseoli* (Figure 1).

The greatest similarity was correlated with the region of origin, regardless of the isolate race. With 0.98 similarity, races 83*1 and 87*1 and races 337*2 and 337*3 were from the Zona da Mata Region. Races 65*4 and 337*4 (0.97 similarity) are also from the Zona da Mata, as were races 81*1 and 81*5 (0.97 similarity) from Alto Paranaíba. Races 81*1 and 81*5 (0.97 similarity) were from the Alto Paranaíba Region, and races 81*6 and 81*7 (0.96 similarity) and 73*2 and 89*1 (0.94 similarity) were from the Sul de Minas region. Group VI was the least similar of all of the other groups (0.28 similarity). The isolates in this group were each isolated from a single ascospore of *Glomerella cingulata* f. sp. *phaseoli*, the sexual stage of *Colletotrichum lindemuthianum*. The lack of similarity of these isolates to others in this study may be due to recombination events during sexual reproduction.

In this study the effect of region of origin seemed to increase as the similarity among the isolates decreased. The interchange of seeds infected with *C. lindemuthianum* among regions may contribute to isolates from different regions grouping together in several specific examples. For example, among races 65*3, 81*3, 83*1 and 87*1 (0.94 similarity) three isolates are from the Zona da Mata and one from the Sul de Minas (81*3), which may indicate the transport of isolates from more distant regions to the Sul de Minas. Other isolates also presented less similarity even when from the same region and race (65*9 and 65*2 with 0.30 similarity).

The lack of correlation between the *C. lindemuthianum* isolate origin and the cluster was observed in the study by Fabre *et al.* (1995) with isolates from Latin America present in all groups. In the pathosystem wheat x *Drechslera tritici-repentis* the RAPD analysis of 12 isolates of this pathogen, from three different regions of Rio Grande do Sul State, also showed the lack of relationship among the genotypes and the region of origin (Santos *et al.*, 2001). Lack of relationship among the groups formed by RAPD and the geographic origin of those isolates may be attributed to the breeding methods, which are based on the introduction and assessment of many lines of different origins. This may

TABLE 2 - Relation of primers used which presented polymorphic and similar band patterns in each of the two replicate reactions

Primers	Polymorphics Bands	Sequences 5’– 3’
OPAA01	10	AGACGGCTCC
OPAA16	11	GGAACCCACA
OPBB12	7	TTCGGCCGAC
OPAT 09	10	CCGTTAGCGT
OPAT18	10	CCAGCT GTGA
OPAT19	14	ACCAAGGCAC
OPZ03	12	CAGCACCGCA
OPZ06	11	GTGCCGTTCA
OPZ07	16	CCAGGAGGAC
OPZ10	13	CCGACAAACC
OPZ19	19	GTGCGAGCAA

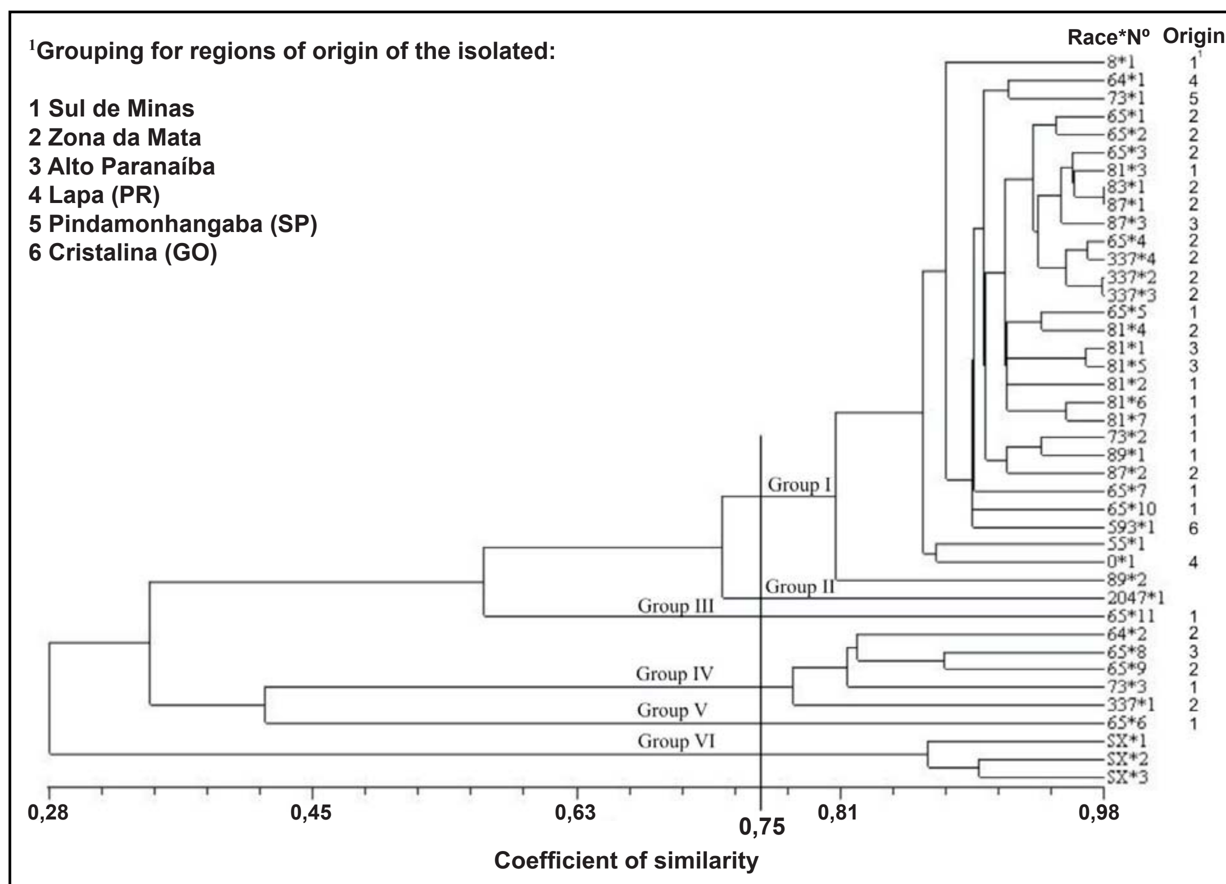

FIG. 1 - Genetic similarity between races of *Colletotrichum lindemuthianum.* SX: *Glomerella cingulata* f.sp. *phaseoli.*

result in the high variability of the pathogen (Alzate-Marin *et al.*, 1997; González *et al.*, 1998).

González *et al.* (1998) suggested a certain tendency to group isolates by geographic origin using RAPD data, but these authors obtained better performance with AFLP data, which showed a clear association between the region of origin and genetic distance. According to the same authors AFLP data did not correlate with race. Both RAPD and AFLP data randomly sample the entire genome and may not sample regions associated with pathogenicity.

Specific bands for single isolates of races 73 and 65 were observed in the study by Mesquita *et al.* (1998) using the OPAT09 and OPAT18 primers, respectively. In the present study, the OPAT09 and OPAT18 primers were tested and specific bands for races 73 and 65 were not observed. Alzate-Marin *et al.* (2001) tested these primers to verify the presence or absence of the specific bands reported and did not find the existence of specific bands either. According to Alzate-Marin *et al.* (2001), the lack of repeatability in the results among the studies may be explained by greater sampling of isolates within the same race when compared to the study by Mesquita *et al.* (1998) that used only a single isolate from each race. Thus sampling within races and geographic locations, as well as the number of races sampled, are critical to the interpretation of results. Completely different results may be obtained when only a few isolates belonging to few races are compared with analyses of larger number of isolates per race. Relationships between a large number of isolates belonging to the same race may also not be comparable to analyses of races represented by a single individual (Alzate-Marin *et al.*, 2001).

Another important finding was the lack of relationship between RAPD clusters and the races identified in the differential cultivars system, corroborating the results obtained by Vilarinhos *et al.* (1995), Thomazella *et al.* (2002), Balardin, (1997) and Alzate-Marin *et al.* (1997, 2001). In these studies, using the same RAPD technique, these authors placed the races in groups different from those defined by the differential cultivars. The amplified portions of the pathogen genome may not have correlated with the avirulence alleles because the primers searched random regions in the genome (Alzate-Marin *et al.*, 1997; Maclean *et al.*, 1993). This fact alone may justify the lack of close relationship between the grouping by RAPD markers and their race based on differential cultivars.

Many factors may be responsible for this finding. *G. cingulata* f.sp *phaseoli* have been observed a few times after attempted crosses in laboratories, but it is difficult for this to occur in nature (Bryson *et al.*, 1992); nevertheless, high variation is observed at their asexual stage. There are reports

of variation occurring in *C. lindemuthianum* monosporic isolates when kept and cultivated in the laboratory (Rocca, 2004). Mutation is a basic source of genetic variation for asexually reproducing fungi (Hastie, 1981). However, observations of nuclear migration between two or more conidia connected by anastomosis may explain some of the variability detected in *C. lindemuthianum*. In this event, many data can be obtained or lost during the asexual cycle by recombination (Roca, 2004). Other factors may be linked to the genetic variability in any given study, such as the number of isolates selected for analysis, the type of organism studied, the selection pressure and migration (Santos *et al.*, 2002). Phytopathogenic organisms are constantly subject to extinction and re-colonization and are rarely found in equilibrium (Peever & Milgroom, 1994). The process of co-evolution resulting from selection pressure exerted by the plant and the pathogen is considered to be a potential mechanism acting on virulence diversity.

The high variability of the *C. lindemuthianum* makes it difficult to breed for anthracnose resistance. The use of genetic resistance as a method to control the disease economically is of great interest. Therefore, information about the variability of the fungus in each region is the basis for resistance breeding programs (Balardin, 1997). The use of pathogenicity and molecular analysis together will lead to a better understanding of the variability present in *C. lindemuthianum*. This information is critical for anthracnose management strategies, selection of resistance genes and development of resistant commercial bean cultivars (Mahuku & Riascos, 2004).

This study permitted the authors to verify that the use of differential cultivars is still of the most importance for checking the variability of the pathogen and classifying its isolates in races within the regions. The pathogenicity and RAPD markers corroborated the broad genetic diversity of *C. lindemuthianum* and can be used as tools to improve the investigation into the fungus variability and, thus, help breeding programs in choice the source of resistance for each region.

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