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Vegetative compatibility and genetic analysis of *Colletotrichum lindemuthianum* isolates from Brazil

Q.L. Barcelos[1], E.A. Souza[2] and K.J. Damasceno e Silva[3]

[1]Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brasil
[2]Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brasil
[3]Embrapa Meio-Norte, Teresina, PI, Brasil

Corresponding author: E.A. Souza
E-mail: easouza@dbi.ufla.br

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ABSTRACT. The causal agent of common bean anthracnose, *Colletotrichum lindemuthianum*, has considerable genetic and pathogenic variability, which makes the development of resistant cultivars difficult. We examined variability within and between Brazilian pathotypes of *C. lindemuthianum* through the identification of vegetative compatibility groups (VCGs) and by RAPD analysis. Two hundred and ninety-five *nit* mutants were obtained from 47 isolates of various pathotypes of the fungus collected from different regions, host cultivars and years. In complementation tests, 45 VCGs were identified. Eighteen RAPD primers were employed in the molecular analyses, producing 111 polymorphic bands. Estimates of genetic similarities, determined from the Sorence-Dice coefficient, ranged from 0.42 to 0.97; the dendrogram obtained by cluster analysis revealed 18 groups of isolates.

RAPD and VCG markers presented high genotypic diversity. The number of significant associations (P = 0.05) between RAPD, VCG and pathogenicity markers ranged from 0 (VCG) to 80% (pathogenicity). The test of multilocus association (r_d) for RAPD markers was significantly different from zero (P < 0.001), suggesting linkage disequilibrium. However, the results for VCG markers show the presence of recombination mechanisms. In conclusion, RAPD markers and VCGs were useful for detecting genetic variability among isolates of *C. lindemuthianum*. We found considerable diversity among isolates from the same geographic origin within a short interval; this suggests rapid evolution. There is a need for further studies to elucidate the population structure of this pathogen in agro-ecosystems.

Key words: Anthracnose; Common bean; RAPD analysis; Vegetative compatibility group; *nit* mutants

INTRODUCTION

The causal agent of anthracnose, one of the most devastating diseases of the common bean (*Phaseolus vulgaris*), is the phytopathogen *Colletotrichum lindemuthianum*. Under suitable environmental conditions, this fungus can cause dramatic losses in crop production. Brazil is the largest producer of dry beans in the world; anthracnose is controlled in this country mainly by cropping cultivars that are genetically resistant to this phytopathogen (Damasceno e Silva et al., 2007). However, durable long-term resistance in *P. vulgaris* has not been achieved so far due to the high diversity in the *C. lindemuthianum* population.

Diversity in the phytopathogen has been demonstrated mainly through the identification of numerous pathotypes, using the binary race classification system proposed by the Centro Internacional de Agricultura Tropical (CIAT, 1990), and by application of molecular (Ansari et al., 2004; Mahuku and Riascos, 2004; Talamini et al., 2006; Damasceno e Silva et al., 2007) and morphological (Souza et al., 2007) markers. Mahuku and Riascos (2004) identified 90 different *C. lindemuthianum* pathotypes amongst 200 isolates originating from Andean and Mesoamerican countries, although pathogenicity and molecular analyses revealed no association between the isolates and the genomes of the host cultivars. A group of 30 pathotypes was identified in cultivar pathogenicity tests of 74 isolates of *C. lindemuthianum* collected from Central and South American, European and African countries (Ansari et al., 2004). Using amplified fragment length polymorphism analyses, these authors demonstrated an association between the genetic diversity of the fungal isolates and the country of origin, while no association was found between genetic group and pathotype classification.

Pathogenicity studies conducted in Brazil have resulted in the identification of more than 50 pathotypes of *C. lindemuthianum*, the most common of which are 65, 73 and 81 (Rava et al., 1994; Balardin et al., 1997; Sartorato, 2002; Talamini et al., 2006; Damasceno e Silva et al., 2007). Randomly amplified polymorphic DNA (RAPD) analyses

revealed that intra-regional molecular diversity amongst these pathotypes is greater than inter-regional variability (Talamini et al., 2006; Damasceno e Silva et al., 2007).

An alternative technique for studying genetic diversity in populations of *C. lindemuthianum* involves the identification of vegetative compatible groups (VCGs) during the asexual reproductive phase of the fungus, since such groups can exchange genetic information via heterokaryosis (Brooker et al., 1991). Hyphae that are capable of anastomosis to form stable heterokaryons are considered to be vegetatively compatible; thus, isolates that form heterokaryons are often included in the same VCG (Leslie and Summerell, 2006).

In order to characterize and classify fungal isolates according to VCGs, it is necessary to develop mutant strains that are both resistant to chlorate and unable to use nitrate as the sole source of nitrogen, the so-called "nitrate non-utilising" or *nit* mutants. The characterization of VCGs has been employed successfully in various species of *Colletotrichum* (Brooker et al., 1991; Skroch et al., 1992; Wasilwa et al., 1993; Correll et al., 1993; Chacko et al., 1994; Beynon et al., 1995; Gichuru et al., 2000; Nitzan et al., 2002; Varzea et al., 2002; Abang et al., 2004; Pereira, 2005), and nitrate non-utilizing mutants have been used to demonstrate heterokaryosis and parasexual segregation in *C. lindemuthianum* isolates (Castro-Prado et al., 2007). However, little could be inferred about vegetative compatibility in this species, because the *nit* mutants were obtained from only five isolates. Therefore, there are no reports concerning the application of such mutants in the classification of populations of *C. lindemuthianum*.

Anastomosis formation among hyphae of different isolates of *C. lindemuthianum* has been evaluated (Rodríguez-Guerra et al., 2003; Ishikawa et al., 2008), allowing classification into several anastomosis groups. Anastomosis is, however, only the first step in the formation of a heterokaryon, since heterokaryotic cells formed by the fusion of isolates that are not in the same vegetative compatibility group either grow at a very much reduced rate or undergo apoptosis and cell death. The vegetative compatibility group status of an isolate depends on a number of genes, known as *vic* genes, involved in vegetative incompatibility control. Detailed information concerning vegetative compatibility group could be obtained by complementation with auxotrophic *nit* mutants, a technique that can provide unambiguous proof of compatibility.

We classified *C. lindemuthianum* isolates into distinct VCGs in order to determine the association between the various groups and pathogenicity, and examined the genetic similarities of the isolates by using RAPD markers.

MATERIAL AND METHODS

Origin of *C. lindemuthianum* isolates

Forty-seven *C. lindemuthianum* isolates belonging to 13 different pathotypes were collected from naturally infected bean cultivars produced in various regions of Brazil from 2000 to 2006 (Table 1). Small sections of infected plant tissue (leaves, stems and pods) were surface-sterilized, placed onto Petri dishes (90 mm in diameter) containing M3 culture medium (Junqueira et al., 1984) and incubated in the dark at 22°C. Each isolate was purified using the single-spore isolation technique.

Table 1. Descriptions of the isolates of *Colletotrichum lindemuthianum*.

	Isolate	Pathotype	Origin	Year		Isolate	Pathotype	Origin	Year
1	LV5	81	Lavras (MG)	-	25	LV67	321	Pinhão (PR)	2004
2	LV11	81	Viçosa (MG)	2001	26	LV68	81	Lavras (MG)	2004
3	LV13	81	Viçosa (MG)	2001	27	LV69	81	Lambari (MG)	2004
4	LV17	81	Viçosa (MG)	2001	28	LV70	81	Viçosa (MG)	2003
5	LV18	87	-	-	29	LV72	81	Lambari (MG)	2004
6	LV24	64	-	-	30	LV73	65	Ijaci (MG)	2004
7	LV28	65	Lavras (MG)	2001	31	LV75	81	Heliodora (MG)	2003
8	LV29	65	Lavras (MG)	2001	32	LV76	73	Lavras (MG)	-
9	LV32	73	-	2002	33	LV77	81	Lavras (MG)	-
10	LV33	337	Viçosa (MG)	2002	34	LV80	65	Lavras (MG)	2004
11	LV43	81	-	2002	35	LV84	73	Lavras (MG)	-
12	LV44	81	-	2002	36	LV86	337	Lavras (MG)	2005
13	LV46	81	Lambari (MG)	2002	37	LV88	337	Lavras (MG)	2005
14	LV48	65	Coromandel (MG)	2002	38	LV89	65	Lambari (MG)	2005
15	LV49	81	-	-	39	LV90	65	Ijaci (MG)	2005
16	LV50	72	Lavras (MG)	-	40	LV94	65	Lambari (MG)	2005
17	LV51	73	Lavras (MG)	2004	41	LV95	81	Guarapuava (PR)	2006
18	LV54	81	Lambari (MG)	2004	42	LV96	71	Campinas (SP)	2006
19	LV55	65	Ijaci (MG)	2004	43	LV97	64	Campinas (SP)	2006
20	LV57	65	Lambari (MG)	2004	44	LV99	73	Lambari (MG)	2006
21	LV58	65	Nepomuceno (MG)	2004	45	LV100	-	Campinas (SP)	2006
22	LV59	65	Ijaci (MG)	2004	46	LV101	8	Turvo (PR)	2006
23	LV61	65	Ijaci (MG)	2004	47	CL837*	65	Buritis (MG)	2000
24	LV66	89	-	-					

*Provided by EMBRAPA Arroz e Feijão (State of Goiás, Brazil); (-) = information not available; MG = Minas Gerais; PR = Paraná; SP = São Paulo (States of Brazil).

Isolation and characterisation of *nit* mutants

The methods described by Brooker et al. (1991) were applied to the development of *nit* mutants. Briefly, mycelial plugs (3 mm in diameter) were removed from the edges of the colonies and transferred to Petri dishes (60 mm in diameter) containing minimal medium (MM) supplemented with potassium chlorate at concentrations of 15, 20 or 25 g/L, with L-asparagine (1.6 g/L) or L-threonine (1.4 g/L) (Klittich and Leslie, 1989). For each isolate, 10 mycelial plugs were incubated under the conditions described above and the colonies were examined weekly in order to detect formation of dense sectors. Such sectors were transferred to basal medium (BM) supplemented with sodium nitrate at a concentration of 2 g/L. Isolates presenting poor mycelial growth on this medium were considered to be *nit* mutants, while isolates presenting robust growth, similar to that of the wild-type cultures grown on MM + chlorate, were discarded (Leslie and Summerell, 2006).

Following identification, the *nit* mutants were transferred to Petri dishes containing BM separately supplemented with the nitrogen sources ammonium tartrate (1.0 g/L), sodium nitrite (0.5 or 0.4 g/L) or hypoxanthine (0.5 g/L). Plates were incubated under the conditions described above for seven days and mutants were characterized phenotypi-

cally and classified as *nit1*, *nit2*, *nit3*, or *nitM* according to their growth parameters. BM media containing ammonium or nitrate salts were used as positive and negative controls, respectively (Leslie and Summerell, 2006).

Vegetative complementation tests

The vegetative self-compatibilities of different *nit* mutants of a single isolate and the cross-compatibilities between *nit* mutants of different isolates were tested by pairing mycelium plugs in order to determine the capacity for anastomosis (Leslie and Summerell, 2006). Forty-six isolates produced *nit3* mutants; these were paired against a *nit1* mutant from each of the seven isolates that produced both *nit1* and *nit3* mutants and also against the *nitM* mutant derived from the remaining isolate. A mycelial plug of a *nit1* (or *nitM*) mutant was placed at the center of a Petri dish (90 mm in diameter) containing BM + nitrate, and four mycelial plugs of *nit3* mutants were arranged around the edge of the dish at a distance of 1 cm from the central plug. Each combination of mutants was repeated twice, and the dishes were incubated under the conditions described above for at least four weeks. Growth of aerial mycelia in the contact zone between the two colonies and formation of heterokaryons were monitored weekly. Paired isolates that exhibited dense aerial mycelia at the intersection between colonies of *nit3* and *nit1* (or *nitM*) mutants of the other isolate were included in the same VCG, while paired isolates that exhibited sparse growth at these intersections were included in different VCGs. Positive controls presented robust growth of aerial mycelia at the intersection between *nit3* and *nit1* (or *nitM*).

DNA extraction and RAPD analysis

Mycelia from each of the 47 isolates were placed onto Petri dishes containing M3 medium (Junqueira et al., 1984) and incubated as described above for 12 days. Agar plugs taken from the actively growing margins of the colonies were transferred to Erlenmeyer flasks containing 125 mL M3 liquid medium and incubated for seven days under constant agitation (110 rpm) in the dark at 20°C. The mycelial mass was filtered through cheesecloth, washed with 0.05 M EDTA, and stored at -20°C until required. DNA was extracted from the isolates according to a modified version of the method of Raeder and Broda (1985), except for seven isolates that failed to grow in liquid medium and could not be included in the analyses.

RAPD reactions were carried out using 17 arbitrary primers (Table 2; Operon Technologies, Alameda, CA, USA). Amplification reactions were performed using an Eppendorf (Hamburg, Germany) MasterCycler Gradient 5331 in a total volume of 12 µL containing 4.49 µL water, 25 ng genomic DNA, 50 µM of each dNTP, 0.4 µM of each oligonucleotide primer, 50 mM Tris-HCl, pH 8.0, 2.0 mM $MgCl_2$, 20 mM KCl and 0.4 units Taq DNA polymerase. The amplification program consisted of one initial denaturation cycle of 2 min at 94°C, followed by 40 cycles of 2 min at 94°C, 15 s at 37°C and 1 min at 72°C, and a final extension step of 2 min at 72°C. Amplicons were separated by electrophoresis on 1% agarose gels, bands were visualized under UV light using a Fotodyne (New Berlin, WI, USA) ultraviolet trans-illuminator and photographed with an Eastman Kodak (Rochester, NY, USA) EDA -290 camera.

Table 2. Primers used in RAPD analysis (Operon Technologies, Alameda, CA, USA).

Primers	Sequences	Primers	Sequences
OPAQ12	5'CAGCTCCTGT3'	OPAS15	5'CTGCAATGGG3'
OPAR06	5'TGGGGCTCAA3'	OPAS17	5'AGTTCCGCGA3'
OPAR12	5'GGATCGTCGG3'	OPAS18	5'GTTGCGCAGT3'
OPAS03	5'ACGGTTCCAAC3'	OPAT08	5'TCCTCGTGGG3'
OPAS06	5'GGCGCGTTAG3'	OPBB07	5'GAAGGCTGGG3'
OPAS08	5'GGCTGCCAGT3'	OPBB09	5'AGGCCGGTCA3'
OPAS10	5'CCCGTCTACC3'	OPBB10	5'ACTTGCCTGG3'
OPAS12	5'TGACCAGGCA3'	OPBB16	5'TCGGCACCGT3'
OPAS14	5'TCGCAGCGTT3'		

Location within the manuscript: Material and Methods, subsection "DNA extraction and RAPD analysis".

Data analysis

Each individual isolate was scored according to the presence (1) or absence (0) of DNA amplicons; only polymorphic bands were considered. The genetic similarity between isolates *i* and *j* (sg_{ij}) was estimated from the Sorensen-Dice coefficient as given by the expression $sg_{ij} = 2a / (2a + b + c)$, where *a* represents the presence of a determined band in *i* and *j*, *b* represents the presence of the band in *i* and the absence in *j*, and *c* represents the absence of the band in *i* and the presence in *j*. A dendrogram was produced from the similarity matrix thus generated, using the unweighted pair-group method with arithmetic means (UPGMA) with the assistance of the NTSYS-PC 2.1 software (Rohlf, 2000). The errors associated with each similarity were estimated according to the following expression modified from Skroch et al. (1992):

$$\text{estimated standard error } (s_{sg}) = \sqrt{sg_{ij}\frac{1-sg_{ij}}{n-1}} \qquad \text{(Equation 1)}$$

where *n* is the sum of *a*, *b* and *c* for each isolate pair. Genetically different isolates were identified in the dendrogram on the basis of the estimated maximum significant similarity value (sg_m), which was determined from:

$$sg_m = 1 - (t \times \bar{s}_{sg}) \qquad \text{(Equation 2)}$$

where *t* is the tabulated value of the Student *t* distribution at the 1% level of probability with *n* - 2 degrees of freedom, and $\bar{s}_{sg}$ is the mean error of the comparisons considered in the dendrogram.

The exact test of digenic linkage disequilibrium for haploid data, which is an extension of the Fisher exact test for contingency tables (Slatkin, 1994), was applied using the Arlequin software version 2.0 (Schneider et al., 1997). RAPD locus, VCG and pathogenicity were used to test the genetic differentiation for each nonrandom association among pairs of these different markers. A pathotype was identified as a combination of virulence factors of a strain on the set of 12 differentials.

The MULTILOCUS software V2.1 (2003) was used to investigate multilocus linkage disequilibrium. The index of association (I_A) (Brown et al., 1980; Smith et al., 1993), which tests the importance of clonal or sexual reproduction within each population, was calculated. The I_A is calculated by measuring the distances between all pairs of loci, and comparing them

to the expected value (zero), assuming no linkage disequilibrium. Unfortunately I_A is sensitive to the number of loci sampled, hence we also used the linkage disequilibrium measure (r_d) developed by Agapow and Burt (2001), which is independent of the number of loci sampled, and the significance of which is also tested by its deviation from the expected value (zero), assuming no linkage disequilibrium. The r_d value was calculated and 1000 randomizations were performed. RAPD haplotypes were used for multilocus analysis and a haplotype was defined as a combination of RAPD alleles at all polymorphic loci.

RESULTS

Generation of *nit* mutants

A number of isolates of *C. lindemuthianum* formed dense sectors when cultivated on MM + chlorate, although the frequency of formation varied between isolates, with some presenting a single sector per colony and others forming two or more sectors per colony (Figure 1). Sectors could be observed after three weeks in culture, although most emerged only after five to six weeks of incubation. The frequency of formation of chlorate-resistant mutants was greater in MM supplemented with 20 g/L than with 15 g/L potassium chlorate, while a concentration of 25 g/L of the salt was highly toxic for some isolates and completely inhibited the growth of mycelia. Chlorate-resistant mutants were transferred to BM + nitrate, and those that presented poor mycelial growth were considered to be *nit* mutants.

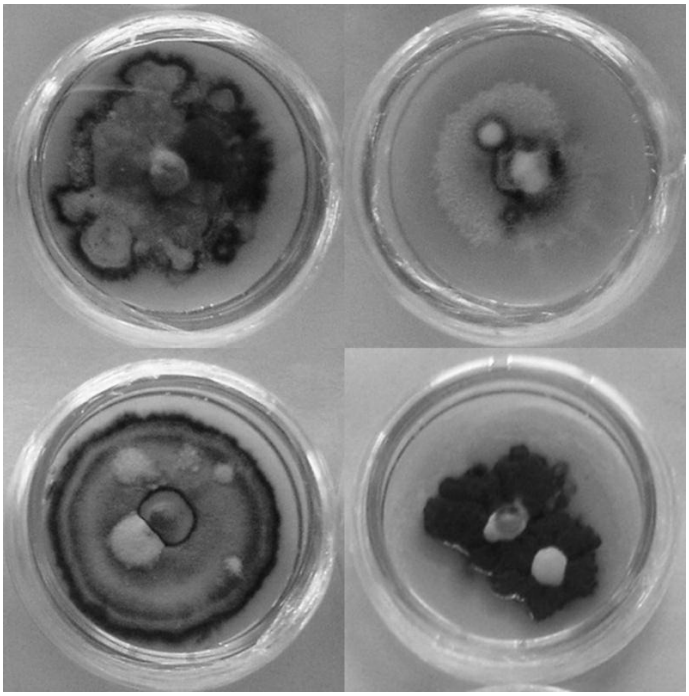

Figure 1. Formation of chlorate-resistant sectors in *Colletotrichum lindemuthianum* cultured on minimal medium containing potassium chlorate.

The 295 *nit* mutants recovered from the 47 *C. lindemuthianum* isolates were incubated separately on selective medium (BM + tartrate, nitrite or hypoxanthine), and 279 *nit3* mutants were identified, together with 15 *nit1* mutants and one *nitM* mutant. Seven of the 47 isolates of *C. lindemuthianum* produced *nit1* and *nit3* mutants, 39 isolates produced only *nit3* mutants and one isolate produced a single *nitM* mutant. A large number of mutants were initially classified as *nit3* when BM + 0.5 g/L sodium nitrite was employed as the selective medium, but when the concentration of the salt was reduced to 0.4 g/L, four *nit3* mutants changed phenotype to *nit1*. Supplementation of MM + chlorate with the amino acid L-threonine did not significantly increase the number of *nitM* mutants formed.

Vegetative compatibility

Six of the 47 *C. lindemuthianum* isolates were observed to be self-incompatible since the development of aerial mycelia and the formation of heterokaryons were not observed at the line of intersection between colonies of *nit3* and *nit1* mutants derived from the same isolate. One isolate (LV97), however, exhibited weak self-compatibility, as shown by the sparse mycelium growth at the intersection between *nit* mutants.

The results of cross-compatibility tests allowed classification of the *C. lindemuthianum* population into 45 separate VCGs, among which three isolates (LV13, LV68 and LV48) were grouped into one VCG, while the other isolates were incompatible and constituted 44 different VCGs. A strong cross-complementation was observed between *nit3* mutants derived from isolates LV13 (C) and LV68 (D) (Figure 2). The dense line of growth corresponds to the formation of heterokaryons. In contrast, only weak complementation was observed between the isolate pairs LV70/LV51, LV68/LV51 and LV97/LV73, hence each isolate was classified in a separate VCG. For most of the complementing isolates, the formation of heterokaryons could be observed only after the second week of incubation, but in the case of isolates LV13 and LV68, heterokaryons appeared within the first week. No complementation was observed between *nit3* and *nitM* mutants.

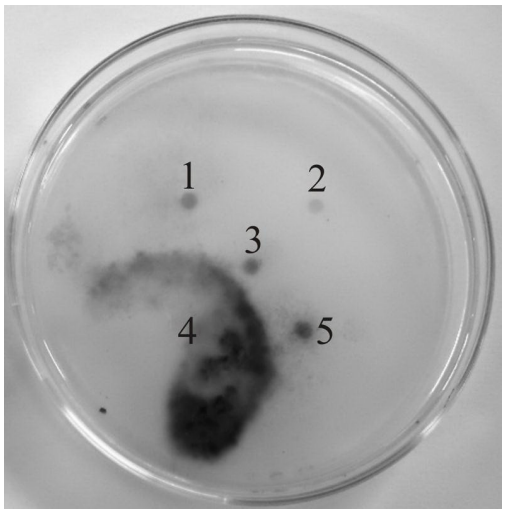

Figure 2. Compatibility assays between *nit* mutants: the mycelial plug (**3**) at the center of the plate is a *nit1* mutant from isolate LV48 (**1**) and the four *nit3* mutants on the periphery of the plate are from isolates LV48 (**1**), LV69 (**2**), LV13 (**4**), and LV68 (**5**). The dense line of growth corresponds to the formation of heterokaryons.

Genetic similarity

In order to establish genetic similarities between isolates of *C. lindemuthianum* through the analysis of RAPD markers, 111 polymorphic bands were analyzed; an average of 6.2 bands were generated by each primer. The coefficients of genetic similarity within the fungal population varied between 0.42 and 0.96, with a cut-off point of 0.889 (Figure 3). Based on the dendrogram, the *C. lindemuthianum* population could be grouped into 18 genetically similar clusters. Some associations were observed between pathogenicity, RAPD groups and geographic origin. Group II (Figure 3), including isolates LV44, LV49 and LV77, belongs to pathotype 81. Isolates LV17 and LV70, which also belong to pathotype 81, were collected in the same geographic region and were grouped together (group III, Figure 3). Both isolates from group XI (LV32 and LV51) and group XII (LV76 and LV84) belong to pathotype 73 and were collected in Lavras, MG. An association was observed between RAPD groups and VCG, when the isolates LV13 and LV48 were grouped together according to genetic similarity; they were also included in the same VCG. Additionally, isolates LV28, LV29, LV80, LV89, LV90, LV95, LV54, LV59, LV61, LV50, and LV43 were classified in separate genetic groups and in distinct VCGs; most of these isolates belong to pathotype 65, showing intra-specific genetic variation.

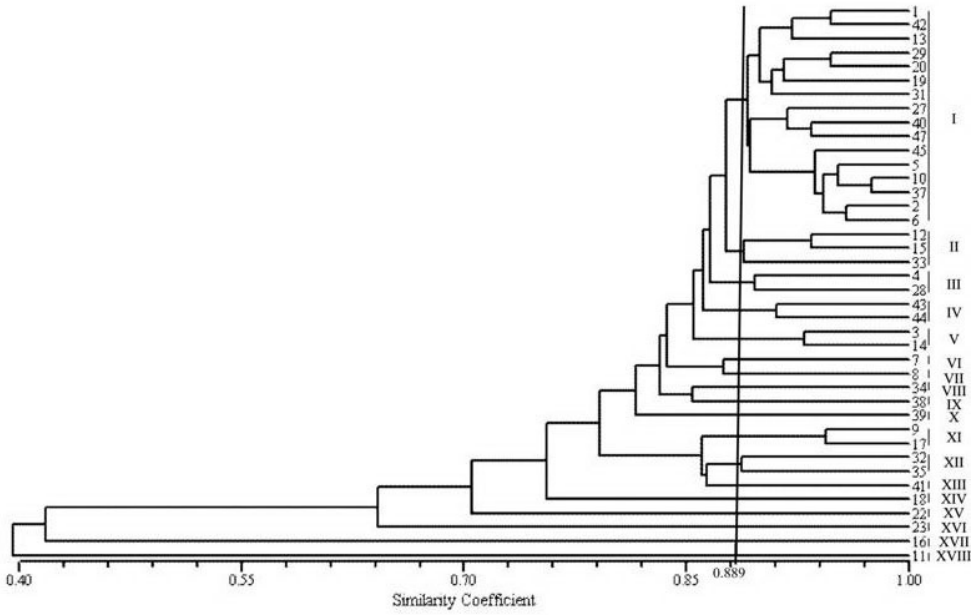

Figure 3. UPGMA analysis of population data showing the relationship between the 40 isolates of *Colletotrichum lindemuthianum*.

Linkage disequilibrium

The number of polymorphic loci scored was 101 for the RAPD markers, six for pathogenicity markers and 39 for VCG markers. The number of significant (P = 0.05) associations between RAPD, VCG and pathogenicity markers ranged from 0 (VCG) to 80% (pathogenicity)

(Table 3). All multilocus genotypes occurred only once. The multilocus association (I_A and r_d) for RAPD markers was significantly different from zero ($P < 0.001$), confirming that there is linkage disequilibrium in this population. The I_A and r_d values observed were 10.71 and 0.10, respectively.

Table 3. Number and percentage of significant nonrandom associations between pairs of random-amplified polymorphic DNA (RADP), pathogenicity and vegetative compatibility group (VCG) markers in *Colletotrichum lindemuthianum*.

Marker	Nº of significant[a]/No. of calculated combinations		VCG
	RAPD	Pathogenicity	
RAPD	3020/5050 (60%)	164/606 (27%)	946/3838 (25%)
Pathogenicity	-	12/15 (80%)	148/228 (65%)
VCG	-	-	0/703 (0%)

[a]$P < 0.05$.

DISCUSSION

The high frequency of *nit3* mutant that we observed was also found in previous research on *Colletotrichum* species (Brooker et al., 1991; Beynon et al., 1995), although some studies reported a predominance of *nit1* mutants (Nitzan et al., 2002; Pereira, 2005; Fávaro et al., 2007). The recovery of *nit* phenotypes (especially *nitM*) from *Colletotrichum* species is clearly not straightforward and sometimes is not possible (Beynon et al., 1995; Varzea et al., 2002).

Self-incompatible isolates were observed within the *C. lindemuthianum* population; this phenomenon had already been described for other *Colletotrichum* species (Brooker et al., 1991; Wasilwa et al., 1993; Beynon et al., 1995; Varzea et al., 2002; Pereira, 2005). Incompatibility between most of the *C. lindemuthianum* isolates may be explained on the basis of the observed self-incompatibility, since such isolates do not normally form heterokaryons with other isolates (Leslie, 1993). In *Aspergillus flavus*, Papa (1986) suggested that the lack of complementation observed between some isolates could be due to a double mutation in some of the *nit* mutants. Another explanation stated that the lack of complementation resulted from the inability of the isolates to form hyphal anastomoses (Correl et al., 1993), a deficiency also detected in *C. lindemuthianum* (Ishikawa et al., 2008). In the latter case, it was shown that some isolates of *C. lindemuthianum* (namely, LV58, LV61 and LV73) could be classified into the same anastomosis group, but they belonged to different groups based on the criterion of genetic similarity in RAPD analysis. Although these isolates were genetically different they had the ability to undergo anastomosis, which is the first step in the parasexual cycle. In our study, however, isolates LV58, LV61 and LV73 were included in different VCGs, suggesting that the alleles controlling pre-fusion events are similar, while the alleles controlling vegetative compatibility are different. Thus, although these isolates were able to form anastomoses, they could not form heterokaryons. Since isolate LV73 was self-incompatible, the alteration must have occurred in the *het* genes controlling self- and non-self recognition (Leslie and Summerell, 2006).

Although the sexual structures of *C. lindemuthianum* have not been found in nature (Mahuku and Riascos, 2004), the sexual form has been observed frequently in the laboratory,

from lesions isolated from common bean fields in Brazil (Camargo Jr. et al., 2007; Damasceno e Silva et al., 2007). Moreover, the organization and genetic diversity of this species is more comparable with populations that are derived sexually than from asexual reproduction (Brygoo et al., 1998). If a sexual phase occurs, considerable genetic variability, together with the formation of various VCGs, may be expected, since the possible combinations between *vic* loci are numerous (Leslie, 1993).

In our study, RAPD markers and VCGs were useful for detecting high genetic variability among isolates of *C. lindemuthianum*. Defining the genetic structure of populations is a logical first step in studies of fungal population genetics because the genetic structure of a population reflects its evolutionary history and its potential to evolve (McDonald, 1997). The results demonstrate considerable diversity within isolates from the same geographic origin within a short time, and this suggests rapid evolution. Capelle and Neema (2005) showed local adaptation at the scale of the individual common bean plant and indicated that fine-scale dynamics has evolutionary consequences in this pathosystem. However, the origin of these patterns could involve weak dispersal ability of the spores, founder effects, greater evolutionary potential of the fungus, and/or selection pressure by the resistance genes of the host. Moreover, the common bean is grown during three seasons in Brazil; therefore, the pathogen populations evolve, adapting to constant changes in environmental conditions.

In our study, the digenic test for linkage disequilibrium showed a low to high percentage (0-80%) of pair-wise comparisons in disequilibrium (Table 3). The high level of linkage disequilibrium (80%) for pathogenicity markers may be due to the large number of isolates belonging to the same pathotype and the small number of different pathotypes. Moreover, this trait is under selection mainly by host-resistant genes, which leads to linkage disequilibrium. VCG markers showed lack of linkage disequilibrium (0%), and this suggests that recombination is occurring. RAPD markers showed high level of linkage disequilibrium (60%). However, the r_d value was low (considering an r_d value of 1.0 = clonal, and 0 = no linkage) confirming that it is possible that a level of recombination occurs in this pathogen. Actually, there is evidence of asexual recombination through conidial anastomosis tube fusion (Roca et al., 2004) and of the parasexual cycle (Castro-Prado et al., 2007) in *C. lindemuthianum*. Moreover, there are reports of sexual reproduction in this pathogen in Brazil (Damasceno e Silva et al., 2007; Camargo Jr. et al., 2007; Souza et al., 2007). There are some hypotheses that try to explain this high level of linkage disequilibrium. RAPD is a selectively neutral marker, and a sporadic sexual cycle followed by several generations of asexual reproduction would lead to the linkage disequilibrium. Another explanation could be a reminiscent linkage disequilibrium caused by a founder effect because the main inoculum source of *C. lindemuthianum* is seed-born contamination; also, there is weak dispersal ability of the spores in the field.

The level and distribution of genetic variability within and between populations of plant pathogens provide an indication of the adaptability of a pathogen in overcoming the effects of artificial and natural stresses on the population. Such genetic changes can neutralize the measures employed to control the pathogen, such as application of fungicides and use of genetically resistant cultivars (McDonald et al., 1989; Adachi et al., 1993; McDonald and Linde, 2002). We affirm the difficulties incurred in achieving long-term resistance against anthracnose in the common bean and emphasize the need for studies to elucidate the population structure of this pathogen in agro-ecosystems.

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