Sexual recombination in *Colletotrichum lindemuthianum* occurs on a fine scale

E.A. Souza[1], O.A. Camargo Jr.[2] and J.M.A. Pinto[1]

[1]Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brasil
[2]Departamento de Agricultura, Universidade Estadual do Centro-Oeste do Paraná, Guarapuava, PR, Brasil

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

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ABSTRACT. *Glomerella cingulata* f. sp *phaseoli* is the sexual phase of the fungus *Colletotrichum lindemuthianum*, the causal agent of common bean anthracnose. This fungus is of great concern, because it causes large economic losses in common bean crops. RAPD markers of five populations of *G. cingulata* f. sp *phaseoli* from two Brazilian states were analyzed to determine if this population possesses the sexual reproductive potential to generate the genetic variation that is observed in this phytopathogen. We identified 128 polymorphic bands, amplified by 28 random primers. The estimates of genetic similarity in this analysis ranged from 0.43 to 1.00, and the dendrogram generated from analysis of all genotypes displayed five principal groups, coinciding with the five populations. Genetic differentiation was observed between the populations (G_{ST} = 0.6455); 69% of the overall observed genetic variation was between individual populations and 31% of the variance was within the sub-populations. We identified significant levels of linkage disequilibrium in all populations. However, the values of the disequilibrium ranged from low to moderate, indicating that this pathogen maintains a genetic structure consistent with sexual reproduction. The mean contribution of sexual reproduction was determined by comparison of the amplitudes of genetic similarity of isolates from sexual and asexual phases. These results support the

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hypothesis that recombination plays an important role in determining the amplitude of variability in this pathogen population and that this determination occurs on a fine scale.

Key words: Anthracnose; Common bean; RAPD markers; Genetic variability

INTRODUCTION

The common bean (*Phaseolus vulgaris*) is a species of great agronomic interest, especially in tropical and subtropical developing countries. With 26.9 million hectares devoted to this crop, Brazil is the world's largest common bean producer (Paula Junior et al., 2008). Diseases are responsible for significant yield losses, and anthracnose is one of the most severe diseases of this crop, causing losses of up to 100% when contaminated seeds are sown under conditions that favor disease development (Silva et al., 2007).

The fungus *Colletotrichum lindemuthianum*, the causal agent of anthracnose of the common bean, has been found in many countries in the Americas, Europe, Africa, and Asia; it has considerable pathogenic variability. More than 100 pathotypes of this fungus have been described (Silva et al., 2007). One of the most common and least expensive strategies employed to control anthracnose is the use of cultivars carrying resistance alleles. The success of adopting resistance as a disease control strategy depends on knowledge of the level of variability between and within populations of the pathogen. Information about the processes responsible for high population variability is particularly important for this effort.

It is likely that various separate and simultaneous mechanisms are involved in the amplification of genetic variability of this pathogen. Sexual recombination is an important part of this process (Rodríguez-Guerra et al., 2005; Camargo Jr. et al., 2007). Relevant research has been done on the parasexual cycle (Roca et al., 2003), mutation (Tu, 1992) and transposons (Kistler and Miao, 1992) in this pathogen. Studies on the population structures of both host and pathogen, their respective recombination systems and their interactions in space and time can provide relevant information about the epidemiology of this disease, the relative importance of each mechanism responsible for generating variability and the process of pathogen-host co-evolution (Bock et al., 2005).

The pathogen *Glomerella cingulata* f. sp *phaseoli* (the sexual phase of the fungus *C. lindemuthianum*), first described by Shear and Wood (1913), has been identified in field isolates from Brazil (Camargo Jr. et al., 2007). Sexual compatibility studies performed in the laboratory have revealed both homothallic and heterothallic behaviors in different isolates of *G. cingulata* f. sp *phaseoli* (Mendes-Costa, 1996; Rodríguez-Guerra et al., 2005; Camargo Jr. et al., 2007).

Studies of the population structure of *C. lindemuthianum* have demonstrated that although reproductive processes in this species are predominantly asexual, the mating patterns of the populations reflect those of random species. In other words, the populations exhibit gametic equilibrium, suggesting that at some points during the host cycle, the pathogen undergoes sexual reproduction (Rodríguez-Guerra et al., 2003). Sexual reproduction allows for the generation of new combinations of alleles in each recombination cycle, leading to a high level of genetic diversity in the pathogen's population. Consequently, periodic occurrence of sexual reproduction can explain much of the variability found in *C. lindemuthianum* populations.

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We evaluated the genetic diversity both within and between populations of *G. cingulata* f. sp *phaseoli* isolated from Brazil by random amplified polymorphic DNA (RAPD) analyses. Our objective was to examine the potential for sexual reproduction in this species to generate genetic variability.

MATERIAL AND METHODS

Collection and maintenance of the isolates and procurement of the monoascosporic cultures

Five populations of *G. cingulata* f. sp *phaseoli* were isolated from stems, leaves and pod lesions of the common bean (*P. vulgaris*). All isolates were obtained without the use of inducers. Material was collected during each of the three seasons of the common bean crop from both experimental and commercial production fields. Three populations were sampled in the State of Minas Gerais, two populations in Lavras (Valente and Majestoso) and one in Lambari (VCU); two populations were sampled in the State of Paraná, Guarapuava and Turvo. The isolates were maintained in M_3 media (Junqueira et al., 1984). The methodology described by Camargo Jr. et al. (2007) was used to obtain monoascosporic cultures. Forty random ascospores were isolated from each population, and the cultures were grown in an incubator at a controlled temperature (21°C) and under controlled light conditions (a 12-h light period per day).

Characterization of *G. cingulata* f. sp *phaseoli* pathotype

The 12 differentiated cultivars, as proposed by the International Center of Tropical Agriculture (Centro Internacional de Agricultura Tropical, CIAT, 1990) to identify different pathotypes of *G. cingulata* f. sp *phaseoli*, were inoculated with spore suspensions of each strain. Sterile pods were inoculated and incubated at 22°C for 10 days in the dark to obtain inocula with concentrations of 1.2×10^6 spores/mL. The ascospore suspensions were sprayed 10 days after sowing (4 mL per plant), followed by incubation in mist chambers at 22°C, 12-h photoperiod and 98% relative humidity for 48 h. The plants were transferred to a greenhouse, and they were evaluated for symptoms seven days after inoculation, according to the descriptive scale recommended by Rava et al. (1993).

Sexual compatibility test

Paired crosses were made to examine the sexual behavior (homothallic or heterothallic) of the populations. Ten individuals from each population were used, providing 900 interpopulation combinations. The crosses were performed on Petri dishes containing 20 mL M_3 culture medium, as described by Camargo Jr. et al. (2007).

Obtaining mycelial masses and DNA extraction

Two hundred monoascosporic isolates were cut into pieces and transferred to Erlenmeyer flasks with 150 mL M_3 liquid medium and chloramphenicol (0.1 mg/mL). The cultures were maintained on an automatic shaker at a speed of 110 rpm and 22°C for five days. Dehydration of the

mycelial masses was performed with a vacuum pump. Total genomic DNA was extracted from lyophilized mycelium, according to the methodology described by Raeder and Broda (1985). DNA concentrations were estimated with a Hoeffer Scientific TKO100 fluorometer.

RAPD analyses

RAPD reactions were performed with 200 µM dNTPs, 0.6 units Taq DNA polymerase, and 0.4 µM primer, in a reaction buffer at pH 8.0 (50 mM Tris, 2.0 mM $MgCl_2$, 20 mM KCl, 250 µg/mL bovine serum albumin, 1% Ficoll 400, and 1 mM tartrazine), with 20 ng genomic DNA, in a final volume of 15 µL. The mixture was amplified in a thermocycler programmed for two cycles of 91°C for 60 s, 42°C for 70 s and 72°C for 70 s; then 38 cycles that differed from the first in the denaturation time (1 s) plus one cycle of 4 min at 72°C. Three hundred random primers were tested, and only those repeatedly presenting medium- or high-intensity bands were selected for further analyses. To analyze the DNA fragments, the amplified products were separated on 1.0% agarose gels in TBE buffer (Tris, boric acid and EDTA) with an electrical current of 100 V for 2.5 h. The amplified fragments were treated with ethidium bromide (0.5 µg/mL) for 30 min and were observed under ultraviolet light.

Data analyses

All RAPD bands were annotated using a binary system with codes of 1 or 0 for the polymorphic bands when they were present or absent, respectively. The estimation of genetic similarity (sg_{ij}) between each pair of strains was calculated using the Nei and Li coefficient (1979), and the analyses were performed using the NTSYS-PC 2.1 program (Rohlf, 2000). Dendrograms were generated for all populations simultaneously and for each population individually using the method of similarity averages (UPGMA).

The errors associated with each similarity were estimated according to the following expression, modified from Skroch et al. (1992):

Estimated standard error: $(s_{sg}) = \sqrt{sg_{ij}\frac{1-sg_{ij}}{n-1}}$

where n is the sum of a, b and c for each isolate pair.

The genetically different isolates were identified in the dendrogram based on the estimate of the maximum significant similarity value (sg_m). The sg_m was estimated by the t-test, using the following expression: $sg_m = 1-(t.\bar{s}_{sg})$

where t is the tabled 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 number and percentage of polymorphic loci were determined, as were the expected heterozygosity (Nei, 1973) and the Shannon index (1948); the latter was used to estimate the diversity within the populations. This value was also used to determine the diversity attributed to components within the populations. The analyses were performed using the GDA program (Genetic Data Analysis), version 1.0 (http://lewis.eeb.uconn.edu/lewishome/software.html).

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The ARLEQUIN 2.000 program (Schneider et al., 2000) was utilized for analysis of the molecular variance (AMOVA), considering a hierarchical structure in which isolates derived from the same location were analyzed as a single population. The digenic linkage disequilibrium within the five populations of *G. cingulata* f. sp *phaseoli* was also calculated using the ARLEQUIN 2.000 program. RAPD bands were considered as unique loci, and the presence or absence of the bands was considered as an allele. The probability for each locus association was tested using the Fisher test with Markov chain methodology (Raymond and Rousset, 1995). Associations with probability less than 0.05 were considered to be significantly different from zero. MULTILOCUS 1.3 (Agapow and Burt, 2001) was used to calculate multilocus connection disequilibrium, $\bar{r}_d$, a modification of the association index, I_A (Brown et al., 1980). The estimate of I_A tests the importance of the clonal and sexual reproduction modes within each population by calculating the distance between all loci pairs and comparing these distances to the linkage equilibrium. However, due to the fact that this estimate is influenced by the number of sample loci, the estimate of $\bar{r}_d$ is preferred, as it is independent of the number of sampled loci (Burt et al., 1999). The $\bar{r}_d$ values were calculated, and a total of 1000 permutations were completed for each population.

RESULTS

Characterization of pathotypes and crosses between the strains

The *G. cingulata* f. sp *phaseoli* isolates were classified as pathotype 0 when artificially inoculated in the 12 differentiating cultivars, since none of the differentiating cultivars presented a compatibility reaction.

No fertile crosses were identified among the crosses between strains of different populations. However, when the strains developed from these populations were grown in isolation on Petri dishes using M_3 medium, they produced perithecia with asci and ascospores.

Characterization of the RAPD markers

Among the 300 random primers tested, 28 repeatedly generated medium- or high-intensity bands in the RAPD amplifications of the isolates evaluated. These 28 primers (OP M-1, OP M-2, OP M-3, OP M-4, OP M-5, OP M-6, OP M-7, OP M-8, OP M-9, OP M-10, OP M-11, OP M-12, OP M-13, OP M-14, OP M-15, OP M-16, OP M-17, OP M-18, OP M-19, OP M-20, OP L-1, OP L-2, OP L-3, OP L-4, OP L-5, OP L-6, OP L-7, OP L-8) from the Operon kit were used for further analyses. In total, 128 polymorphic bands were obtained from these primers, an average of 4.57 bands per primer. The OP M-7 and OP M-14 primers were the most efficient at detection of polymorphism. The polymorphic locus average was 65%, with the highest percentage of polymorphisms observed in the Valente population (73%) and the smallest percentage observed in the Turvo population (55%; Table 1).

Statistical analyses

The cut-off line represents the maximum similarity value (sg_m) at the right of which the strains are considered to be similar. The sg_m at the 1% probability level was 0.886. The gen-

eral dendrogram showed the formation of five groups, with the formation of three major clades in the Valente population. The genetic similarity estimates between the strains involving all five populations presented similarity amplitudes from 0.43 to 1.00 (Figure 1). The dendrograms generated for each population independently indicated that the isolates of *G. cingulata* f. sp *phaseoli* displayed significant intra-population diversity, with many haplotypes. Among the 200 strains, 57 genotypes did not group with any others. The Valente population presented the highest amplitude of similarity and formed the largest number of groups. Specifically, 34 of the 40 individuals of this group did not cluster with any other individuals (Table 1).

Table 1. Number and percentage of polymorphic loci, range of genetic similarity, Shannon index (H), heterozygosity (HE), and number of groups formed by the five *Glomerella cingulata* f. sp *phaseoli* populations from Brazil.

Population	Number of polymorphic loci	Percentage of polymorphic loci (%)	Similarity amplitude	Number of groups	H	HE
Guarapuava	91	71.1	0.59-0.97 (0.38)	16	0.2010	71.09
Majestoso	85	66.4	0.60-1.00 (0.40)	9	0.1348	66.41
Turvo	70	54.7	0.80-1.00 (0.20)	7	0.0827	54.69
Valente	93	72.7	0.77-0.99 (0.22)	7	0.3171	72.66
VCU	80	62.5	0.54-0.97 (0.43)	34	0.1452	62.50

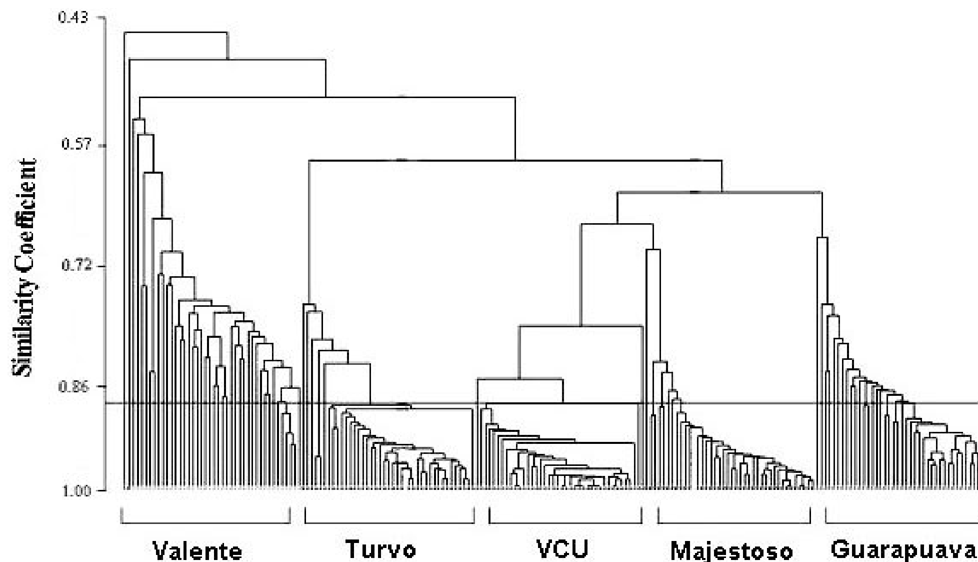

Figure 1. Cluster analyses of 200 *Glomerella cingulata* f. sp *phaseoli* isolates by UPGMA based on the genetic distance of Nei and Li (1979).

In order to measure the average contribution of sexual reproduction to genetic diversity presented in the evaluated populations, we compared published amplitudes of genetic similarity of asexual phase isolates (Table 2) with the amplitudes of genetic similarity of the sexual phase populations that we found (Table 1). We also calculated, the average contribution of the sexual phase to the amplification of genetic diversity of the pathogen (Table 3).

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Table 2. Summary of the number and origin of the isolates, the types of markers used in the analyses and the rate of similarity found in asexual phase populations of *Colletotrichum lindemuthianum*.

Number of isolates	Origin of isolates	Marker used	Similarity amplitude	Reference
24	Mexico	RAPD	0.60-0.87	González et al., 1998
49	Mexico	AFLP	0.92-1.00	Rodríguez-Guerra et al., 2003
40	South America	AFLP	0.55-0.75	Ansari et al., 2004
30	Brazil	RAPD	0.80-0.98	Talamini et al., 2006
48	Minas Gerais (Brazil)	RAPD	0.70-0.99	Silva et al., 2007
19	Paraná (Brazil)	RAPD	0.62-0.98	Silva et al., 2007
49	Brazil	RAPD	0.90-0.99	Silva et al., 2007

RAPD = random amplified polymorphic DNA; AFLP = amplified fragment length polymorphism.

Table 3. Estimated mean contribution of sexual reproduction to the expansion of genetic variability in *Glomerella cingulata* f. sp *phaseoli* populations compared to asexual population phases of the pathogen.

Populations	General averages	MG averages	PR averages	MG/PR averages
Guarapuava	0.17	-	0.02	0.06
Majestoso	0.19	0.11	-	0.08
Turvo	-0.01	-	-0.16	-0.13
Valente	0.01	-0.07	-	-0.11
VCU	0.22	0.14	-	0.11

Averages calculated by subtracting total genetic similarity amplitude (Table 2) from the mean amplitude of the asexual phase (Table 1). Minas Gerais (MG), Paraná (PR) and Minas Gerais/Paraná States.

The index of genetic diversity varied from 0.0827 to 0.3171 (Table 1). The lowest degree of diversity was detected in the Turvo population, and the greatest diversity was observed in the Valente population. The expected heterozygosity was also greater in the Valente population and smaller in the Turvo population (Table 1). The genetic diversity of the Nei value was decomposed, and we found that the total diversity between populations was 0.3647 (H_T), indicating that 36% of the isolates are unique. Partition analyses of the genetic diversity for all 128 polymorphic loci showed significant genetic differentiation between the populations (G_{ST} = 0.6455).

Based on AMOVA, the genetic differentiation detected between sample populations was significant (Φ_{ST} = 0.69, P < 0.000), indicating that 69% of the genetic variation is between populations and 31% of the variation is found within the populations (Table 4).

Table 4. Summary of analysis of molecular variance of five *Glomerella cingulata* f. sp *phaseoli* populations from Brazil evaluated with random amplified polymorphic DNA markers.

S.V.	d.f.	SS	Variance components	Total (%)	Φ_{ST}	P
Among populations	4	3022.25	18.68	69.03	0.69	0.000
Within populations	195	1633.90	8.38	30.97		
Total	199	4656.15	27.06	100		

S.V. = sources of variation; d.f. = degrees of freedom; SS = sum of squares.

The largest and smallest gametic disequilibria were detected for the Majestoso and Turvo populations, respectively. The gametic disequilibrium analyses, based on the Fisher test, generated values significantly different from zero, although the observed levels of linkage disequilibrium were low to moderate for all populations (Table 5). The number of combi-

nations of loci in these analyses was 128*(128 - 1) / 2 = 8128. The multilocus association tests were also significant, showing that there is linkage disequilibrium between the five populations. However, considering that a value of $\bar{r}_d$ = 1.0 indicates individual clones, the values determined in this study can be considered of low magnitude, as the largest $\bar{r}_d$ value calculated was 0.1307 (Majestoso; Table 5).

Table 5. Digenic linkage disequilibrium test and association index for the disequilibrium test of five *Glomerella cingulata* f. sp *phaseoli* populations from Brazil.

Populations	Pairwise digenic linkage disequilibrium (%)	Indices of multilocus association	
		I_A	$\bar{r}_d$
Guarapuava	21.37	4.8747	0.05642
Majestoso	27.32	10.2112	0.1307
Turvo	11.66	3.6629	0.05707
Valente	15.31	2.4999	0.0278
VCU	15.62	4.6826	0.0658

DISCUSSION

Anthracnose of the common bean is characterized by characteristic symptoms, including depressed and dark-colored oval lesions in pods and stems and dark stains along the veins of leaves. However, absence of such symptoms in plants inoculated with *G. cingulata* f. sp *phaseoli* isolates has been reported (Camargo Jr. et al., 2007; Ishikawa et al., 2009); the symptoms in the sexual phase are milder than those presented during the asexual phase.

Although no fertile crosses were obtained from the combinations of isolates that we used, recombination between *G. cingulata* f. sp *phaseoli* isolates has been reported (Rodríguez-Guerra et al., 2005; Camargo Jr. et al., 2007). In fact, atypical sexual behavior in *Colletotrichum* species, with both heterothallic and homothallic forms, has been described (Rodríguez and Owen, 1992; Cisar and TeBeest, 1999; Vaillancourt et al., 2000).

The general dendrogram demonstrated five distinct groups within the sampled populations. These groups correspond to the five populations that we analyzed, even though these populations were isolated from nearby regions. This form of local adaptation has been reported in several host-pathogen interactions (Thompson, 1994; Kaltz and Shykoff, 1998). Coevolution can become evident in complex hierarchical levels, when several plants are sampled at different times of the year, and also in more simple hierarchical levels, such as in populations of a few plants, a single plant, or even in a single lesion, when these events occur in pathogens with reduced genetic flow between geographic units. The evolutionary advantage of the pathogen in relation to its host is characterized by the fact that the pathogen adapts to changes in the local host population, principally because the *C. lindemuthianum* life cycle is up to 10 times shorter than that of the wild common bean (Capelle and Neema, 2005).

We analyzed populations originating from a few common bean plants derived from agroecosystems; this permitted clear separation of the five populations. This population structure indicates that this pathogen is dispersed at a fine scale, a fact that directly interferes with genetic flow and recombination between populations. Capelle and Neema (2005), who studied asexual phase isolates and wild populations, reported that this population structure is typical for this pathogen. Even during heavy storms, maximum dispersal of *G. cingulata* f. sp *pha-*

seoli spores is a few meters (Tu, 1992). It is probable that the dispersal of the spores occurs preferentially between different parts of the same plant and occasionally between adjacent plants. Thus, diversity is generated and maintained in simple hierarchic levels.

In spite of the results demonstrating these evolutionary dynamics in the *G. cingulata* f. sp *phaseoli* x *P. vulgaris* interaction on a reduced scale, the origin of the genetic diversity that was found remains unclear. It is believed that selection pressure by the host and the alternation of sexual and asexual phases are keys to explaining this diversity (Ruey-Shyang and McDonald, 1996). An entirely asexual population has high frequencies of clonal individuals, with a few genotypes at an especially high frequency. In our study, the *G. cingulata* f. sp *phaseoli* populations were characterized by considerable genetic diversity. Comparing the data obtained from sexual populations with published data on asexual populations, we can infer the capability of sexual reproduction to amplify genetic variability in this pathogen. Considering data from Silva et al. (2007) in an analysis of the asexual phase of the pathogen in populations from the State of Minas Gerais, Brazil, the extent of similarity ranged from 0.70-0.99, with 29% dissimilarity. The *G. cingulata* f. sp *phaseoli* strains that we collected from Minas Gerais presented similarities of 0.54-0.97 and dissimilarity of 43%. This difference in the diversity values between the sexual and asexual populations indicates that recombination plays a fundamental role in the expansion of variability in populations of this pathogen. In addition to the characterization of variability in the populations, we identified few clonal lines. The Turvo population had the largest number of clonal lines, a result that is expected based on its low degree of similarity and reduced number of groups formed in the dendrogram. This implies that the Turvo population originated from several cycles of asexual reproduction, which may have obscured the contributions of sexual reproduction (Ruey-Shyang and McDonald, 1996). Larger-scale studies characterizing the population structure of this pathogen with regards to its sexual and asexual phases are needed to resolve these questions.

Analysis of the partition of diversity of this species supports the hypothesis that the spore of this pathogen disperses only a short distance from the source (Table 4). One possible reason for the different levels of genetic diversity observed in each of the populations that we examined is that the pathogen interacts with the different host cultivars at each location and consequently interacts with different genetic backgrounds. In this case, the host diversity would influence the maintenance of the genotype diversity of the pathogen (Rodríguez-Guerra et al., 2003). The differences observed between populations are consistent with the fact that they did not produce compatible crosses, suggesting reproductive isolation between the five populations, which resulted in significant variability between the different sample locations.

Results of analyses of digenic and multilocus gametic disequilibrium demonstrate significant levels of linkage disequilibrium, although these levels may be considered low to moderate in comparison with the disequilibrium presented by a clonal population. Based on these results, some level of clonality exists in the *G. cingulata* f. sp *phaseoli* population, though recombination is still possible. However, the data demonstrate a genetic structure consistent with sexual reproduction for this pathogen (Schmale et al., 2006). In addition, the estimates generated by multilocus associations for other species with a known sexual phase (Rau et al., 2003) are similar to those calculated in our study.

Colletotrichum lindemuthianum have been described as an asexual pathogen. However, regional-scale studies have demonstrated levels of linkage disequilibrium consistent with recombination, suggesting that sexual reproduction is more frequent under field conditions

than previously thought (Capelle and Neema, 2005). This observation can be explained by a low capacity for dispersion of spores, founder effects and selection pressure exerted by host-resistance genes. In Brazil, the common bean is cultivated in three distinct seasons, an approach that pressures the pathogen towards sexual recombination in order to respond to environmental changes. It is likely that the sexual cycle is triggered at some stage of the pathogen's population development, resulting in an expansion of genetic variability. Following this expansion, several asexual reproduction cycles would take place, as the asexual phase of the pathogen is responsible for the epidemiological phase of infection.

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