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RESEARCH ARTICLE / *ARTIGO*

Effect of crop rotation on specialization and genetic diversity of *Macrophomina phaseolina*

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ABSTRACT

Charcoal rot, caused by *Macrophomina phaseolina*, is one of the most important diseases of soybean. Genetic variability among soybean isolates has been observed but the effect of host specialization on genetic variability has not been reported. In this work, isolates from soybean, corn and sunflower were evaluated based on cross inoculations and number of microsclerotia/g of roots. The highest and lowest significant (P<0.005) production of microsclerotia was obtained in soybean (863 microsclerotia/g of roots) and sunflower (578 microsclerotia/g of roots), respectively, regardless of the origin of the isolate. Additionally, the effect of a 20-year crop rotation on genetic variability based on RAPD was studied. Eighty-nine isolates from five populations were obtained from soil samples under four crop rotation systems and an uncropped soil. Seven clusters were obtained considering a similarity level of 85%. Analysis indicated that *M. phaseolina* is a highly diverse species and also revealed a strong effect of the rotation system on genetic diversity. AMOVA was conducted for the RAPD data. From the total genetic variability, 21% (P<0.0001) could be explained by the differences between populations while 79% could be explained by differences within populations (Φ_{st} = 0.2110; P<0.0001). The mean coefficient of gene differentiation (G_{ST}) estimated among the five populations indicated 27% of differentiation between populations similar to the AMOVA results where Φst= 0.2110. Total gene diversity estimated indicated high levels of variability (H_T =0.3484). Results suggest that genetic differentiation of *M. phaseolina* can be altered by crop rotation.
Keywords: RAPD, polymorphism, adaptability.

RESUMO

Efeito da rotação de culturas na especialização e diversidade genética de *Macrophomina phaseolina*

Podridão de carvão, causada por *Macrophomina phaseolina* é uma das mais importantes doenças da soja. Variabilidade genética tem sido observada entre isolados. Entretanto, o efeito de especialização deste fungo, de acordo com a planta hospedeira, não foi ainda relatado. Isolados de soja, milho e girassol foram avaliados, em inoculações cruzadas, quanto à capacidade de formação de microescleródios em raiz. A maior e menor produção significativa de microescleródios (P<0.005) foram observadas em raízes de soja (863 microescleródios/g de raiz) e girassol (578 microescleródios/g de raiz), respectivamente, independente da origem do isolado. Além disso, estudou-se a variabilidade de isolados do fungo, obtidos de sistemas de rotação de culturas estabelecido há 20 anos utilizando-se RAPD. Oitenta e nove isolados, oriundos de cinco populações foram coletados em amostras de solo de quatro sistemas de rotação e uma amostra de solo virgem. Sete agrupamentos foram observados com 85% de similaridade. As análises indicaram que *M. phaseolina* é uma espécie com alta variabilidade genética com forte efeito do sistema de rotação. AMOVA foi realizada com os dados de RAPD. Da variabilidade genética total, 21% (P<0.0001) foi explicada pelas diferenças entre populações e, 79% foi explicada por diferenças dentro das populações (Φ_{st} = 0.2110; P<0.0001). A média do coeficiente de diferenciação gênica (G_{ST}) estimada entre as cinco populações foi de 27%, similar aos resultados da AMOVA (Φst= 0.2110). A diversidade genética total mostrou que as cinco populações têm alto grau de variabilidade (H_T =0.3484). Os resultados sugerem que a diferenciação genética de *M. phaseolina* pode ser alterada pela rotação de culturas.
Palavras-chave: RAPD, polimorfismo, adaptabilidade.

INTRODUCTION

Charcoal rot of soybean is caused by *Macrophomina phaseolina* (Tassi) Goid., a soilborne fungus that infects over 500 plant species and is one of the most important soybean diseases worldwide (Hartman *et al.*, 1999). It is seedborne (Andrus, 1938) and responsible for extensive damage in Brazilian soybean fields where disease losses were first reported in the Southern States (Lehman *et al.*, 1976). Since then, the disease has been considered one of the most prevalent soybean pathogens in Brazil (Almeida *et al.*, 2005). It is most important during dry weather or when unfavorable environmental conditions stress the plant. Charcoal rot of soybean leads to early maturation, chlorosis and incomplete pod filling.

To date, genetic resistance to charcoal rot has not been identified. Due to the wide host range of the pathogen, the disease is difficult to control with crop rotation. Reduced

incidence of the disease was observed in no-tillage fields where straw from the winter crop left on the soil surface contributed to decreasing the temperature and increasing the moisture (Almeida *et al.*, 2001). No-tillage is a management system that is steadily increasing in Brazil and is commonly associated with crop rotation.

A twenty-year-old no-tillage and crop rotation system was used to determine the effect of these practices on concentration of *M. phaseolina* propagules in the soil (Almeida *et al.*, 2003). Results showed that soil samples from conventional cropping system contained more propagules and also a higher incidence of infected roots than soil from no tillage system. A possible explanation was the substantial amount of crop residue left on soil surface, which could reduce temperature and increase moisture, two factors that suppress charcoal rot (Olaya & Abawi, 1996), or the improvement of conditions that favoured mycoparasitism (Chet *et al.*, 1981).

Morphological differences between *M. phaseolina* isolates have raised questions about potential differences in pathogenicity and genetic diversity in the pathogen related to different rotation systems. This question arose after the report of Su *et al.* (2001), who found that isolates from a given host were genetically similar to each other but distinct from those obtained from other hosts. All the isolates used grouped together and were obtained from a monocropping system where each crop was cultivated for 15 years in the same soil, without rotation. Therefore, the question of whether the use of different crop rotation systems could increase the genetic diversity of this pathogen has been unanswered.

The objective of this study was to determine the effect of crop rotation on the genetic diversity of *M. phaseolina* and to compare genetic diversity of isolates obtained from cultivated and native uncropped soils.

MATERIAL AND METHODS

Evaluation of adaptability

Soil sampling and fungal density. A four-year no-tillage crop rotation field has been monitored since 1986 in Campo Mourão county (24º 02´ 45´´S, 52º 22´58´´W, at 630 m altitude), State of Paraná, Brazil. The experiments were located on an oxisol (Rhodic Eutrudox, USA classification). Climate is defined as subtropical (Cfa, according to Koppen´s classification). The schedule of crop rotation was established for the southern region of Brazil where a double-cropping system exists. This system consists of: summer (November-March) and winter (April-August) plantings (Table 1). Soybean and corn are sown in summer while wheat, lupins, canola, and oilseed radish are grown in winter (April-August) as cover crops. Traditionally, soybean is grown in the summer followed by wheat in the winter. Soil samples were randomly obtained from ten different points inside each plot (7 m x 11 m) from each of the four treatments using a five–cm-diameter by 15-cm-deep core (Table 1). In 2004, composite soil samples of six-cm-diameter by 15-cm-deep cores were collected from the two center rows of each plot. Microsclerotia densities in each plot were determined according to Kendig *et al.* (2000) from soil samples dried to 5% moisture. Samples were mixed thoroughly and stored at 4°C. Additionally, soil samples from native uncropped soil located 300 m from the crop rotation field was also used and constituted the first treatment. Bulk soil samples obtained from each treatment were air dried and passed through a 20-mesh sieve and used for estimating fungal density (Kendig *et al.*, 2000). Plates were incubated in darkness at 28ºC for nine days. The number of colony-forming units (CFU) was counted with a stereoscopic microscope to determine the number of CFU/g of air-dried soil or root tissue.

Fungal isolation and adaptedness tests

Adaptability was evaluated based on the concentration of microsclerotia formed/ g of root. Isolates were obtained from roots of soybean, corn and sunflower collected in commercial fields and thoroughly washed and dried at room temperature. From each root, small pieces were excised, sterilized in sodium hypochloride and transferred to PDA containing streptomycin (0.1 mg/ml). Plates were incubated at 26ºC in the dark for 7 days. Small pieces of medium containing microsclerotia were examined under the stereoscopic microscope and a single microsclerotium was collected and transferred to PDA slants. Three single-microsclerotium isolates were obtained from each host. From

TABLE 1 - Double crops used in a four-year rotation system

	2002		2003		2004		2005		2006	
Treatment	**W***	**S***	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**
1- Native soil	-	-	-	-	-	-	-	-	-	-
Rotation 2	L	C	O	So	Wh	So	Wh	So	L	C
Rotation 3	L	C	Mt	So	Wh	So	Wh	So	L	C
Rotation 4	CN	So	N	So	Wh	So	Wh	So	CN	So
Rotation 5	Wh	So	Wh	So	Wh	So	Wh	So	Wh	So

*W=winter crop; S=summer crop
CN= canola; C=corn; L=lupins; Mt=millet; N= oilseed radish; O=oat; So=soybean; Wh= wheat

each colony, single discs were transferred to potato-dextrose broth for 14 days at 26ºC when mycelium and microsclerotia were collected, washed in distilled water and dried at room temperature. Fungal structures were ground and stored at 6ºC. Sterilized field soil was divided in three 18-kg samples. Each soil sample was mixed with 100 g of each isolate stored at 6ºC to yield 48-55 colony-forming units (CFU)/ g of soil, a value in the range found in a previous experiment (Almeida *et al*., 2001) with naturally infested soils (47± 9.6 CFU/g soil). The mixture was used to fill nine 2.0 kg plastic pots (22 cm diameter). Soybean, sunflower and corn were sown keeping two plants/pot and three pots/isolate in greenhouse conditions (28ºC ± 3.7ºC). Three months later and until maturation, watering was reduced to 75% of field capacity. After this, pots were kept in total dryness for ten days to help loosen roots from the soil. Roots were washed in running water, dried and ground in a 20-mesh mill (Manesco & Ranieri®, São Paulo, SP). A sample of 25 mg from each plant root was used to evaluate the concentration of propagules (CFU/g root) in specific medium for *M. phaseolina* (Kendig *et al.*, 2000). The experimental design consisted of factorial design with three host species (soybean, corn and sunflower), nine isolates (three from each plant species) and three replications/ treatment.

Analysis of genetic diversity

DNA isolation. Isolates of *M. phaseolina* were obtained from the soil dilutions (Kendig *et al*., 2000) and transferred to PDA. Isolates from each crop rotation system were considered a population. A total of eighty-nine cultures from the five treatments (Table 1) were selected and prepared for evaluation of genetic variability. Cultures were kept at 4ºC on PDA slants. A total of eighty-nine isolates were used for this study and formed five populations. Each isolate was grown in liquid potato-dextrose broth at 26ºC for 2 weeks. Mycelia were centrifuged at 3,000 x g for 10 min and the pellet was washed in sterile water. Partially dried mycelium was stored at -80º C for further DNA extraction. Mycelium was ground in liquid nitrogen and treated with CTAB extraction buffer (50mM Tris-HCl, pH 8.0, 700 mM NaCl, 10 mM EDTA, 10% hexadecyltrimethyl-ammonium bromide - CTAB), followed by phenol/chloroform purification and precipitation with ethanol. DNA concentration was estimated by spectrophotometer and the samples were stored at -80ºC (Almeida *et al.*, 2003).

RAPD-PCR analysis. Genomic DNA from the isolates was analyzed by the random amplified polymorphic DNA (RAPD) method (Williams *et al.*, 1990), using nine random oligonucleotide (10-mer) primers (OPAO-08, OPAO-09, OPAB-03, OPAB-12, OPAC-03, OPAC-07, OPM-02, OPM-04, OPU-13 and OPU17, Operon Technologies, Inc., Alameda, CA, USA). Reactions were carried out in a volume of 25 µL volumes containing 10 mM of Tris-HCI (pH 8.3), 50 mM of KCl, 2 mM of $MgCl_2$, 100 µM of each deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP), 0.4 µM of primer, one unit of *Taq* DNA polymerase and 30 ng of template DNA. Amplification was programmed for 45 cycles in a thermocycler 9600 (Perkin-Elmer, Norwalk, CT, USA). Each cycle consisted of a denaturation step at 94ºC for 30 sec, a primer annealing step at 35ºC for 30 sec, and a primer extension step at 72ºC for 1 min. Amplicons were resolved by gel electrophoresis in 1.3% agarose gels stained with ethidium bromide, and photographed over UV light (302 nm). To test reproducibility of the markers two independent DNA extractions were performed from the isolates 9.9, 4.4 and 11.4, which were used for PCR with the primers OPAO-08, OPAC-03, OPAC-07, OPM-02, and OPU-13.

Data analysis

Amplified fragments were scored across isolates for their presence or absence. Polymorphisms in loci with faint bands were not scored. Data analysis was performed with the NTSYS program version 2.02j (Exeter Software, Setauket, NY, USA). A pair-wise similarity matrix was generated using Dice´s similarity coefficient (Dice, 1945). Cluster analysis was performed with the cluster program SAHN using the unweighted pair-group method with arithmetic averages (UPGMA). To evaluate the repeatability of the inferred groups, data was subjected to bootstrapping using the WinBoot software program (Yap & Nelson, 1996). The dendrogram was reconstructed 1000 times by repeated sampling with replacement, and the frequency with which each cluster is represented in the consensus phenogram was used as a criterion for confidence of each inferred cluster. Analysis of molecular variance (AMOVA) was performed to estimate variance components by partitioning variation within and between populations from different rotation systems, wirh ARLEQUIN software (Schneider *et al.*, 2000), using dominant markers (Huff *et al*., 1993). Φ_{st} was tested statistically by nonparametric randomization analysis.

The significance of the variance components was tested by a permutational approach (Excoffier *et al.*, 1992). Vectors for the presence of RAPD markers ("1" for the presence of a marker and "0" for the absence), for each isolate, were used to compute the genetic distance between each pair of isolates. The parameter used was the Euclidean metric measurement (*E*) (Excoffier *et al.,* 1992), defined by Huff *et al.* (1993) as follows: $E=\varepsilon^2_{xy}=n(1-2n_{xy}/2n)$, where $2n_{xy}$ is the number of markers shared by two strains and n is the total number of polymorphic sites.

Nei´s unbiased genetic distances (Nei, 1978) between all isolates were estimated using allele frequencies to determine whether a population was genetically differentiated from others. Nei´s genetic diversity (h) and Shannon index (Lewontin, 1964) was estimated for each population. The correlation between the Shannon index and the percentage of polymorphic loci was also determined. The total genetic diversity (H_T), genetic diversity within each population (H_S) and genetic diversity of populations (D_{ST}) was calculated according to Nei (1986).

The genetic coefficient of differentiation among five populations was estimated according to Nei (1986) using the formula $Nm = \frac{1}{4}\left(1 - \frac{1}{G_{ST}}\right)$ of Wright (1951). The software POPGENE version 1.32; (http://www.ualberta.ca/~fyeh/) was used in this analysis, and its results were interpreted according to Wright (1978), Slatkin (1987) and McDermott & McDonald (1993).

RESULTS

Adaptedness tests. All nine randomly chosen isolates from roots of soybean, sunflower and corn produced characteristic symptoms of charcoal root rot (darkened roots and gray to black masses of microsclerotia under root epidermis). The number of microsclerotia developed on roots of soybean, corn and sunflower ranged from 578 to 863 CFU/ g of root (Table 2). ANOVA showed a significant interaction ($P<0.05$) between the origin of the isolate and the host. Isolates from each crop (soybean, corn and sunflower) colonized roots of all three species but roots of sunflower always produced significantly fewer microsclerotia than soybean or corn, showing that sunflower is the least adapted. The highest significant concentration of CFU was observed in roots of soybean and corn ($P<0.005$) regardless of the host-isolate origin.

RAPD polymorphism. Nine RAPD primers were used to amplify the DNA from 89 *M. phaseolina* isolates. The fragments ranged from 120 bp to 720 bp. Only those fragments > 200 bp were scored, totaling 39 putative loci. RAPD-PCR analysis revealed many polymorphisms among isolates. A similarity matrix was generated by Dice´s coefficients for all pairwise combination among isolates, showing values ranging from 0.44 to 1.0 (data not shown). The matrix was used in cluster analysis. UPGMA clustering produced a dendrogram that separated the isolates into seven clusters at the level of 85% similarity (Figure 1). The total number of RAPD bands observed over all nine primer combinations varied between populations, ranging from 176 bands for population 1 to 278 bands for population 3. Number of isolates, in each population, belonging to a specific cluster is shown in Table 3. Clusters A, B, C and D have 25, 21, 28 and 7 isolates, respectively. Clusters E, F and G have two, three and three isolates, respectively.

The two populations that exhibited less genetic variability among their isolates were collected in rotation system 5 (double crop soybean-wheat) and native uncropped soil (treatment 1). The other treatments had higher variability, a fact that contributed to the presence of seven clusters.

The largest genotypic variation was found among isolates obtained from soil of treatment 3, suggesting that diversity of *M. phaseolina* was altered by cultivation and crop rotation (Table 3). Similar clusters could also be found in several rotations. However, few clusters (3) were formed when the rotation system was a double-crop type (soybean in the summer and wheat in the winter).

AMOVA was conducted for the RAPD data based on five populations (non-cultivated soil and populations 1, 2, 3 and 4) (Table 4). From the total genetic variability, 21% ($P<0.0001$) could be explained by the differences between populations while 79% could be explained by the differences within populations ($\Phi_{st} = 0.2110$; $P<0.0001$).

Nei´s genetic distance between populations ranged from 0.023 to 0.245 (Table 5). The shortest value for genetic distance was observed between population 1 and 2. From the 39 RAPD loci the number and the percentage of polymorphic loci for each population were deduced (Table 6). The gene diversity values (*h*) as well as the Shanon index (I) were smaller for populations 2 and 3. Nei´s genetic diversity between populations ranged from 0.22 to 0.31 while Shannon's index varied from 0.34 to 0.45 (Table 6). A positive correlation was detected between the Shannon index and the percentage of polymorphism for all populations ($R^2=0.96$; $P=0.001$) suggesting that RAPD markers were unevenly distributed in the populations.

The total gene diversity (Nei, 1978) obtained showed that these five populations indicated high levels of total genetic diversity ($H_T=0.3484$). Further, an H_S value of 0.2541 was obtained, a fraction of the total of genetic diversity within each population and D_{ST} the fraction of the diversity within populations (Table 7). This result indicates that most of the genetic diversity is within populations, as was also pointed out by AMOVA.

The mean coefficient of gene differentiation (G_{ST}) obtained from five populations was 0.27, indicating 27% of differentiation between populations. Also, this fact supports the result obtained by AMOVA, where $\Phi st= 0.2110$, very close to G_{ST}.

Gene flow (Nm) between populations was 0.6732 and it was estimated from G_{ST} value as Wright, 1951 where Nm is the average number of migrants among the populations (Table 7). According to Wright (1951), the Nm value < 1 indicates the existence of restricted gene flow among populations.

TABLE 2 - Mean number of colony-forming units/g of roots of *Macrophomina phaseolina* inoculated on three different hosts

Isolate	Crop			
	Soybean	Corn	Sunflower	Average
Soybean	980,6* aA	874.6 aA	554.4 cA	803,2 A
Corn	873,2 aA	814.3 aA	567.4 bA	751,6 A
Sunflower	736,6 aB	726.7 aB	612.0 bA	691,8 B
Mean	863,5 a	805,2 a	578,0 b	
C.V. = 18.2%				

* Mean number of CFU formed in one g of roots. Values followed by small letters in the lines and capital letters in the columns are not significantly different according to Tukey´s test ($P<0.05$).

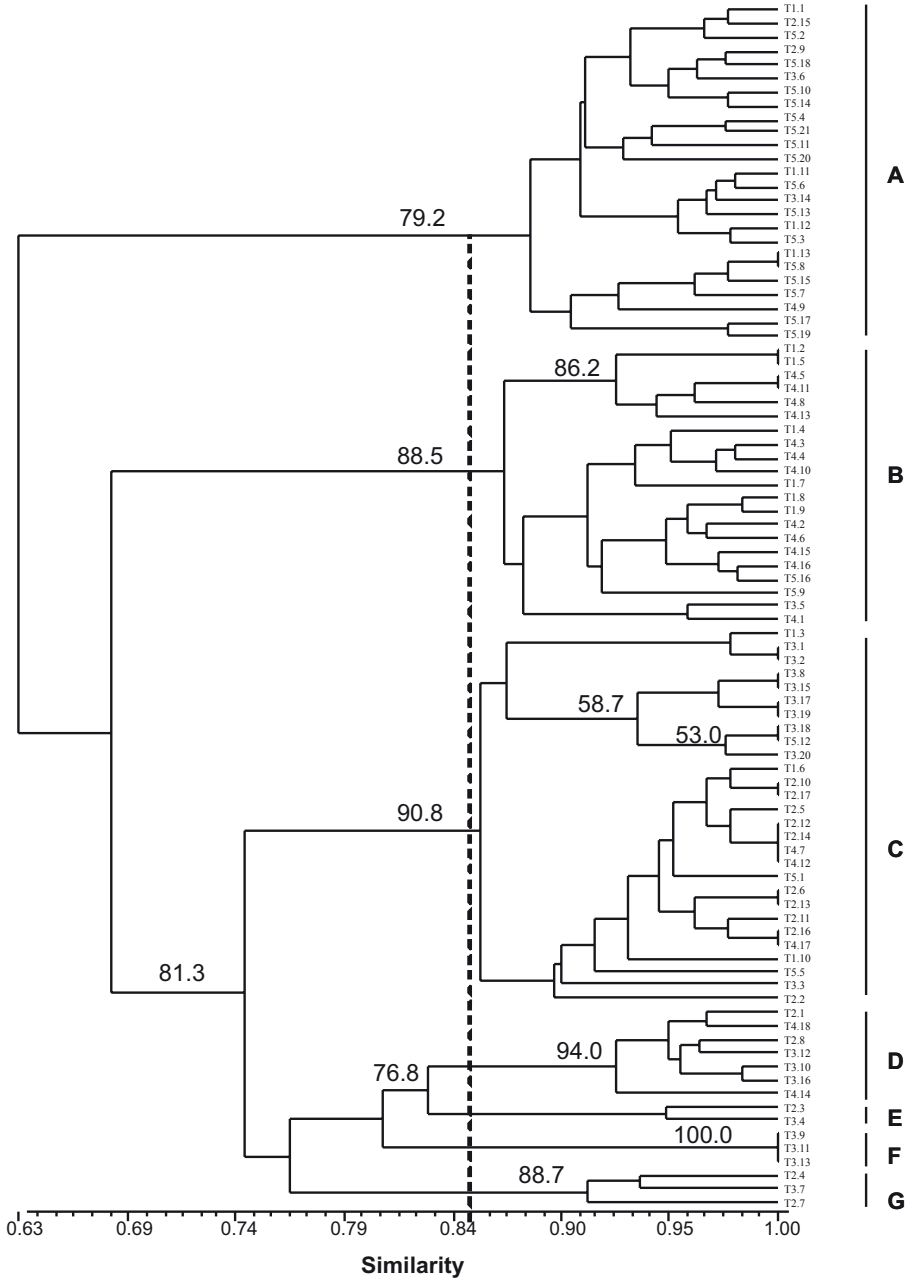

FIG. 1 - Genetic similarity among *Macrophomina phaseolina* isolates sampled from different crop rotation systems and native uncropped soil. Dendrogram was estimated by clustering a similarity matrix (Dice coefficient) of binary random amplified polymorphic DNA data using the unweighted pair group method of arithmetic means in the NTSys program. Letters A to G indicate the seven clusters of isolates found. Only bootstrap values of 50% or greater are shown. Identification: the first letter and number means the rotation system; the second number after period means the number of the isolate in the population; T1= non-cultivated soil; T2= rotation system 2; T3 = rotation system 3; T4 = rotation system 4 and T5 = rotation system 5.

DISCUSSION

In this work, the host specialization of *M. phaseolina* was not detected since all isolates, regardless of their origin, were able to infect all host species. However, it was observed that colonization of sunflower roots produced fewer microsclerotia ($P<0.005$) per gram of root than roots of corn and soybean either infected with its own isolates or from cross infection. It is clear from these data that soybean and corn isolates were more adapted than the isolate

TABLE 3 - Frequency of isolates of *Macrophomina phaseolina* from non-cultivated soil and four different crop rotation systems in each cluster based on 85% of similarity coefficient

		No. of isolates/cluster							
Treatment	**N° isolates**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**No. clusters**
1- Native soil	13	4	6	3	0	0	0	0	3
Rotation 2	17	2	0	10	2	1	0	2	5
Rotation 3	20	2	1	9	3	1	3	1	7
Rotation 4	18	1	12	3	2	0	0	0	4
Rotation 5	21	16	2	3	0	0	0	0	3
Total	89	25	21	28	7	2	3	3	
%		28.08	23.59	31.46	7.86	2.24	3.37	3.37	

TABELA 4 - Analysis of molecular variance (AMOVA) for 89 isolates of *Macrophomina phaseolina*. The total data set contains isolates from five populations

Source of variation	**d.f.**	**Sum of squares**	**Variance component**	**% total variance**	**P-value**	**Fixation index Φ_{st}**
Among populations	4	122.06	1.42 Va	21.11	<0.0001	0.2110
Within populations	84	447.08	5.32 Vb	78.89	<0.0001	

TABLE 5 - Nei´s unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) between populations of *Macrophomina phaseolina* obtained from four systems of crop rotation and non-cultivated soil

Population	**Native soil**	**Rotation 1**	**Rotation 2**	**Rotation 3**	**Rotation 4**
Native soil	-	0.91	0.92	0.96	0.91
Rotation 1	0.091	-	0.97	0.85	0.87
Rotation 2	0.073	0.023	-	0.88	0.87
Rotation 3	0.037	0.151	0.123	-	0.78
Rotation 4	0.088	0.133	0.134	0.245	-

TABLE 6 - Genetic variation of five populations of *Macrophomina phaseolina* from non-cultivated soil, four different rotation systems and total

Population		**na***	**ne**	**h**	**I**	**% Polymorphic loci**
Native soil	13	1.74 (0.44)**	1.57 (0.39)	0.31 (0.20)	0.45 (0.28)	74.3
Rotation 1	17	1.69 (0.46)	1.36 (0.32)	0.22 (018)	0.34 (0.26)	69.3
Rotation 2	20	1.84 (0.36)	1.48 (0.37)	0.28 (0.18)	0.42 (0.24)	84.6
Rotation 3	18	1.79 (0.40)	1.40 (0.32)	0.24 (0.17)	0.38 (0.24)	80.0
Rotation 4	21	1.74 (0.44)	1.37 (0.34)	0.23 (0.18)	0.35 (0.25)	74.3
Total***	89	1.84 (0.36)	1.58 (0.35)	0.32 (0.18)	0.47 (0.25)	-

**Standard deviation
na = Observed number of alleles; ne = Effective number of alleles (Kimura & Crow, 1964);
h = Nei's (1973) gene diversity; I = Shannon's Information index (Lewontin, 1972)
*** Total considering only one population without structure

from sunflower. This result is similar to those of Su *et al.* (2001) who observed more production of microsclerotia in isolates of soybean than in isolates of corn, sorghum and cotton. The effect of crop rotation in this study, with crops changing annually in the winter and/or summer for 20 years, could have affected the pathogen's specialization and the adaptability levels observed. In contrast, the isolates used by Su *et al.* (2001) were collected from fields where crops were

TABLE 7 - Genetic structure of five populations of *Macrophomina phaseolina* from four different rotation systems and non-cultivated soil

Estimative	H_T	H_S	G_{ST}	Nm
Mean	0.3484	0.2541	0.2708	1.3466
St. dev.	0.0222	0.0147	-	-

* Nm = estimate of gene flow from $Nm = \frac{1}{4}\left(1 - \frac{1}{G_{ST}}\right)$ (Wrigth, 1951) H_T = total genetic diversity in the pooled populations; H_S = mean diversity within each population (Nei, 1978).

cultivated continuously (monoculture) for 15 years, without any challenge. Also, the concentration of microsclerotia/g of soil used in this study was in accordance with the average values found in cultivated soils in southern areas of Brazil (215 microsclerotia/g of soil), differently from that used by Su *et al.* (2001) who found an average of 10 microsclerotia/g of soil.

Molecular analysis based on RAPD data indicated a strong effect of the rotation system on genetic diversity. Since microsclerotia can survive in soil for several years (Short *et al.*, 1980) and their germination and adaptation to specific crops may occur over a period of years, it is acceptable to have an increase in variability. It is important to consider that this study was conducted in a double-crop system, typical of southern regions of Brazil. Cumulative effect of fungus variability could be due to the double crop. Su *et al.* (2001) detected the effect of continuous crop on the adaptation of the fungus to their hosts, but in our study, the results demonstrated that annual rotation in a double-crop system stimulated the fungus to diversify its adaptation, permitting several genotypes to survive independently in the same field.

Among the 89 isolates, those from population 3 and 4 had the highest similarity coefficient, forming the most consistent groups supported by bootstrap values of 88.5% and 79.2%, respectively. Genetic variation among isolates of *M. phaseolina* had been previously reported by Fuhlbohm (1997) in Australia, Su *et al.* (2001) in USA and Almeida *et al.* (2003), in Brazil. A total of seven clusters were identified among the 89 isolates studied at 85% of similarity. It was not evaluated, however, in terms of time, when this diversification began to occur. The amount of genetic diversity therefore appeared considerably higher in treatments that involved more diversified crop rotation (populations 2, 3 and 4) than in continuous double crop of soybean and wheat (population 5) and native uncropped soil (Population 1). Genetic variability among isolates of *M. phaseolina* could be due to fusion of vegetative cells favoring heterokarions or parasexual recombination between nuclear genes (Carlile, 1986). Despite the asexual nature of this pathogen, the levels of genetic variability observed here were responsible for the occurrence of clusters. An important observation came from Chen & McDonald (1996) who demonstrated that a *Mycosphaerella graminicola* population was able to change within three years from its original population, a condition that could be attributed to the sexual nature of the pathogen and also to the use of various different host genotypes. Probably the same conclusion can be drawn here, replacing genotypes with plant species. The occurrence of different hosts with variable levels of resistance to the pathogen might subject the pathogen population to high selection pressure, leading to an increase in pathotypes, as observed in this work. According to Goodwing *et al.* (2001), host specificity played an important and crucial role in speciation within the genus *Cercospora*. Additionally, Bhat and Browne (2007) demonstrated that much of the genetic variation observed in a population of *Phytophthora citricola* was associated with the host.

The management used to control a polyphagous and soilborne fungus, such as *M. phaseolina,* does not consider crop rotation. Researchers have evaluated the number of formed microsclerotia in roots as an indicator of tolerance. Knowledge of genetic variability helps conclusions to be made, since most farmers use the no-tillage system associated with crop rotation.

This study confirmed that *M. phaseolina* is a genetically variable species and can be affected by crop rotation. It also shows that there are differences between natural (non-cultivated soil) and agricultural habitats, with undisturbed areas having lower genetic diversity than the cultivated areas.

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