RAPD ANALYSIS FOR THE CHARACTERIZATION OF *Cercospora sojina* ISOLATES

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ABSTRACT

Sixteen isolates of *Cercospora sojina* Hara, which causes the frog-eye leaf spot in soybean (*Glycine max* (L.) Merrill), collected in different regions of the state of Minas Gerais, Brazil, were characterized by RAPD analysis. Seventeen random oligonucleotide primers allowed for the amplification of 89 loci, 26 of them being polymorphic. Genetic distances among the isolates based on these data varied between 6 and 76%. Isolates collected in the same area were not necessarily clustered in the same group. The data demonstrate the extensive variability of *C. sojina* in the state of Minas Gerais, and opens up the possibility of a systematic classification of this pathogen by DNA-based molecular markers.

Key words: soybean, DNA, frog leaf spot, *Glycine max*.

RESUMO

Análise de RAPD para a caracterização de isolados de *Cercospora sojina*

Dezesseis isolados de *Cercospora sojina* Hara, o agente causador da mancha olho de rã em soja, coletados em diferentes regiões do estado de Minas Gerais, Brasil, foram caracterizados por análise RAPD. Dezessete diferentes oligonucleotídeos iniciadores aleatórios usados nas reações de amplificação geraram 89 bandas de DNA, sendo 26 delas polimórficas. As distâncias genéticas calculadas com base nesses dados variaram entre 6 e 76%. Isolados coletados na mesma região não foram necessariamente incluídos no mesmo grupo. Os dados demonstram a extensa variabilidade de *C. sojina* no estado de Minas Gerais e abrem a possibilidade de uma classificação sistematizada deste patógeno por meio de marcadores moleculares de DNA.

INTRODUCTION

Frog-eye leaf spot, an important fungal disease caused by *Cercospora sojina* Hara, affects soybean in different growing regions of Brazil. The disease affects plant performance as well as seed quality (Lucena *et al.*, 1983). High humidity associated with high temperatures favor the development of the pathogen. The recent shift of soybean frontiers to the *cerrado* region (savanna-like region) and to the northern parts of Brazil and the use of susceptible cultivars have facilitated considerably the spreading of the disease. This problem has become so serious that susceptible cultivars can no longer be registered (Yorinori, 1989).

The development of resistant varieties requires a previous knowledge of the variability of soybean germplasm, the mode of inheritance of the resistance genes, and the variability of the pathogen (Pereira *et al.*, 1985). Twenty-two physiological races of *C. sojina* have been identified in Brazil. The identification of these races has traditionally been made by inoculation of a differential cultivar series with different isolates of the pathogen. However, direct comparison of data obtained by different research groups may be misleading due to the lack of uniformity of the differential series (Yorinori, 1989).

DNA-based molecular marker analyses such as RAPD (Random Amplified Polymorphic DNA) have proven to be a powerful tool for the characterization and identification of

isolates of *Colletotrichum graminicola* (Guthrie *et al.*, 1992), *Metarhizium* sp. (Cobb & Clarkson, 1993), and *Agrobacterium* sp. (Dong *et al.*, 1992).

In this work we used the RAPD technique to characterize 16 isolates of *C. sojina* collected in different regions of the state of Minas Gerais, Brazil.

MATERIAL AND METHODS

Fungal isolates. All isolates were collected in the state of Minas Gerais and maintained at the "Departamento de Fitotecnia (UFV)" (Table 1). Monosporic cultures were grown on solid medium and agar plugs were used to inoculate 50ml of supplemented minimum medium [(K_2HPO_4, 7; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4.2H_2O$, 1; glucose, 10; and yeast extract, 1g/l; (autoclaved separately)] in 250 ml Erlenmeyer flasks. After inoculation, the flasks were kept under agitation for 10 days at room temperature. The mycelia were collected, washed with distilled water and frozen at -80 °C until use.

TABLE 1 - Origin of the 16 isolates of *Cercospora sojina* collected in the state of Minas Gerais, Brazil.

Isolate	Pathotype[1]	Origin (City)
1	Cs-04	São Gotardo
2	Cs-22	Viçosa
3	Cs-22	Presidente Olegário
4	–	Presidente Olegário
5	Cs-17	Paracatu
6	–	Monte Alegre
7	Cs-19	Presidente Olegário
8	Cs-06	Presidente Olegário
9	–	Viçosa
10	Cs-19	Presidente Olegário
11	Cs-17	Florestal
12	–	Coromandel
13	–	Viçosa
14	Cs-22	Presidente Olegário
15	–	Capinópolis
16	–	São Gotardo

[1] Silva Neto *et al.* (*in press*).
– Not classified.

DNA extraction. Total DNA was extracted from the mycelia by a modified cetyltrimethylammonium bromide (CTAB) method (Schäfer & Wöstmeyer, 1992). Briefly, 200 mg of mycelium were pulverized with liquid nitrogen in a mortar and pestle. The powder was transferred to a 1.8-ml microfuge tube and extracted for 1h at 65 °C with 0.8 ml of pre-warmed (65 °C) extraction buffer (100mM Tris-HCl, pH 8, 20mM EDTA, 1.4M NaCl, 2% CTAB, 1% 2-mercaptoethanol). Proteins were then extracted with one volume of chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with one volume of cold isopropanol, washed with 70% ethanol, vacuum-dried, resuspendend in a TE buffer (10mM Tris-HCl, pH 8, 0.1mM EDTA) containing RNAase A (40 µg/ml), and incubated for 30 min at 37 °C for RNA degradation. DNA concentration was estimated by visual comparison with DNA standards in agarose gels.

DNA amplification. Amplification reactions of 25 µl contained: 10mM Tris-HCl, pH 8, 50mM KCl, 2.5mM $MgCl_2$, 0.1mM of each deoxynucleoside triphosphate, 0.1mM of one oligonucleotide decamer primer (Operon Technologies, CA, USA), 30 ng of mycelial DNA, and one unit of Taq DNA polymerase. The reactions were conducted in a thermocycler model 9600 from Perkin-Elmer Cetus (Norwalk, CT, USA) programmed for 40 cycles, each consisting of one denaturation step (94 °C for 15 sec), one annealing step (35 °C for 30 sec), and one extension step (72 °C for one min). After the 40th cycle one extra extension step was performed for 7 min at 72 °C. Amplification products were then loaded on 1.2% agarose gels containing ethidium bromide, examined under UV light and photographed with polaroid film type 667. The 11 primers that revealed polymorphisms in the amplification reactions and their respective sequences (5' to 3') are as follows: OPA-01 (CAGGCCCTTC), OPA-07 (GAAACGGGTG), OPA-11 (CAATCGCCGT), OPB-07 (GGTGACGCAG), OPE-14 (TGCGGCTGAG), OPE-15 (ACGCACAACC), OPG-14 (GGATGAGACC), OPH-05 (AGTCGTCCCC), OPH-12 (ACGCGCATGT), OPK-12 (TGGCCCTCAC), and OPL-14 (GTGACAGGCT).

Data analysis. Only the most intense and reproducible DNA bands were considered for analysis. They were scored as 1 (for presence) and 0 (for absence). Pairwise genetic distances among the isolates were calculated by the complement (1 - S_{ab}) of the Dice similarity index (Dice, 1945) which is defined by:

$$S_{ab} = \frac{2n_1}{2n_1 + n_2 + n_3}, \text{ where:}$$

S_{ab} = similarity between individuals a and b
n_1 = number of bands present in a and b
n_2 = number of bands present in a and absent in b
n_3 = number of bands present in b and absent in a.

These data were used to cluster the isolates into defined groups based on the single linkage method with the statistical software STATISTICA for Windows, release 5.0.

RESULTS AND DISCUSSION

PCR amplification reactions of DNA samples from the 16 isolates of *C. sojina* with 17 different oligonucleotide primers generated a total of 89 loci (not shown). The eletrophoretic pattern of DNA amplification products obtained with primer OPA-11 is presented (Figure 1). Eleven of these primers were informative, revealing 26 polymorphic loci which allowed for the identification of each isolate by a specific amplification pattern similar to a bar code (Table 2). These patterns not only identify the isolate but also give an idea about the pathogen variability within a certain area. Several research groups have successfully used the RAPD technique for characterization and differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races (Assigbetse *et al.*, 1994), the entomopathogenic fungus *Metarhizium* sp. (Cobb & Clarkson, 1993), pathogenic strains of *Agrobacterium* (Dong *et al.*, 1992), isolates of *Colletotrichum graminicola*

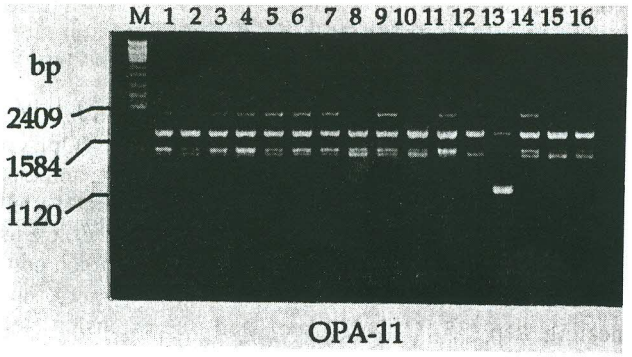

FIG. 1 - Electrophoretic pattern of DNA amplification products of 16 isolates of *Cercospora sojina* obtained from primer OPA-11. Lanes are as follows: M, lambda phage DNA digested with *Bam*HI, *Hin*dIII and *Eco*RI (size markers), 1 through 16, isolates as identified on Table 1.

(Guthrie *et al.*, 1992), isolates of the oilseed rape pathogen *Phoma lingam* (*Leptosphaeria maculans*) (Schäfer & Wöstmeyer, 1992), and others.

The amplification products were used to calculate the genetic distances among the isolates (Table 3). These distances varied from 6 to 76%. The most divergent among the genotypes was isolate 13, an isolate collected in Viçosa, MG, which has not been classified into a pathotype group. The shortest genetic distance between this isolate and the other materials was 50% and the largest, 76% (Table 3).

To better understand the genetic relationship among the isolates, they were clustered into defined groups based on the genetic distances among them (Figure 2). This type of analysis confirmed that isolate 13 is, indeed, distinct, forming a group apart from the other genotypes. Unfortunately only nine out of 16 analyzed isolates had been classified into pathotypes with a differential soybean cultivar series (Table 1). This limit prevented a proper comparison among the groups define the RAPD analysis and the ones defined by convention means. Despite this limitation, our data clearly demonstrate the extensive variability within *C. sojina* in the state of Minas Gerais even with the reduced number of isolates analyzed. They also demonstrate that, in many instances, isolates collected in the same area (e.g. isolates 2, 9, and 13) are genetically more diverse than isolates collected in distinct areas (e.g. isolates 2 and 7) (Table 3 and Figure 2).

Comparisons among DNA patterns of *Fusarium oxysporum* f. sp. *vasinfectum* isolates belonging to the same physiological race have already allowed for the identification of primers which are able to precisely distinguish among different races (Assigbetse *et al.*, 1994). The use of molecular markers will certainly be a more rational and precise method to identify pathotypes without the need for time consuming inoculation procedures.

TABLE 2 - Polymorphic amplification products obtained from 11 different primers.

Primer / band	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
OPA-01/a	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
OPA-01/b	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OPA-01/c	1	1	1	0	0	1	0	1	1	0	1	0	1	0	0	1
OPA-07/a	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1
OPA-07/b	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
OPA-07/c	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
OPA-11/b	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OPE-14/a	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OPE-14/b	0	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1
OPE-15/a	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OPG-14/c	0	1	0	0	0	0	1	1	0	0	1	1	0	0	1	0
OPG-14/d	0	1	0	1	1	0	0	0	0	1	0	0	0	0	1	1
OPH-05/a	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OPH-12/a	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
OPH-12/b	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
OPH-12/c	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
OPH-12/d	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0
OPH-12/e	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
OPK-12/a	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1
OPL-14/a	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
OPL-14/b	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

(1) - presence of DNA band.
(0) - absence of DNA band.

TABLE 3 - Pairwise genetic distances (%) among isolates of *Cercospora sojina* based on RAPD data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
2	20														
3	10	9													
4	17	15	12												
5	24	15	12	6											
6	19	23	13	27	20										
7	21	6	10	16	16	24									
8	10	9	6	12	19	20	10								
9	14	12	10	10	16	24	13	10							
10	26	23	20	7	13	36	24	20	17						
11	21	19	16	16	16	17	20	10	13	24					
12	28	31	29	29	36	23	26	21	33	31	26				
13	61	53	51	59	65	70	57	52	50	63	64	76			
14	11	16	7	7	13	21	10	7	10	14	17	23	56		
15	33	23	27	13	13	36	24	20	24	7	17	31	70	21	
16	21	12	16	10	16	31	20	16	7	10	20	33	50	17	17

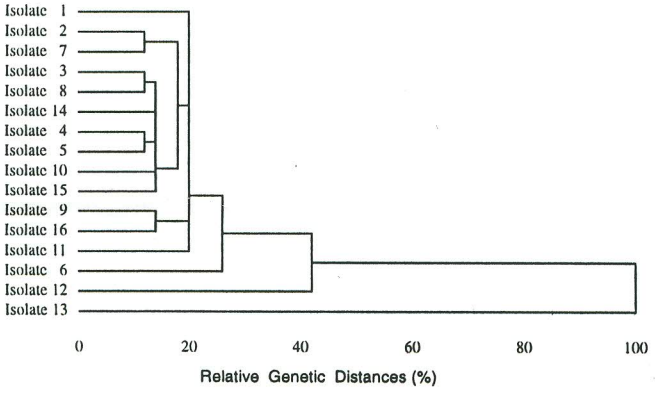

FIG. 2 - Cluster analysis based on pairwise genetic distances among isolates of *Cercospora sojina*.

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