

DIFFERENTIATION OF *COLLETOTRICHUM TRUNCATUM* ISOLATES BY RANDOM AMPLIFIED POLYMORPHIC DNA

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ABSTRACT

Isolates of *Colletotrichum truncatum*, the causal agent of soybean anthracnose, were collected at distinct areas in Brazil, from soybean stems (4 isolates) and crotalaria (1 isolate). Purified DNA from each isolate was amplified by the random amplified polymorphic DNA (RAPD) technique with the aid of arbitrary oligonucleotide primers. Amplification products visualized in agarose gels

showed specific patterns (fingerprints) for each isolate. Genetic dissimilarities and cluster analyses showed that the five isolates could be separated into three distinct groups. These results confirm RAPD as a useful technique to study genetic variability among isolates of *C. truncatum*.

Key words: *Colletotrichum truncatum*, anthracnose, soybean, RAPD, molecular markers.

RESUMO

Diferenciação de isolados de *Colletotrichum truncatum* através da amplificação ao acaso de DNA Polimórfico (RAPD).

Cinco isolados de *Colletotrichum truncatum* foram obtidos de hastes infectadas de soja (4) e crotalária (1), coletadas em Londrina (PR), Ponta Grossa (PR) e Cândido Mota (SP). Após isolamento e desenvolvimento em meio líquido (batata-dextrose), o micélio foi utilizado para extração de DNA. Amostras de DNA de cada isolado foram amplificadas utilizando-se "primers" de sequências arbitrárias, pela técnica de amplificação ao acaso de DNA polimórfico (RAPD). Géis de agarose, após separação eletroforética dos produtos de amplificação, mostraram

bandas polimórficas que permitiram avaliar a dissimilaridade genética entre os cinco isolados. Estes podem ser agrupados em três grupos distintos de acordo com os perfis apresentados. Os isolados GL-614 e Preto apresentaram uma distância genética de 6% entre si; a distância genética entre os isolados Ponta Grossa e Cândido Mota foi de 8%. O isolado Crotalária diferiu de todos os outros, apresentando uma distância média em torno de 21% com relação aos outros isolados.

INTRODUCTION

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Soybeans are attacked in the field by a number of pathogens that may cause yield reduction as well as seed decay. More than 30 diseases have already been described

on this crop, causing severe losses to farmers in several regions of the world.

The most common pathogen associated with soybean anthracnose is *Colletotrichum truncatum* (Schw.) Andrus & Moore (Sinclair, 1988). However, the disease may be

caused by other species of *Colletotrichum* such as *C. destructivum* O'Gara [teleomorph *Glomerella glycines* (Hori) Lehman & Wolf], *C. gloeosporioides* (Penz.) Sacc. (teleomorph *G. cingulata* (Ston.) Spauld. & Scherenk), and *C. graminicola* (Ces.) Wilson (teleomorph unknown).

Soybean anthracnose is considered an important disease in tropical and sub-tropical regions of Brazil (Henning, 1984; Almeida, 1990). This disease is severe in these areas, especially when precipitation and relative humidity is high. In addition to yield reduction, *C. truncatum* may affect seed quality (França Neto and West, 1989). Common symptoms during the pod stage include lesions on the stems and petioles, pod abortion and seed decay.

Breeding programs to solve this problem have found difficulties due to lack of information on the genetic variability of the pathogen, which may interfere with the screening for resistant genotypes.

Tests based on pathogenicity to classify pathotypes do not guarantee that individuals in the same group are genetically homogeneous. A modern technique, described by Mullis & Faloona (1987) based on the polymerase chain reaction (PCR) made possible the distinction between aggressive and non-aggressive isolates of *Phoma lingam* (Schäfer & Wöstemeyer, 1992).

Since its introduction, PCR has undergone modifications that improved the technique and expanded its application. A constraint in the use of PCR was the need of previous knowledge of specific sequences in the target DNA required to specify the primers. A modification in this technique was described by Williams *et al.* (1990) and called random amplified polymorphic DNA (RAPD) which overcame this problem and has added an important contribution as a diagnostic tool. It has been demonstrated that RAPD is an effective and reliable approach to study genetic variability of fungi, bacteria, plants and animals (Schäfer & Wöstemeyer, 1992; Dong *et al.*, 1992; Guthrie *et al.*, 1992).

The main goal of this study was to establish the genetic variability among five isolates of *C. truncatum*, in Brazil, for which morphological differences in culture were demonstrated previously (not published).

MATERIALS AND METHODS

Fungal isolates. Soybean stems showing typical lesions of anthracnose were collected in the states of Paraná and São Paulo, Brazil. Stem pieces measuring 3 to 5 mm were surface sterilized and transferred to Petri dishes containing PDA medium and streptomycin (1.2 ppm). Four cultures were established for this work. An additional culture was isolated from *Crotalaria sp.* and also identified as *Colletotrichum truncatum*. Agar plugs containing small pieces of the fungal colonies were then transferred to a liquid culture medium (potato, dextrose, streptomycin) and incubated in a rotatory shaker, at room temperature, for 10 to 12 days. Mycelia were harvested, blot dried in filter paper, and kept at -20 °C until use.

DNA extraction. DNA was extracted based on a modified cetyltrimethylammonium-bromide (CTAB) procedure (Schäfer and Wöstemeyer, 1992). The mycelium (200 mg) was ground under liquid nitrogen in a prechilled mortar and pestle. The resultant powder was mixed with 800 µl of a preheated solution (65 °C) containing 2% CTAB, 100 mM Tris-HCl (pH 8.0), 20mM EDTA, 1.4 M NaCl and 1% 2-mercaptoethanol. After incubation for 1 h at 65 °C, proteins were extracted twice with one volume of chloroform-isoamyl alcohol 24:1 (v/v). The aqueous phase was transferred to a clean tube, and the nucleic acids were precipitated with one volume of cold 2-propanol. After 1 h incubation at -20 °C the sample was centrifuged for 10 min at 12,000 g. The pellet was washed once with 70% (v/v) ethanol, vacuum-dried, resuspended in 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA containing RNase (40 µl/ml), and incubated at 37 °C for 20 min. DNA concentration was estimated by comparing band intensities with known DNA standards on an agarose gel.

DNA amplification. Forty-three different oligonucleotide decamers (Operon Technologies Inc., Alameda, CA, USA) were used to prime fungal DNA synthesis. Amplification reactions contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, 0.1 mM each of dATP, dCTP, dGTP, and dTTP, 0.4 µM of a single primer, ca. 30 ng of DNA, and 1 unit of Taq DNA polymerase in a total volume of 25 µl. Reactions were conducted in a thermocycler model 9600 (Perkin Elmer Cetus, Norwalk, Conn., USA) programmed for 40 cycles, each consisting of a denaturation step of 15 sec at 94 °C, an annealing step of 30 sec at 35 °C, and an elongation step of 1 min at 72 °C. After the 40th cycle, an extra elongation step of 7 min at 72 °C was performed. Amplification products were analyzed electrophoretically in 1.2% agarose gels in TBE buffer (90 mM Tris-borate, 1 mM EDTA). Gels were stained with ethidium bromide and photographed under UV light.

Data analysis. DNA bands corresponding to the amplification products were scored as 1 (presence) or 0 (absence), and a matrix (isolate x amplified loci) was generated. Only reproducible bands were considered, normally the most intense ones. A statistical software (SAEG) developed at the Federal University of Viçosa was used to group the isolates in clusters based on the genetic distances among them.

RESULTS AND DISCUSSION

Identification of resistant soybean genotypes to anthracnose and transfer of resistant genes to adapted cultivars are dependent on knowledge about the causing agent of this disease. Several pathotypes of *C. truncatum* have been identified so far (Henning, 1984; Almeida, 1990). However, this classification is based on few morphological features and on pathogenicity tests which often have poor resolution. As an initial effort to systematize the classification of *C. truncatum* into different races, we used the RAPD technique and determined genetic

distances among five isolates of *C. truncatum* collected in different regions in Brazil.

DNA extracted from mycelia of each isolate was amplified with the aid of 43 random primer decamers. Two hundred and thirteen reproducible DNA bands were obtained, an average of 5 bands per primer. Figure 1 illustrates typical patterns obtained for DNA amplification with primers OPD-08, OPH-08, OPH-14, and OPH-19. Seventy-two of the amplified bands were polymorphic between at least two of the isolates (Table 1). These differences allowed the calculation of pairwise genetic distances among the isolates by dividing the number of distinct bands between two given isolates and the total number of amplification products (Table 2). The genetic distance between isolates Preto and GL-614 was 6% (Table 2), with only 12 different loci among 213 scored (Table 1). Sixteen loci were distinct between isolates Ponta Grossa and Cândido Mota, i.e., a genetic distance of 8% (Table 2). On the other hand, isolate Crotalária differed from all the others, with an average genetic distance of 21% in relation to the other four isolates (Tables 1 and 2). Cluster analyses

based on the genetic distances (Table 2) separated the 5 isolates into 3 distinct groups (Figure 2).

These data confirm the usefulness of the RAPD technique for studies with fungi as pointed by Elliot *et al.*

TABLE 2. Genetic dissimilarities between the five *C. truncatum* isolates. Matrix shows the number of polymorphic bands between two isolates divided by the total number of bands scored. Results are given in percentage.

	1	2	3	4	5
1. Preto	—				
2. Cândido Mota	16	—			
3. GL-614	6	17	—		
4. Ponta Grossa	21	8	20	—	
5. Crotalária	19	22	21	23	—

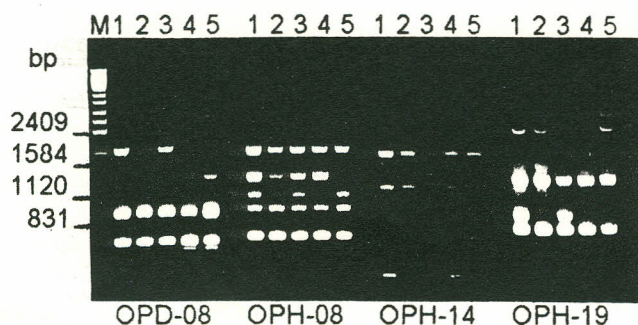


FIG. 1. Amplification of genomic DNA from five isolates of *C. truncatum* with random decamer primers OPD-08, OPH-08, OPH-14, and OPH-19. Lanes are as follows: M, DNA size markers; 1, isolate Preto; 2, isolate Cândido Mota; 3, isolate GL-614; 4, isolate Ponta Grossa; 5, isolate Crotalária. Four of the size markers within the range of the amplified fragments are indicated on the left side of the figure (bp).

TABLE 1. Pairwise differences between the *C. truncatum* isolates. Matrix shows the number of distinct DNA bands between each pair of isolates obtained by the RAPD technique.

	1	2	3	4	5
1. Preto	—				
2. Cândido Mota	35	—			
3. GL-614	12	37	—		
4. Ponta Grossa	45	16	43	—	
5. Crotalária	40	47	44	49	—

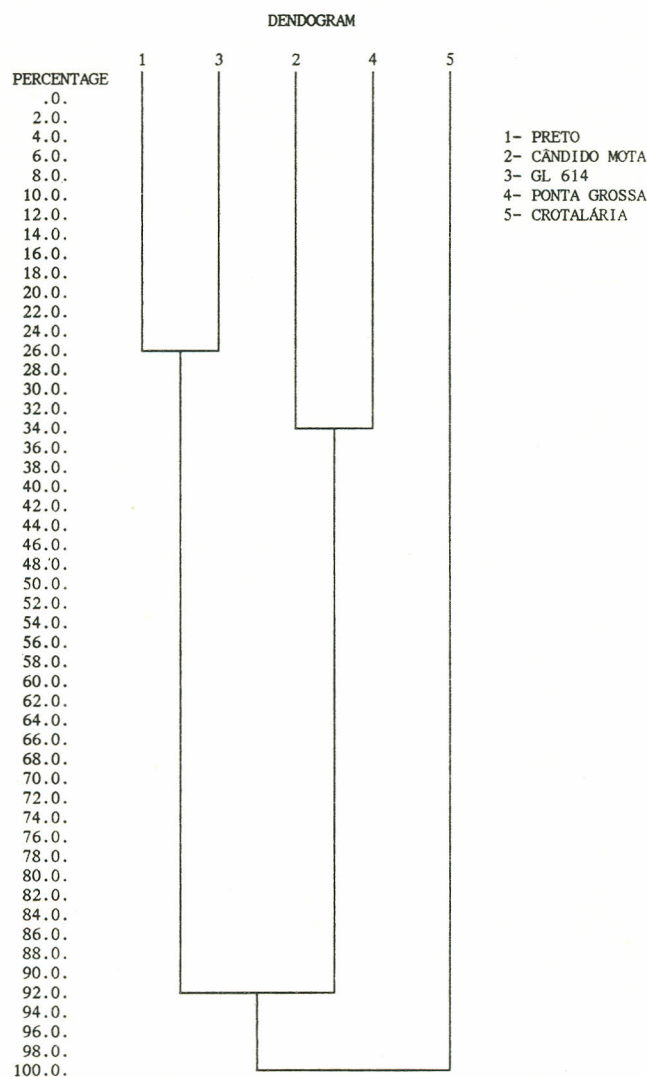


FIG. 2. Dendrogram showing relative genetic distances among the five *C. truncatum* isolates.

(1993) and Doudrick *et al.* (1993). The high resolution and speed of the technique associated with classical approaches used so far should enable a systematic classification and study of several races of pathogens which affect different plant species.

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