Pathogenic and Molecular Characterization of Brazilian Isolates of *Sphaceloma manihoticola*

E. Alvarez, J. F. Mejía, and T. Losada
International Center for Tropical Agriculture (CIAT), A.A. 6713, Cali, Colombia; Universidad Nacional de Colombia (UNAL), A.A. 237, Palmira, Colombia;
Instituto Agronómico de Campinas (IAC), A.A. 28 - 13001 - 970, Campinas (SP) - Brasil.

INTRODUCTION

Superelongation, caused by the fungus *Sphaceloma manihoticola*, sexual stage *Elsinoe brasiliensis*, is a major cassava disease in Central America, Colombia, Brazil, and Venezuela. Typical symptoms include weak stems; dieback; defoliation; necrotic leaf spots; cankers on leaf veins, petioles, and stems; leaf and stem distortion; and internode elongation in severely affected plants. Crop losses can be as high as 80%. By using molecular techniques, we aimed to determine (1) the pathogenic variability of fungal isolates from central-southern Brazil, and (2) their genetic variability.

MATERIALS AND METHODS

Twenty isolates were collected in central-southern Brazil from cassava plants affected with superelongation disease and from *Euphorbia heterophylla* , another Euphorbiaceae species. Collection sites were Paranavaí (Paraná); Campo Grande (Mato Grosso do Sul); and Campos Novos, Assis, Platina, Cândido Mota, Echaporã, Conchal, and Campinas (all in São Paulo) (Table 1). Isolates were conserved at 4ºC in inclined vials containing natural PDAn (natural).

Pathogenicity analysis. The cassava varieties M Bra 703 (susceptible) and M Bra 12 (resistant) were inoculated with these isolates and incubated at 30°C and 100% relative humidity for 5 days, then at 30°C and 98% until evaluations at 7, 14, and 28 days after inoculation. An experimental design with eight replicates was used, where the main plots were varieties and subplots were isolates, and the experimental unit two plants. Isolates were clustered into four groups according to disease severity on the two inoculated varieties, using the Ward minimum variance analysis. Area under the disease progress curve (AUDPC) was also calculated.

DNA extraction. Isolates were placed in a liquid medium (obtained by filtering V8 juice), then incubated under constant agitation for 15 days at 25°C. Colonies were harvested according to the Lee and Taylor protocol, modified as follows: 400 ml solution of phenol, chloroform, and isoamyl alcohol (25:24:1) was used to precipitate the DNA, which was then centrifuged at 10,000 rpm for 15 min. The pellet was resuspended in 100 µl of TE and incubated with 10 µl of ribonuclease (10 mg ml^{-1}) at 37°C for 30 min. DNA concentration was determined with a fluorometer (Hoefer DyNA Quant 200).

Amplifying rDNA by PCR. The internal transcribed spacer (ITS) region of the gene 5.8S from ribosomal DNA was amplified, using a thermocycler (MJ Research, Watertown, MA), adjusted to the following program: (1) 95°C for 3 min; (2) 57°C for 30 s; (3) 72°C for 2 min; (4) 95°C for 30 s; (5) 24 cycles of steps 2 to 4; (6) 50°C for 30 s; and (7) 72°C for 10 min. The amplified segments were analyzed by electrophoresis in a gel comprising 1.5% agarose, TBE 0.5× buffer (Trizma-base, boric acid, EDTA, pH 8.0), and ethidium bromide at 10 mg ml^{-1}. The electrophoresis chamber was maintained at a constant 90 V for 90 min. A 100-bp marker was included in each gel. A photo was taken under ultraviolet light, using an Eagle eye II image analyzer (Stratagene, La Jolla, CA).

Restriction enzymes. For restriction analysis, 11 enzymes were used. A sample of 15 µl of the product from the PCR reaction was taken, and 2 µl of a buffer of 10× enzyme and 1 µl of restriction enzyme were added. The suspension was incubated for 16 h at an average temperature of 37°C, after which 2 µl of running buffer was added. A gel, comprising 1.5% agarose, TBE 1× buffer, and ethidium bromide at 10 mg ml^{-1}, was then placed in an electrophoresis chamber for 2 h at a constant 90 V.

Random amplified polymorphic DNA (RAPD). The RAPD technique was identical to that described for the PCR but, instead of using ITS primers, 12 decaprimers that randomly amplified genome fragments of DNA samples of *S. manihoticola* were used.

Data analysis. The electrophoretic patterns of DNA were quantified, using as criterion the presence or absence of bands. To estimate genetic relationships between isolates, a phylogenetic tree was constructed, using the UPGMA method with the SAHN and TREE options of the NTSYS-pc 2.01 (F.J. Rohlf, Exeter Software, New York).

RESULTS

Symptoms appeared on both cassava cultivars M Bra 703 (susceptible in the field) and M Bra 12 (resistant). M Bra 703 was susceptible to 68.4% of pathogen isolates, intermediately resistant to 26.3%, and tolerant of 5.3%. M Bra 12 was tolerant of 36.8% of isolates, intermediately resistant to 36.8%, and susceptible to 26.4% (Figure 1).

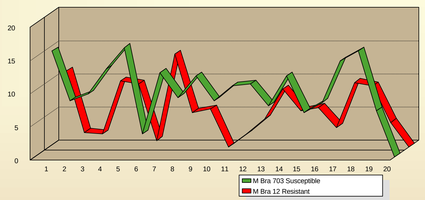

Figure 1. Two varieties of cassava (*Manihot esculenta* Crantz) compared for their susceptibility to superelongation disease. AUDPC = area under the disease progress curve.

The ITS region was amplified with primers ITS4 and ITS5. For all isolates, a homogeneous band of about 645 bp (Figure 2) was observed. The product generated by the PCR technique was digested with the endonucleases *Cfo*I, *Msp*I, *Hinf*I, *Hae*III, and *Taq*I, presenting similar patterns of bands for all isolates and for each enzyme (Figure 3). Twelve primers were evaluated, of which OPA-01, OPA-02, and OPA-03 were selected because they showed reproducible bands in most isolates (Figure 4). A total of 120 bands were read, and a dendrogram constructed, based on 41 polymorphic bands. The statistical analysis showed the population of 20 isolates clustering into three genetic groups (Dice similarity index of 0.60). Clusters were fused, using the method of the unpaired group mean average. The similarity scale corresponded to the average similarity at which clusters fuse (Figure 5).

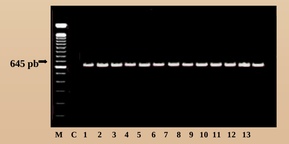

Figure 2. Amplification of the ITS4 and ITS5 regions of ribosomal DNA from the fungus *Sphaceloma manihoticola*. M = marker (100 bp); C = control; lanes 1 to 13 = isolates from Campos Novos, Paranavaí, Assis, Platina, Candido Mota, Conchal, and Echaporá, Brazil; lane 13 = Campos Novos.

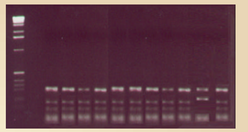

Figure 3A. Electrophoresis of ITS fragments digested with the endonuclease *Msp*I. M = marker (1 Kb); C = control; lanes 1 to 11 = isolates from Campos Novos, Paranavaí, Candido Mota, Conchal, and Echaporá, Brazil.

Figure 3B. Electrophoresis of ITS fragments digested with the endonuclease *Cfo*I. M = marker (100 bp); C = control; lanes 1 to 11 = isolates from Campos Novos, Paranavaí, Candido Mota, Conchal, and Echaporá, Brazil.

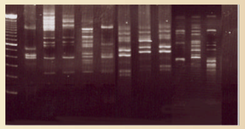

Figure 4. Patterns of bands obtained with the primers OPA-01 (lanes 1 to 4), OPA-02 (lanes 5 to 7), and OPA-03 (lanes 8 to 11). Isolates were collected from Campos Novos, Paranavaí, Conchal, and Platina, Brazil. M = marker (100 bp); C = control.

Table 1. Origins of *Sphaceloma manihoticola* isolates used to study the fungus's genetic and pathogenic diversity. The fungus causes superelongation disease in cassava (*Manihot esculenta* Crantz).

Isolate	Germplasm	Plant part	Geographic origin[a]
1	Euphorbia heterophylla	Stem	Campos Novos, SP
2	Fibra	Leaf	Campos Novos, SP
3	IAC-13	Leaf	Campos Novos, SP
4p	IAC-13	Petiole	Paranavaí, PR
5	IAC-13	Leaf	Assis, SP
6	Euphorbia heterophylla	Stem	Conchal, SP
7h	Roxinha	Leaf	Platina, SP
7p	Roxinha	Petiole	Platina, SP
8	IAC-13	Leaf	Candido Mota, SP
9	IAC-12	Leaf	Conchal, SP
10	B. S. Catarina	Leaf	Conchal, SP
11p	Fibra	Petiole	Conchal, SP
12	IAC-13	Leaf	Conchal, SP
13h	Roxinha	Leaf	Echaporá, SP
14	Roxinha	Leaf	Campos Novos, SP
15	B. S. Catarina	Leaf	Campos Novos, SP
Bh	Unknown	Leaf	Campo Grande, MS
25h	F 1047	Leaf	Campinas, SP
26h	Clone under selection	Leaf	Campinas, SP

a. Brazilian states: SP = São Paulo; PR = Paraná; MS = Mato Grosso do Sul.

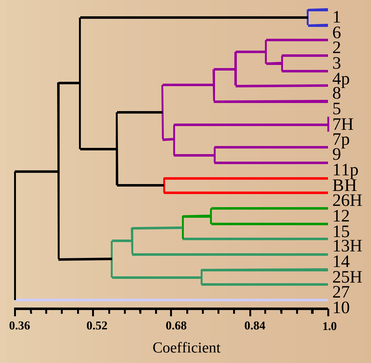

Figure 5. Similarity dendrogram of 20 isolates of *Sphaceloma manihoticola*, a fungus that causes superelongation disease in cassava (*Manihot esculenta* Crantz).

BIBLIOGRAPHY

Alvarez E; Molina ML. 2000. Characterizing the *Sphaceloma* fungus, causal agent of superlongation disease in cassava. Plant Dis 84(4):423–428.

Lee SB; Taylor JW. 1990. Isolation of DNA from fungal mycelia and single spores. In: PCR protocols. p 282-287.

Zeigler RS; Alvarez E; Lozano JC. 1983. Characteristics of cassava resistance to superelongation disease (*Elsinoe brasiliensis*). Trop Pest Manage 29(2):148–158.