Assessing Virulence and Genetic Variability of *Sphaceloma manihoticola*, causal agent of superelongation in cassava (*Manihot esculenta*), in Brazil and Colombia, using RAMS and AFLP

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INTRODUCTION

Sphaceloma manihoticola is the causal agent of superelongation, one of the diseases that most limits cassava production. Studies have demonstrated pathogenic and genetic variability between isolates of Brazil and Colombia using RFLP and RAPD. The level of polymorphism can be visualized more precisely, however, with molecular markers such as amplified fragment length polymorphisms (AFLP) and random amplified microsatellites (RAMS). This study aimed to develop efficient DNA fingerprinting protocols for *S. manihoticola* and to use RAMS and AFLP markers for analyzing the genetic variability in single-spore cultures of *S. manihoticola*.
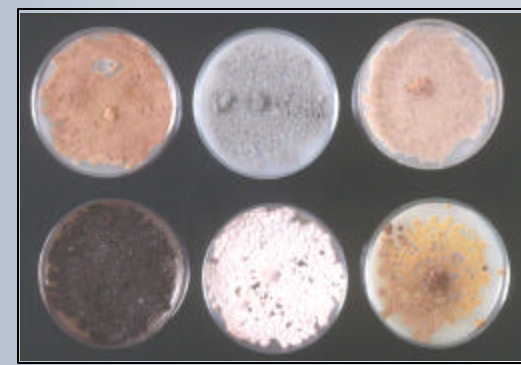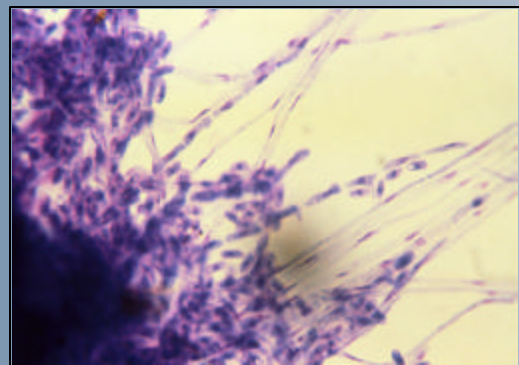
MATERIALS AND METHODS

Isolates. Twenty-five isolates were collected from cassava plants infected with superelongation at different sites in central-south Brazil and Colombia. Isolates were conserved at 4 ºC in inclined vials containing natural PDA. All isolates were pathogenic on cassava genotypes M BRA 12 and M BRA 703.

Virulence. Variation in virulence was determined by inoculating wounded plantlets of 15 cassava genotype differentials. Inocula consisted of six selected isolates from different regions of Brazil. Inoculated plantlets were incubated for 5 days at 95% relative humidity and 27 ºC, then transferred to the greenhouse and observed for symptom development at 7, 14, 21, and 28 days after inoculation. A split-plot experiment design with five replicates was used, where the main plots were the varieties and the subplots, the isolates. Each experimental unit had one cassava plants.

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

RAMS. The reaction was performed in a final volume of 25 µl that contained 0.2 mM of each nucleotide; 3 mM MgCl$_2$; 0.5 µM of each of the primers, 1X PCR buffer; and 0.625 U the enzyme *Taq* polymerase. Everything was diluted in sterilized distilled water. The PCR reactions were carried out under the following conditions: samples were denatured by 10 min incubation at 95 °C, after which 37 cycles of amplification were carried out (30 s denaturation at 95 °C, 45 s annealing at a temperature depending on the primer, and 2 min primer extension at 72 °C). The annealing temperature for each primer was as follows: CCA primer = 64 °C, CGA primer = 61 °C, GT primer = 58 °C, and ACA primer = 45 °C (Hantula *et al*, 1997). After the cycles, the reaction was ended with a 7-min extension at 72 °C. The electrophoresis chamber was maintained at a constant 90 V for 90 min.

AFLP. Isolates were evaluated using three combinations of primers: EAC/MA, EAC/MC and EG/MA (Gibco BRL, AFLP Analysis System for Microorganisms). The amplification products were mixed at equal volume, denatured at 95 °C, and 6 µl loaded into a 6% (w/v) polyacrylamide gel in 1X TBE electrophoresis buffer.

Data Analysis. Isolates were clustered into groups according to area under disease progress curve (AUDPC) for the 15 inoculated varieties, using the Ward minimum variance analysis (SAS Statistical Package, Version 6.0. Cary, North Carolina). To estimate genetic relationships between isolates, a phylogenetic tree was constructed, using the unweighed paired grouped mean arithmetic average method with the SAHN and TREE options of the NTSYS-pc 2.01 (F.J. Rohlf, Exeter Software, New York). *

RESULTS

Twenty-five single-spore cultures of *S. manihoticola* from central-south Brazil and NE Colombia were used to study the variation in virulence and genetics of the fungus.

Virulence. Variation in virulence was determined in the greenhouse by inoculating cassava plantlets of 15 cassava genotype differentials. A high variation in virulence was observed among the six Brazilian isolates because these were grouped into 5 pathotypes (Figure 1).

Five cassava genotypes were identified as differential varieties. Isolate 4p was found to infect 12 of the 15 genotypes and was considered as the most virulent. Isolate Bh was the least virulent, infecting only five differentials. Of the cassava genotypes, M Bra 1044, M Bra 237, and M Col 2215 were ranked as highly susceptible, the most susceptible being M Bra 237. The most tolerant genotype was CM 2177-2.
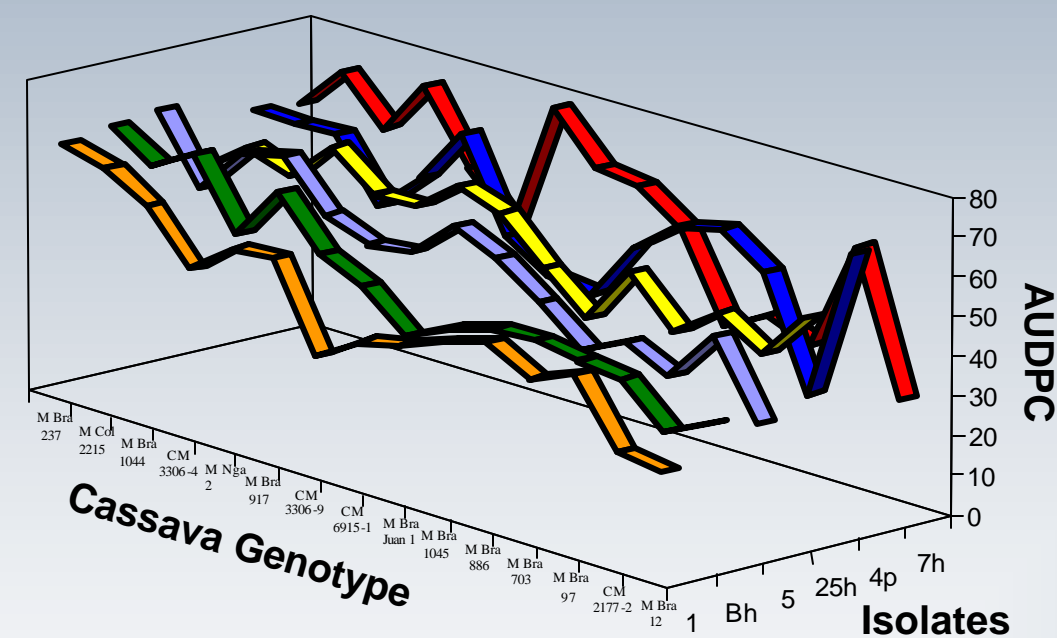

Figure 1. Identification and virulence phenotypes of six isolates of *Sphaceloma manihoticola*, causal agent of superelongation diseases in cassava, used to inoculate 15 cassava genotypes in the greenhouse.

Genetic Variability. AFLP (three primer combinations) and RAMS (four primers) techniques were optimized for *S. manihoticola* and reproducible bands were observed in most isolates (Figures 2 - 4). The results obtained demonstrate polymorphism among isolates, indicating the pathogen's genetic variability. Polymorphism with these single primers differentiated the isolates, and five genetic groups were distinguished among the isolates. Results are shown in Figure 2, where the patterns obtained with the ACA primer are presented. Similar results were obtained with other primers (Figure 3). Based on RAMS and AFLP, Brazilian isolates were not found in Colombia and vice versa. The results suggests all sample sites have their own genetic group of *S. manihoticola*. The correlation observed between geographical origin and polymorphism detected by RAMS and AFLP was +0.83. The dendrogram constructed by both molecular techniques evidences the separation of isolates into five groups with a similarity level of 0.7 (Figure 5).
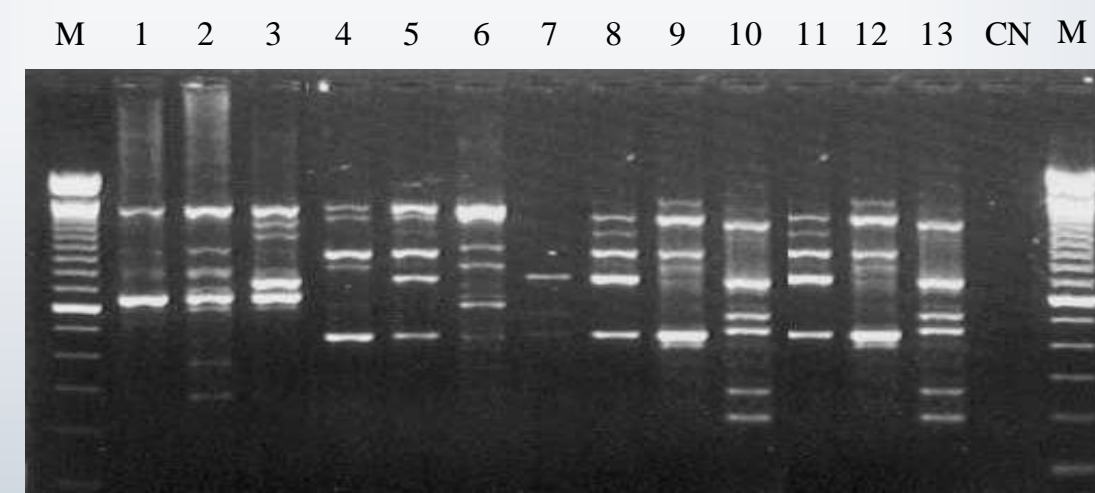

Figure 2. Patterns of bands obtained with the RAMS technique using ACA primer. Isolates were collected from (1 to 7) Campos Novos, Paranavaí, Conchal, and Platina, Brazil, and (8 to 13) Puerto López, Granada, and Carimagua, Colombia. CN = Negative Control; M = marker (100 bp).
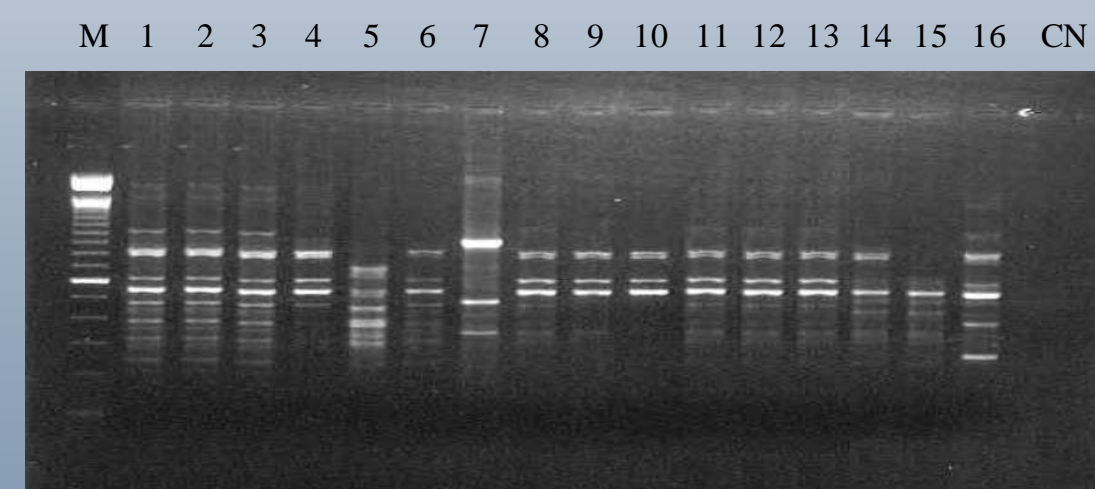

Figure 3. RAMs profiles observed within the isolates of *Sphaceloma manihoticola* with CCA primer. Isolates were collected from (1 to 7) Campos Novos, Paranavaí, Conchal, and Platina, Brazil, and (8 to 16) Puerto López, Granada, and Carimagua, Colombia. CN = Negative Control; M = marker (100 pb).
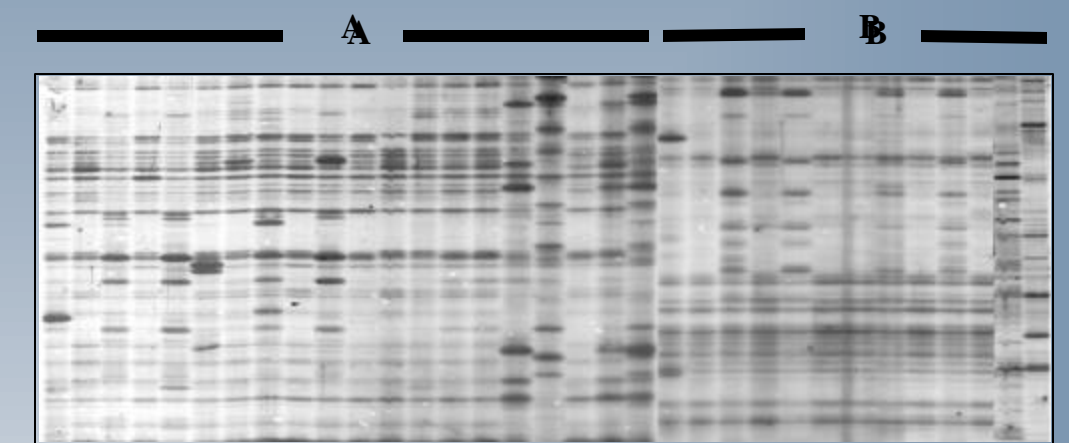

Figure 4. Illustration of *Eco*RI-*Mse*I fragments. AFLP fingerprints of *Sphaceloma manihoticola* DNA were made, using primer combinations with a single selective base for the *Eco*RI primers and two selective bases for the *Mse*I primer. Panels A and B refer to the primer combinations EAC/MA and EAC/MC, respectively.
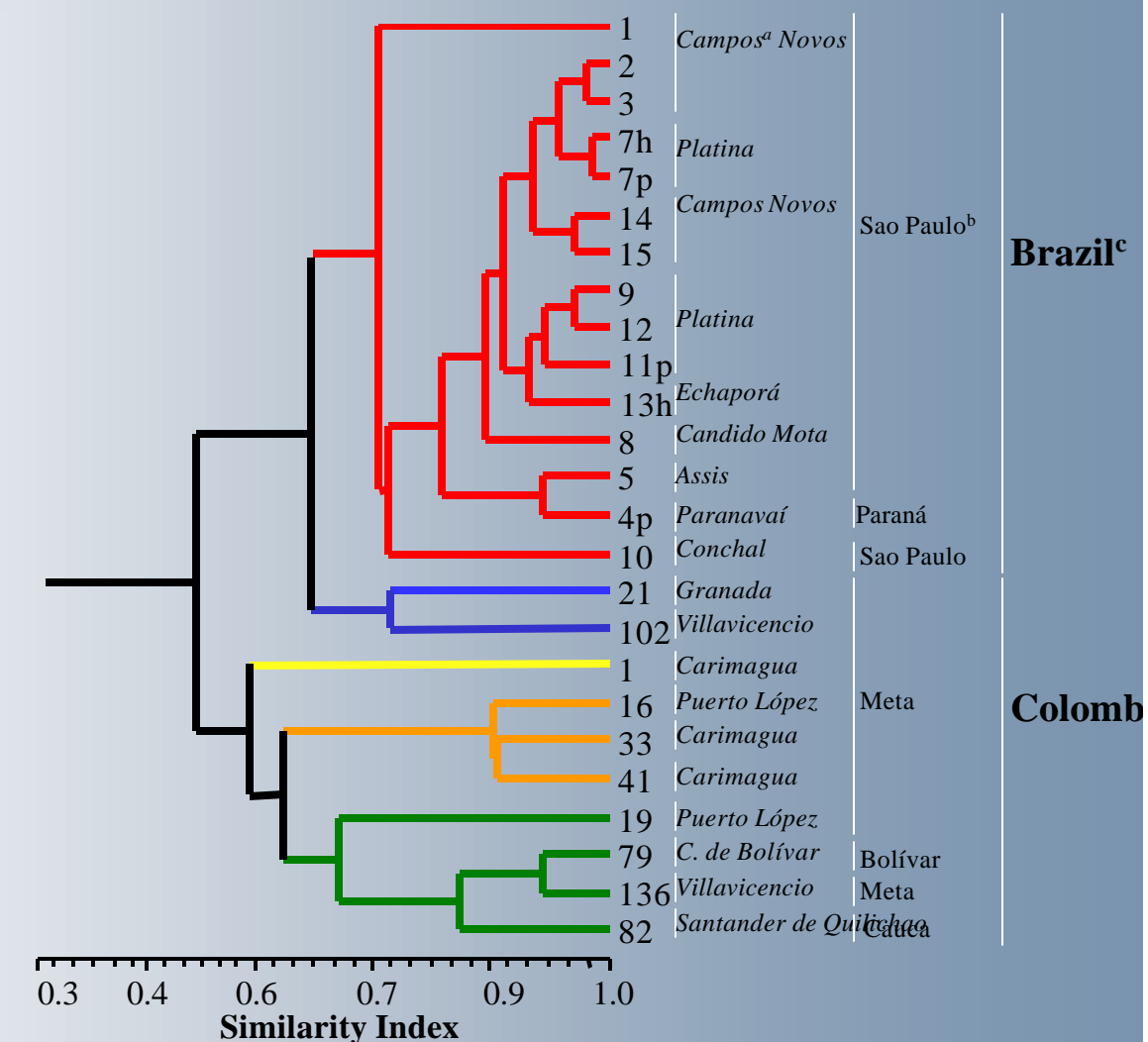

Figure 5. Similarity dendrogram (AFLP-RAMS) of 25 isolates of *Sphaceloma manihoticola*, a fungus that causes superelongation disease in cassava (*Manihot esculenta* Crantz). Bra (Brazil), Col (Colombia). [a] municipality, [b] Department, [c] Country.

CONCLUSIONS

RAMS and AFLP were tested and optimum conditions for PCR amplification were resolved. Both techniques, originally described by Hantula et al. (1997) and Vos et al. (1995), can be applied to *S. manihoticola*.

The results obtained by RAMS and AFLP provided information which was difficult or impossible to obtain on the basis of morphology alone. Wide variability in profiles reflected high polymorphism in the fungal populations. Genetic variation in the Brazilian population was very limited compared with that of Colombia. There was a high correlation between geographical origin (country and municipality) and genetic variation. There was no strong relationship between genetic and virulence markers.

Molecular markers will be useful in gaining a better understanding of the movement of pathogen populations between geographically isolated regions and effectiveness of host resistance. Further isolate sampling will be necessary to determine the pathogen's diversity.

REFERENCES

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

Hantula J; Lilja A; Parikka P. 1997. Genetic variation and host specificity of *Phytophthora cactorum* isolated in Europe. Mycol Res 101:565-572.

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

Vos P; Hogers R; Bleeker M; Reijans M; Van de Lee T; Pornes M; Frijters A; Pot J; Peleman J; Kuiper M; Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23(21):4407-4414.