

Detection of a Phytoplasma Associated with Frogskin Disease in Cassava (*Manihot esculenta* Crantz) in Colombia



E. Alvarez, J.F. Mejía, J. Loke, G. Llano, and L. Hernández
International Center for Tropical Agriculture, A.A. 6713, Cali, Colombia

INTRODUCTION

Frogskin disease (FSD) is an important disease affecting cassava roots, whose causal agent remained unknown for many years. FSD has been reported with increasing frequency in Colombia, Brazil, and Venezuela. In Colombia, for example, incidences of up to 70% have been recorded in commercial cassava fields in the production areas of Valle del Cauca, Cauca, Meta, and the North Coast. Disease symptoms consist of small, longitudinal fissures distributed throughout the root. As the roots increase in diameter, the fissures tend to heal, giving the injuries a lip form. The root cortex or epidermis presents a cork-like appearance and peels off easily. Depending on the severity of symptoms, the depth and number of lesions increase until the root becomes deformed (Figure 1).



Figure 1. (A) Healthy plants, (B) FSD-infected plant, and (C) root presenting disease symptoms.

This study evidences the existence of an association between FSD and phytoplasma. By applying molecular tools and microscopy, phytoplasma was successfully detected in FSD-infected cassava roots, leaf midribs, petioles, and peduncles.

MATERIALS AND METHODS

Plant tissue. Roots, stems, petioles, and leaf midribs of both FSD-infected and healthy cassava plants, grown in the field and greenhouse, were analyzed.

Microscopic analysis. Small pieces of tissue, about 1 mm × 2 mm, were excised and then fixed in 2%-3% glutaraldehyde/0.1M phosphate buffer. Two staining methods were used: DAPI, a nonspecific indication of DNA in sieve elements (Dyer and Sinclair, 1991), and Dienes' stain, which metabolizes the phytoplasma and creates a blue color (Deeley *et al.*, 1979). Ultra-thin sections (16 µm) of leaf midribs, petioles, peduncles, and small roots were prepared for DAPI and viewed under a fluorescence microscope. Samples consisting of ultra-thin sections (60-90 nm) were also prepared and viewed under a transmission electron microscope.

DNA extraction. Total DNA was extracted as described by Gilbertson *et al.* (1991).

Nested PCR analysis. One of the following primer pairs, P1/P7 or R16mF2/R16mR1, was used for the first amplification, with an annealing temperature of 55 °C. For the nested PCR, diluted (1:30) PCR products were used for amplification, with the primer pair R16F2n/R16R2 at an annealing temperature of 50 °C. PCR products were analyzed by electrophoresis on 1.5% agarose gel.

RFLP analyses. The amplified PCR products were digested with the restriction endonucleases *Taq* I, *Rsa* I, and *Alu* I. The restriction products were analyzed by electrophoresis on 5% polyacrylamide gel.

Cloning and DNA sequencing. Purified PCR products were ligated in pGEM-T Easy vector, which was introduced into the *Escherichia coli* strain DH5-α by electroporation at 2.4 kV/cm². Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media. Positive inserts were observed by plasmid restriction with *Eco*RI and electrophoresis in 1.5% agarose gel. Different-sized fragments were selected for sequencing by automated dideoxy sequencing (ABI Prism 377-96 DNA Sequencer), using a DNA-sequencing kit from Applied Biosystems.

Grafting. Cassava stem fragments from the highly susceptible genotype Secundina were grafted on infected cassava plants.

RESULTS

The presence of phytoplasma in different plant tissues of affected plants was confirmed by the DAPI and Dienes' staining methods and by electron microscopy (Figures 2, 3, and 4).

The specific primers R16mF2/R16mR1 and R16F2n/R16R2 were used in a nested PCR assay to detect phytoplasma. Nested PCR revealed 1.3 kb fragments in root, stem, and leaf samples from symptomatic plants (Figure 5). No fragments were obtained from healthy plants.

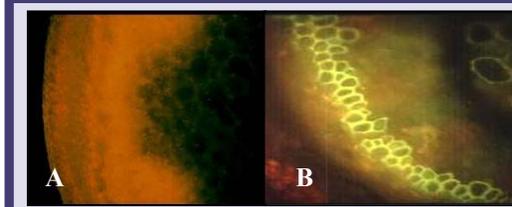


Figure 2. DAPI stain of healthy (A) and infected (B) cassava leaf midribs.

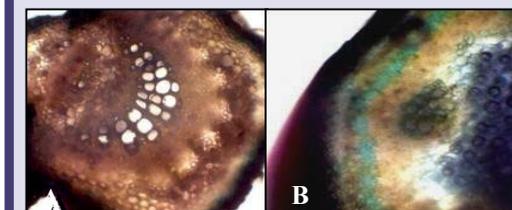


Figure 3. Dienes' stain of healthy (A) and infected (B) cassava leaf midribs.

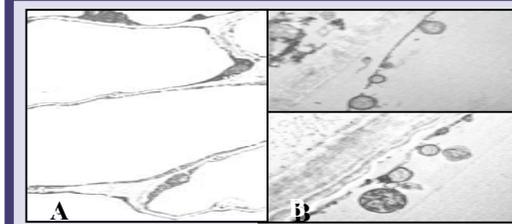


Figure 4. Electron microscopy stain of healthy (A) and infected (B) tissue.

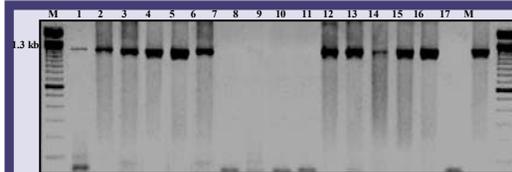


Figure 5. A 1.3-kb fragment was amplified from diseased samples by nested PCR. Lanes 1-2, infected stems; 3-4, infected petioles; 5-6, infected leaf midribs; 7, healthy roots; 8, healthy stems; 9, healthy petioles; 10, healthy leaf midribs; 11-12, infected roots; 13-14, infected stems; 15 and 17, periwinkle (*Catharanthus roseus*); lane 16, negative control; and lane M = 100 pb DNA marker.

Phytoplasma was also detected by PCR in the leaves of grafted stem fragments of infected plants under greenhouse conditions, indicating the successful transmission of the pathogen.

Sequence analysis of a cloned fragment revealed that the cassava phytoplasma was similar to the Chinaberry yellows phytoplasma (GenBank acc. no. AF495657, 16SrXIII Mexican periwinkle virescence group) and *Cirsium* white leaf phytoplasma (GenBank acc. no. AF373106, 16SrIII X-disease group), both with a sequence homology of 100% and 99% in two partial fragments with a total of 1.01 kb (Figure 6).

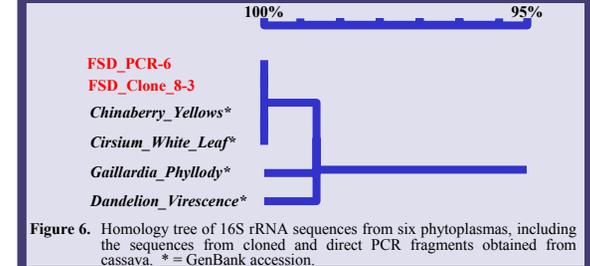


Figure 6. Homology tree of 16S rRNA sequences from six phytoplasmas, including the sequences from cloned and direct PCR fragments obtained from cassava. * = GenBank accession.

Digestion with *Taq* I, *Rsa* I, and *Alu* I of amplified products of different samples showed similar restriction patterns (Figure 7).

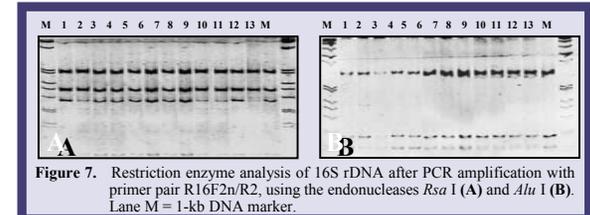


Figure 7. Restriction enzyme analysis of 16S rDNA after PCR amplification with primer pair R16F2n/R2, using the endonucleases *Rsa* I (A) and *Alu* I (B). Lane M = 1-kb DNA marker.

CONCLUSIONS

Phytoplasma was successfully detected in all FSD-infected tissues by the DAPI, Dienes' staining, electron microscopy, and nested PCR techniques. Among the methods used in this study, PCR was the most sensitive for detecting, identifying, and classifying phytoplasma.

Sequence homology from a cloned fragment, obtained from an infected cassava plant, was 100% similar to the Chinaberry yellows phytoplasma and 99% similar to that of *Cirsium* white leaf.

This is the first report of a phytoplasma being associated with FSD in cassava. These results allow us to infer the possible role played by the phytoplasma in this disease.

Future research will involve the evaluation of additional samples with other groups of enzymes as well as sequence analysis to classify the phytoplasmas. Experiments are underway to achieve remission of symptoms with the antibiotic oxytetracycline. Other research topics will include the development of specific primers for pathogen detection, vector identification, and classification of phytoplasmas associated with FSD.

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