VOL. 11, NO. 11, NOVEMBER 2016 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science

www.arpnjournals.com

DETERMINING THE PRESENCE OF THE FIG MOSAIC VIRUS (FMV) IN THREE VARIETIES OF *Ficus carica* L. IN COSTA RICA

Luis Alvarado-Marchena[1,2] Randall Chacón-Cerdas[2], Alexander Schmidt-Durán[2], Carlos Alvarado-Ulloa[2] and Dora Flores-Mora[2]
[1]Laboratorio Institucional de Microscopía, Vicerrectoría de Investigación y Extensión, Instituto Tecnológico de Costa Rica
[2]Centro de Investigación en Biotecnología, Escuela de Biología, Instituto Tecnológico de Costa Rica
E-Mail: lalvarado@itcr.ac.cr

ABSTRACT

Fig is a fruit tree with medicinal and nutritional properties. In Costa Rica, the production of this crop has been promoted in order to stimulate the agricultural diversification. However, conscious of the international problematic caused by the presence of the Fig Mosaic Virus (FMV) assays have been performed to the variety "Brown Turkey", present in Costa Rica, which presented negative results for pathogenicity. Consequently, it was considered necessary to establish standardized protocols which allow the microscopic and molecular diagnostics of other figs varieties in the country. In this context, the present research evaluates the efficiency of using diagnostic tools including diagnosis through symptoms, Transmission Electron Microscopy (TEM) and using molecular assays through PCR with previous retro transcription (RT-PCR) which detected a 302 bp fragment of the FMV RNA-1 in two varieties of fig, corresponding to "Brogiotto Bianco" and "Negro San Juan", using the cultivar "Brown Turkey" as a negative control for the pathogenicity. Through these assays it was possible to determine the differences from a cytological and molecular level in the varieties when compared to the control; evidencing the characteristic symptoms of the studied virus and the amplification of the viral fragment, and hence, indicating that these positive material require a viral removal process before being introduced in the country.

Keywords: fig, fig mosaic disease, electronic microscopy, RT-PCR, phylogenetic.

INTRODUCTION

The Fig Mosaic Disease (FMD), described by Condit and Horne in 1933, is the viral pathology of highest incidence in fig crops (*Ficus carica* L.), and it is related to the presence of Double Membrane Bodies (DMB) denominated Fig Mosaic Virus (FMV), recently classified within the Emaravirus genus of the Bunyaviridae family (Çağlayan *et al.,* 2010).

The mosaic is a characteristic symptoms of the disease, which appears mainly in juvenile leaves and sporadically in fruits (Ishikawa *et al.,* 2012b; Çağlayan *et al.,* 2009); however, there is also presence of chlorotic and necrotic stains, intravessel yellowing, as well as reddish spots associated to damage related to this disease (Alhudaid, 2012; Moreira*et al.,* 2011).

The FMD has been reported in countries from five different continents, with its dissemination caused mainly by vegetative reproduction occurring through cuttings, commonly used by fig growers, as well as the presence of the vector mite (*Aceria ficus*) (Walia *et al.,* 2009).

Due to the increasing demand of the fresh and dried fig fruit in the global market and its nutritional and medicinal properties (Moreira*et al.,* 2011), an interest has grown regarding the study and control of this disease (Çağlayan *et al.,* 2010).

In Costa Rica, this crop is part of an agricultural diversification strategy, hence a phytosanitary restriction for the introduction of propagation material with FMV has been established. Studies related to the presence of FMD in the main variety found in the country has determined the absence of the virus in the tested plants, as well as the absence of *A. ficus* in Costa Rica (Moreira *et al.,*2011).

Regarding this disease, it is important to develop a standardized protocol that allows the analysis of fig plant material before its entrance in the country; in order to ensure the phytosanitary safety of the current material as well as the safe entrance of new varieties and cultivars. Hence, the overall aim of this research is to diagnose the presence of FMV through the macroscopic analysis of its symptoms, Transmission Electron Microscopy analysis, and Reverse Transcription Polymerase Chain Reaction (RT-PCR) in leaf material from two varieties of *F. carica* ("Negro San Juan" and "Brogiotto Bianco"), as well as the predominant variety found in Costa Rica like negative control, "Brown Turkey" and its accessions identified by Castro *et al.* (2015).

MATERIALS AND METHOD

Selection and collection of foliar material

The leaf material from the varieties of *F. carica* ("Negro San Juan" and "Brogiotto Bianco") was obtained from a quarantine section of a greenhouse at the Centro de Investigación en Biotecnología from the Instituto Tecnológico de Costa Rica (ITCR), while the material from the variety ("Brown Turkey"), was collected from another greenhouse in the same location. The samples were gathered during the fruiting period and the subsequent defoliation of the fig trees.

Macroscopic analysis of the symptoms

An analysis of the symptoms with possible viral etiology was performed in the leaves of each variety, establishing comparison or divergence patterns among the leaf material through the execution of a photographic record. The regions of the leaf surface with possible

symptoms related to FMV were processed for their analysis through MET and RT-PCR.

MET Analysis

Symptomatic and asymptomatic fig leaf tissue segments of 0.5 cm^2 were processed according to the standard procedure for biological samples by Çağlayan *et al.* (2010), Elbeaino *et al.* (2009a) and Castellano *et al.* (2007), which consisted of fixing the material for 2 days at 4°C in glutaraldehyde and paraformaldehyde at 2% diluted in 0.15 M phosphate buffer at a pH of 7.4, followed by a post-fixing process using osmium tetroxide at 1%. The samples were dehydrated in gradual dilutions of acetone and were then polymerized using Spurr® low viscosity resin. The ultrathin cuts were dyed with 2% uracil acetate during 15 min, after which they were observed in a Jeol JEM 2100 electron microscope.

Extraction of total RNA, RT-PCR and the bioinformatical analysis of the nucleotide sequence

For total RNA extraction, RNeasy Plant mini kit (Qiagen, GmbH, Germany) was used following the manufacturer specifications. However, before the extraction, the leaf samples were exposed to 95% ethanol during 15 min, and then immersed in liquid nitrogen for 2 min. The tissue rupture was performed in a Retsch® macerator. The RNA concentration and purity was quantified through spectrophotometry, with the absorbance values ranging between 260 and 280 nm.

For the amplification of the E5 fragment from RNA-1 of the FMV genome, a one-step RT-PCR protocol was developed using the E5-s (5'-CGGTAGCAAATGGAATGAAA-3') and E5-a (5'-AACACTGTTTTTGCGATTGG-3') primers, as recommended by Ishikawa *et al.* (2012b) and Çağlayan *et al.* (2009). The RT-PCR reactions with 25 µl were performed using One Step RT-PCR Kit (Qiagen, GmbH, Germany); 0.6 µM of oligos and 2 µg of RNA. The thermal profile used was of 50°C during 30 minutes (inverse transcription), 95°C for 15 minutes, (94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec) x 35 cycles; and a final 7 minute extension at 72 °C. The RT-PCR products were analyzed using a 2% agarose electrophoresis gel dyed with gel Red at 1X.

The amplified products were sent to Macrogen® (USA) for sequencing. They were edited and assembled with the bioinformatics software BioEdit® (Hall, 1999) and CAP3® (Huang y Madan, 1999); finally, the products were compared to the National Center for Biotechnology Information (NCBI) database through a BLAST search. The FMV isolates more closely related to the amplified sequences were used to perform a Maximum Likelihood (ML) phylogenetic reconstruction through the software MEGA v6.0 (Tamura *et al.,* 2011). Through this analysis it was possible to determine the genetic distance between the isolates, based on the number of base substitutions between sequences and removing the positions with missing data using the Tamura-Nei model (1993) and a 500 repetition bootstrap.

RESULTS

Macroscopic analysis of the symptoms

The "Brown Turkey" cultivar presented healthy leaves, with an orderly arrangement of the lobes and the absence of deformities in the foliar sheet (Figure-1 a and b). Meanwhile, the varieties of "Brogiotto Bianco" and "Negro San Juan" showed mosaic patterns, reddish spots associated to the viral lesions and deformities that affected the usual disposition of the lobes in the leaf (Figure-1 c, d, e and f).

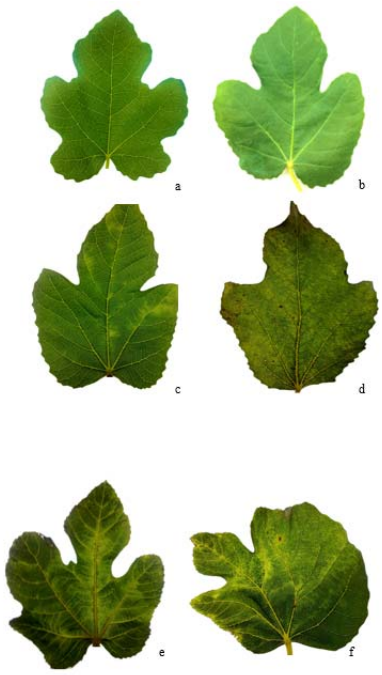

Figure-1. Leaf surfaces from the fig varieties evaluated. (a - b) Asymptomatic var."Brown Turkey". (c – d) var. "Brogiotto Bianco". (e - f) var. "Negro San Juan".

MET Analysis

The histological analysis in mesophyll cells of the "Brown Turkey" variety evidenced a normal behavior of the cells; with healthy chloroplasts presenting large concentrations of grana joined together through the lamellae found within the stroma, and a low concentration of the plastoglobuli (Figures-2 a and b).

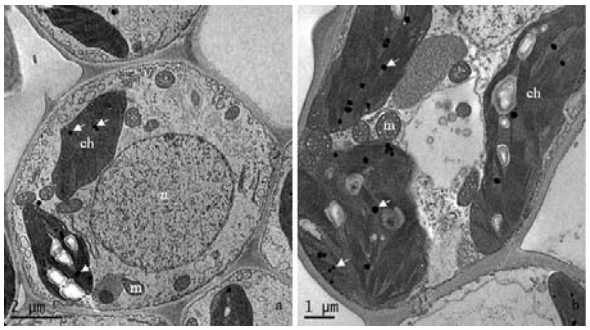

Figure-2. Cytology of the var. "Brown Turkey" leaf mesophyll cells. (ch) chloroplast; (arrows) plastoglobuli; (n) nucleus; (m) mitochondria.

The fig leaf samples from "Negro San Juan" and "Brogiotto Bianco" varieties presented ultrastructural alterations and partial loss of thylakoid grana, associated to the high concentrations of plastoglobuli (Figure3 a andc). On the other hand, the presence of possible DMB close to the cell wall as well as chloroplasts with sizes ranging between 90 nm and 200 nm were observed (Figure-3 b). Additionally, it was possible to identify a large amount of enlarged and partially destroyed membranous bodies (Figure-3b), which in many cases, were immersed in electrodense matrices (Figure-3d).

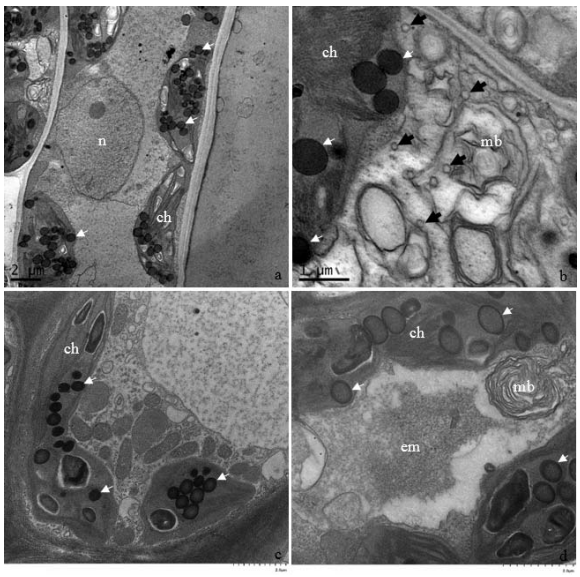

Figure-3. Cytology of leaf mesophyll cells from the fig varieties var. "Negro San Juan" (a and b) and "Brogiotto Bianco" (c and d). (ch) chloroplasts; (n) nucleus; (white arrow) plastoglobuli; (black arrow) possible DMBs; (mb) partially destroyed membranous bodies, (em) electron matrix.

Total RNA extraction, RT-PCR and nucleotide sequence bioinformatics analysis

Through the RNA extraction, an average concentration of 291.5 ng/µl was obtained, with an A260/A280 ratio of 2.06 for the total RNA, obtained from the three different fig varieties used in the study. The RT-PCR amplification, performed with specific primers for the viral RNA 1, generated amplified products 300bp long for the varieties "Negro San Juan" and "Brogiotto Bianco", which was different to the results obtained from the "Brown Turkey" variety (Figure-4).

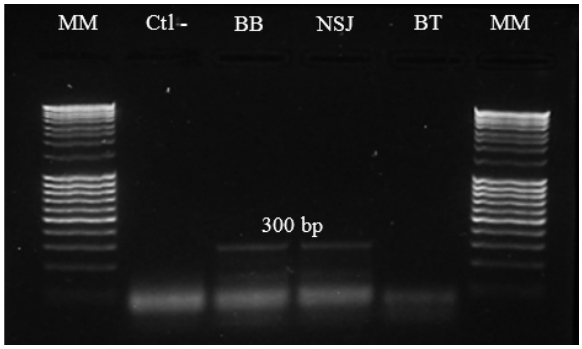

Figure-4. RT-PCR products. (MM) MassRuler^{TM} 1kb DNA Ladder. (Crl -) Negative control of the reaction; (BB) "Brogiotto Bianco"; (NSJ) "Negro San Juan"; (BT) "Brown Trukey".

Through a BLAST search in the GenBank database, it was determined that the viral sequences obtained from "Brogiotto Bianco" (KP796425) and "Negro San Juan" (KP796424) presented, in average, a 99% identity with the sequences AB697836.1 and AB697834.1 of FMV; reported in this genome library by Ishikawa *et al.* (2012b). The data was confirmed through the phylogenetic reconstruction performed with 13 different, closely related FMV sequences (Figure-5).

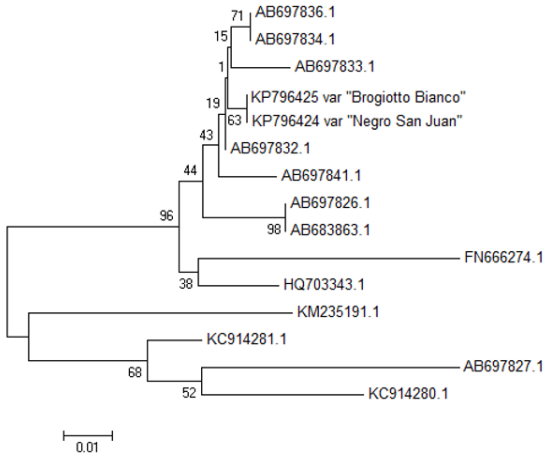

Figure-5. Phylogenetic reconstruction based on the E5 fragment of the FMV's RNA-1 multiparty genome, using the Maximum Likelihood (ML) Method with a 500 bootstrap.

DISCUSSIONS

The results from the leaf comparison matrix evidenced significant changes in "Brogiotto Bianco" and

"Negro San Juan" fig varieties, which were not found in "Brown Turkey" (Figure-1); corresponding to symptoms related to the possible presence of the fig mosaic disease (Alhudaid, 2012; Ishikawa *et al.,* 2012b; Al and Anpoka, 2000).

The common symptoms of the disease also coincide with the fruiting and the plant's defoliation periods. Hence the study of the leaf behavior in the three varieties of *F. carica* was developed in this plant life cycle stages, when the disease symptoms would be more evident (Segarra *et al.,* 2005).

The red spots found on the adaxial surface of the leaves from the varieties "Negro San Juan" and "Brogiotto Bianco", were associated to the damage caused by the FMV in epidermic cells (Figure-1 d andf) (Alhudaid, 2012). This type of symptoms in the fig can be easily confused with other pathologies, mainly rust (*Cerotelium fici*); however, the absence of characteristic structures from this pathogen allow it to be associated to FMD (Flores *et al.,* 2005).

The leaf tissue histological analysis from "Brogiotto Bianco" and "Negro San Juan", showed the presence of a large amount of membranous bodies and certain electrodense regions, located between the chloroplasts and the cell wall, which are typical signs of infections produced by virus from the Bunyaviridae family (Figure-3d) (Elsayed *et al.,* 2011; Pepin *et al.,* 2010). According to Falk (2011), these structures are generated due to metabolic changes within the host cell as a response to the replication processes of the viral particles (Figure-3 b and d); a behavior that is not observed in the samples from the "Brown Turkey" variety.

On the other hand, the chloroplast alterations in the positive plants' parenchyma cells are similar to those described in previous research studies developed by Çağlayan *et al.* (2009) and Elbeaino *et al.* (2009b), where the partial or total grana loss as well as the excessive accumulation of starch granules is related to the presence of the fig mosaic virus (Figure-3 a and c).

The accumulation of starch granules within the chloroplasts is one of the most important functions of these intercellular organelles as well as the production of ATP through photophosphorylation, which is a process performed in the thylakoid membranes (Bussotti *et al.,* 2012). The storage of starch in the chloroplasts occurs during the day, when the photosynthetic machinery is active; while during the night, the starch is hydrolyzed and exported to the cytoplasm for cell growth (Villalobos, 2001). Nonetheless, by reducing the number of thylakoid grana inside the chloroplast's stroma, the photosynthetic processes and the ATP production are both limited, and there is not enough energy required to hydrolyze and export the accumulated starch, causing a metabolic imbalance.

The presence of plastoglobules in the chloroplasts'stroma is still uncertain (Bussotti *et al.,* 2012); however, according to Chacón & Esquivel (2013), they are structures which are released from thylakoid membranes, containing in their interior a large concentration of organic molecules, mainly lipids, whose function is still not well-known. It is likely that the difference between the number of plastoglobules between plants with FMV and the control plants is due to the loss in grana caused by the fig mosaic disease, where the thylakoids disintegrate and form a large amount of plastoglobules. The increase in the plastoglobule concentrations has also been reported by Çağlayan *et al.* (2009).

The use of RT-PCR with specific primers for the RNA-1 fragment from the fig mosaic virus, also used by Ishikawa *et al.* (2012b) and Elbeaino *et al.* (2012 and 2009b), evidenced the existence of a sequence which codes the polymerase RNA dependent of the viral RNA found as part of the total RNA extracted from the symptomatic leaf material of the positive varieties; since it amplified a fragment of approximately 300bp similar to the one reported by these authors in their assays of FMV infection through the use of an insect vector (*A. ficus*) (Figure-4).

As a complement, the use of Blast showed an amplified region of the FMV in the studies varieties, allowing to establish a 99% homology with the isolates AB697836.1 and AB697834.1 of the FMV reported by Ishikawa *et al.*(2012a) in the varieties "Violette de Sollies" and "Lisa" found in Japan.

The phylogenetic analysis of the studies isolates presented a similar behavior to the results obtained through the Blast search (Figure-5), where the viral sequences of "Brogiotto Bianco", "Negro San Juan","Violette de Sollies" and "Lisa" fig varieties were grouped in one same node. According to Mendoza (2012) as more common ancestors are shared between two taxa's, the more closely related they are between each other. This tendency was evidenced by analyzing the genetic distance matrix between the viral isolates, where the divergence between the viral isolates KP796425 and KP796424 in regards to AB697836.1 and AB697834.1 was of 0.005.

This study confirms that the "Brown Turkey" fig variety, found predominantly in Costa Rica, is free of FMV, while the varieties "Brogiotto Bianco" and "Negro San Juan", presented FMD. On the other hand, it was determined that the FMV isolates found in the studied varieties were phylogenetically associated to viral isolates from fig varieties found in Japan.

ACKNOWLEDGEMENTS

The authors would like to thank the Vicerrectoría de Investigación y Extensión from Instituto Tecnológico de Costa Rica for the financial collaboration provided in order to develop this research Project.

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