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Cytological Effect of Viral Infection Associated with Yellow Streak Symptoms on Agave Tequilana

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Abstract

Yellow streak, ring spot and mottle symptoms were observed on young Agave tequilana (agave azul) plants cultivated in commercial nurseries in the state of Jalisco, Mexico. These symptoms have been associated with those possibly caused by an unknown virus. Mechanical inoculation of a host range and serological tests (ELISA) failed to demonstrate the viral nature of agave yellow streak. However, observations made on ultrathin sections of agave tissues with symptoms under a transmission electron microscope showed the consistent presence of flexible rod-shaped virus particles in the cytoplasm and chloroplasts, damage to chloroplasts, and formation of different types of globose membranous inclusions not observed in tissues from healthy plants. Moreover, analysis of Sanger's sequences of nucleic acid and amino acids from amplicons obtain by RT-PCR assays of symptomatic agave plants showed the infections of an unknown virus species of the Order Tymovirales.

Keywords: Viral Particles; Viral Inclusions; Cells; Tequila; Jalisco; Mexico

Introduction

The "agave azul" (Agave tequilana F.A.C. Weber.) is a native Mexican crop [1] that generates the largest source of economic income of agricultural origin for Mexico because is the raw material to produce Tequila (DOT)), the alcoholic beverage with the greatest demand worldwide. 427.2 million liters of tequila (100% Agave) were produced in Mexico in 2023, of which 420 million were exported, with a value of 64 billion dollars [2-4].

However, recently in commercial plots of agave tequilana in the state of Jalisco, Mexico, it is common to observe plants with yellow stripes and irregular mottling on young agave leaves, symptoms typically associated with those caused by viruses, considering the negative results in the diagnosis of other pathogens (Figure 1).



Figure 1: Symptoms of yellow streak and mottle on agave leaves grown in commercial nurseries in Jalisco, Mexico

There are no reports of virus identification in blue agave plants grown in plots and commercial nurseries, nor the economic losses or their distribution in Mexico [5].

We conducted three tests for viral characterization on each of five plants using mechanical transmission by abrasion to a host range of 16 different herbaceous plants [6, 7]. The same plants were subjected to serological detection of viruses by ELISA [8] specific test to Potyvirus, TMV, CMV and AMV (Agdia, Inc). However, no virus was detected or identified by these diagnostic methods. Considering the persistent and evident symptoms of yellow streak and mottle, ultrathin sections of damaged agave tissues were conducted to observed under a transmission electron microscope (TEM) of possible viral particles as a rapid diagnostic method that would show evidence of the presence of viruses in the cells of damaged tissues associated with the symptoms of yellow streak and mottle in A. tequilana [9-11]. Finally, RTPCR assays were conducted with a different set of primers from the most common viruses found in Mexico [7].

Methods

Transmission Electron Microscopy (Tem)

Plants with yellow streak symptoms collected in commercial nurseries in the state of Jalisco, Mexico, and healthy plants produced from seed were selected. Agave tissue samples of 2 x 1 x 1 mm diameter were made with a razor, then isolated and fixed with a 4% paraformaldehyde (PFA) and 2.5% glutaraldehyde (GTA) solution in 0.16 M sodium cacodylate buffer (pH 7.0) [12] and 1% osmium tetroxide (OT). The samples were washed with distilled water and dehydrated in a series of ethanol (10-100%), and a final step with propylene oxide, embedded in EPOXY resin and polymerized in an oven at 60°C [13]. To improve the observation in TEM, OT was used to achieve optimal post-fixation and preliminary contrast. Additional embedding in hydrophilic London Resin White (LRW) of fixed and dehydrated samples were used for observation without OT. Finally, the 60 nm ultra--thin sections were subjected to routine contrast in two steps: 3% uranyl acetate (UA) solution for 20 minutes and 0.3% lead citrate for 10 minutes. Semi-thin and ultrathin sections of 1.5 micrometers and 70 nm, respectively, were made with an ultramicrotome (Ultracut R; Leica). Semi-thin sections were stained with 0.1% toluidine blue in tetraborate solution and examined in bright field AxioScope A.1, coupled to AxioCam (Zeiss) Zen. Wide field and ultrathin sections were observed in TEM ZEISS Libra 120 with GATAN CCD with Digital Micrograph software [14, 15].

RTPCR Assays

To identify the possible viral species involved in agave yellow streak symptoms, total nucleic acid extracts of 100 mg from symptomatic leaves of five plants (Trizol reagent; Gibco BRL Life Technologies, England) were used as template in RT-PCR assays using the set of primers available in our laboratory to genus Potyvirus [16], Potexvirus [17], Tobamovirus [18], Cucumber mosaic virus [19, 20], Alfalfa mosaic virus [22] and Torradovirus [23]. The hypothetical expected amplicons detected were sequenced by Sanger's method, and the obtained nucleotide (nt) sequences and their derived amino acid (aa) sequences were analyzed and compared in the GenBank database [23].

Results and Discussion

Transmission Electron Microscopy (TEM)

The evidence obtained by analysis of the photomicrographs obtained at low magnifications by Transmission Electron Microscopy at low magnifications (2.5k and 8K) showed flexuous, filamentous viral particles 470 to1000 nm long and 12-13 nm in diameter, consistently associated from tissue of agave with streak and mottle yellow symptoms. The structural alteration of cells and their components described above revealed chloroplasts (cl) with alterations in their structure due to redistribution of thylakoid membranes and polarized matrix segregation (2.5K and 8K). and almost total disorganization and destruction of their structure was evident. At these magnifications, globose or spherical cellular inclusions (gci) were located. They were of various sizes and electronic density and detached from a membrane, adding to the surface of the chloroplasts, formation of blister-like folds, membrane deformation (md), and vacuoles (va). On the other hand, flexible rod-shaped virus particles (pvvf) clearly recognized at higher magnifications (25K) within chloroplasts (ch]) and between the membranes (pvvf) (Figure 2) [24]. The distribution of the flexible rod viral particles within the cell and organelles is very broad; they were observed in superficial cells and in internal vascular regions. It was also possible to observe free viral particles in the cytoplasm (cy) in the cross sections associated with globose inclusions (gli) (Figure 3 and 4) and others, crystal-like, clearly associated with globose bodies (Figure 5 A and B) [25-27].



Figure 2: Electron micrograph of blue agave with yellow streak symptoms and speckled (9Ag) (5K) A. Chloroplasts (chl) with flexible rod viral particles (frvp), membrane deformation (md) and vacuole (va) B. Close-up. Flexible rod viral particles (frvp)



Figure 3: Electron micrograph of agave with symptoms of yellow streak and mottle (Ag7) 8k. A. Chloroplasts (chl) with flexible rod viral particles (frvp) and viral particles in cross section (vpc) in cytoplasm. B. chloroplasts (chl) with flexible viral particles and vacuoles (va)



Figure 4: Electron micrograph of agave (4Ag) with symptoms of yellow streak and mottle (8k). A. Chloroplasts (cl) with flexible rod viral particles (frvp). viral particles in cross section (pv), globose membranous vesicles (gmv) B. Close-up. Flexible rod viral particles (frvp). Globose membranous inclusions (gmi)



Figure 5: Electron micrograph of agave with symptoms of yellow streak and mottle (Ag7) 8k. A. Cross section of membranous vesicles (mv) surrounded by viral particles in cross section (vpcs), globose membranous vesicles (gmv) with rod-shaped particles (rp). B. zoom. membranous vesicles surrounded by viral particles in cross section (mvvpcs)

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Figure 6: Electron micrograph of asymptomatic agave leaves. A. Nucleous (N), Cytoplasm (Cyt), Mitochondria (Mit), Cellular wall (Cw). B. Vacuole (V), Immature chloroplasts (Ich). Thylakoids (Thy). C. Mitochondrias (Mit). D. Plastoglobules (pg).

However, no structural alterations of cells and organelles or viral particles were observed in the tissues of agave plants without symptoms used as the control.

The variation in size of flexuous viral particles and filaments (470 to 1000 mm) could indicate species from different orders of plant viruses, such as Tymovirus or Closterovirus, the most numerous species of flexuous and filamentous viruses that could be associated with agave yellow streak and mottle symptoms [28].

Although the symptoms of yellow streaking are evident in young agave tequilana plants, in some commercial plots, these disappear in adult plants, which develop normally until harvesting and processing to produce tequila, and so it is difficult to visually recognize plants with symptoms. On the other hand, the geographical distribution and impact on production of virus infections in agave tequilana in the region are unknown.

RTPCR Assays

RTPCR assays specific for RNA virus groups that infect plants with symptoms were positive only for a RTPCR of RdRp (Po-tex1/Potex5) of a set of primers for the Potexvirus genus, and an amplicon of 600 bp was obtained from agave with yellow streak and mottle symptoms collected directedly from commercial nurseries in Jalisco, Mexico. Sanger-type nucleotide sequenc-

ing of the RTPCR products obtained were analyzed and compared by BLAST with sequences available in the GenBank (NCBI), showing a homology to nucleotide (nc) and amino acid (aa) of 85.56% (Access No. PP266591) with Agave Potexvirus-1 (Access No. MW328740) [23, 29]. Other sequences of different members of Order Tymovirales (data not included), not only of the Potexvirus group, were obtained.

The presence of unknown viruses was indicated, and it will be necessary to identify and characterize these viruses by other methods. Therefore, these results could be the basis for carrying out studies on the confirmation of the identity of the agave viruses, epidemiology and evaluation of the economic losses caused by the viruses in this plant in Mexico, but also in the numerous species of agave that exist in Mexican territory, center of origin of the Agave genus [5].

Conclusions

Flexuous, filamentous viral particles 470 to1000 nm long and 12-13 nm in diameter were consistently observed and associated with the structural alteration of cells and their components in agave azul (Agave tequilana) leaves with yellow streak and mottle symptoms cultivated in commercial nurseries in the state of Jalisco, Mexico, and none were observed in tissue from asymptomatic agave plants. Since no viral infections were detected by mechanical transmission tests in indicator plants, it was not possible to obtain a pure isolate of any virus to use in pathogenicity tests. It is possible that these viruses are not mechanically transmissible to the host range tested, and it will be necessary to use a wider host range or other methods of transmission. The serological tests were also negative; in no case were the viruses in agave yellow streak symptoms associated with the antiserums tested. A possible Potexvirus member of the Order Tymoviral was identified by reverse transcription linked to PCR (RT-PCR) and direct sequencing of its products by Sanger's method. However, the homology of sequences showed the presence of other unknown viruses involved in symptoms of yellow streak and mottle in agave and

it will be necessary to use other methods for identification and characterization of these viruses. Mexico is a center of origin for numerous species of the genus Agave, which have high economic, social, and ecological impact, and viruses in Agave tequilana, could affect other species of Agave or even other susceptible crops.

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