'*Candidatus* Phytoplasma asteris' (16SrI) Y *Dalbulus maidis* ASOCIADOS CON SÍNTOMAS DE HOJA BLANCA EN MAÍZ

'*Candidatus* Phytoplasma asteris' (16SrI) AND *Dalbulus maidis* ASSOCIATED WITH WHITE LEAVE SYMPTOMS IN MAIZE

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SUMMARY

Maize (*Zea mays* L.) has become the most economically important crop worldwide. This cereal belongs to the Poaceae family, is native to Mexico and used for human and livestock consumption. In Mexico, its importance goes far beyond the economy, its influence at a social and cultural level has been reflected in the vestiges of ancient civilizations such as that established by the Mayans, for whom maize had a religious meaning; however, some biotic factors such as bacteria, viruses, fungi and phytoplasmas may limit the production of the various varieties of maize grown in Mexican territory. During the 2021 crop cycle, maize plants were collected from commercial plots in the municipality of Valparaiso, Zacatecas, Mexico, which presented a symptomatology related to the presence of phytoplasmas (dwarfism, color changes in leaves from green to white, general decline and death); in addition, asymptomatic maize plants were sampled as a control, and insects of the species *Dalbulus maidis* were sampled as well. Samples were analyzed by nested polymerase chain reaction (PCR) using the universal oligonucleotides combination P1/Tint, followed by the pair of oligonucleotides R16F2n/R16R2 resulting in sequences of the 16S rDNA gene from phytoplasmas. Phylogenetic and virtual RFLP analyses of the 16S rDNA sequences obtained from the analyzed samples showed 100 % sequence identity to each other and 99.7 % sequence identity with the homologous gene of *Candidatus* Phytoplasma asteris (GenBank accession KMM30790). The incidence of this new disease was 15 % in the sampled crops. These results relate *Dalbulus maidis* and *Candidatus* Phytoplasma asteris as a possible vector and causal agent, respectively, of a new symptomatology in maize.

Index words: *Dalbulus maidis*, *Zea mays* L., molecular diagnostics, nested PCR, phytoplasma.

RESUMEN

El maíz (*Zea mays* L.) se ha constituido como el cultivo de mayor importancia económica a nivel mundial; esté cereal perteneciente a la familia Poaceae, es originario de México y utilizado tanto para consumo humano como ganadero. En México, su importancia va mucho mas allá de lo económico, su influencia a nivel social y cultural se ha visto plasmada en los vestigios de civilizaciones antiguas como la establecida por los mayas, para quienes el maíz tenía un significado religioso; sin embargo, algunos factores bióticos como bacterias, virus, hongos y fitoplasmas pueden limitar la producción de las diversas variedades de maíz cultivadas en territorio mexicano. Durante el ciclo de cultivo 2021, se colectaron plantas de maíz presentes en parcelas comerciales en el municipio de Valparaiso, Zacatecas, México las cuales presentaban una sintomatología relacionada con la presencia de fitoplasmas (enanismo, cambios de color en hojas de verde a blanco, declive general y muerte); además, se muestrearon plantas de maíz asintomáticas como testigos e insectos de la especie *Dalbulus maidis*. Las muestras se analizaron mediante la reacción en cadena de la polimerasa (PCR) anidada utilizando la combinación de oligonucleótidos universales P1/Tint, seguida por el par de oligonucleótidos R16F2n/R16R2 con lo que se logró obtener secuencias del gen 16S rDNA de fitoplasmas. Los análisis filogenético y RFLP virtual de las secuencias de 16S rDNA obtenidas a partir de las muestras analizadas mostraron una identidad de secuencia del 100% entre sí y una identidad de secuencia del 99.7 % con el gen homólogo de *Candidatus* Phytoplasma asteris (accesión del GenBank M30790). La incidencia de esta nueva enfermedad fue del 15 % en los cultivos muestreados. Estos resultados relacionan a *Dalbulus maidis* y a *Candidatus* Phytoplasma asteris como pobible vector y agente causal de una nueva sintomatología en maíz.

Palabras clave: *Dalbulus maidis*, *Zea mays* L., diagnóstico molecular, fitoplasma, PCR anidada.

INTRODUCTION

Mexico is an important producer of maize (*Zea mays* L.) worldwide. In 2022, 6,941,031 ha were planted to maize, and 27,549,000 tons were produced with a value of $ 10.1 USD billion (SIAP, 2024). The state of Zacatecas, located in North-Central Mexico, is gaining importance as a maize producer with 75,805 ha and a production of 325,230 tons (SIAP, 2024). The most significant economic losses caused by microorganisms in maize are due to fungus diseases whose causal agents are members of the genera *Aspergillus*, *Giberella*, *Fusarium* and *Penicillium* (Arispe *et al.*, 2019). Nevertheless, another group of phytopathogens known as phytoplasmas has been reported, which are dispersed in Mexican territory, affecting crops of economic interest, among which maize stands out (Perez-Lopez *et al.*, 2016). Since the 2021 crop season, maize plants have shown symptoms of dwarfism, color changes in leaves from green to white, general decline, and plant death in several commercial fields in Valparaiso, Zacatecas, México (Figure 1).

Figure 1. Maize plants (*Zea mays*) showing symptoms of dwarfism, stunting and white leaves with green stripes and brown edges associated with phytoplasma infection.

Phytoplasmas are small, insect-transmitted, cell-wall-less bacteria that cause numerous diseases in plant species worldwide that are important from an economic and environmental point of view (Bertaccini and Lee, 2018). Phytoplasmas have been found associated with multiple diseases in plants; the symptoms related to their infection may vary depending on the host, phytoplasma strain and environmental factors, and often include yellow/purple discoloration of leaves and shoots, virescence (greening of petals), big bud (conversion of floral organs into leaf-like structures), proliferation of shoots, witches' broom, stunting, general decline, and in some cases plant death (Ermacora and Osler, 2019). Phytoplasma diseases in maize plants have been reported in Bulgaria and Turkey, where *Ca*. Phytoplasma solani (16SrXII) was associated with empty grain bearing and leaf reddening (Çağlar *et al*., 2021; Genov *et al*., 2014); in Mexico and India, the presence of *Ca*. Phytoplasma asteris (16SrI) was related to leaf redness, yellowing, chlorotic stripes, and stunting (Perez-Lopez *et al*., 2016; Rao *et al*., 2017), while *Ca*. Phytoplasma trifolii caused yellowing and dwarfing in Iran (Zibadoost *et al*., 2015); in addition, the presence of maize

plants was reported with bushy growth, short internodes, leaf discoloration, and yellowing disease associated with Bermudagrass phytoplasma (16SrXIV) (Çağlar *et al*., 2019). This study aimed to identify phytoplasmas related to this new disease in maize plants and its possible insect vector.

MATERIALS AND METHODS

Plant samples

A 20-hectare maize commercial field located in the municipality of Valparaiso, Zacatecas, Mexico (22° 46' 0" N, -103° 34' 0" W) was visited during Spring 2021 to find symptoms related to phytoplasma infection (dwarfism, color changes in the leaves from green to white, general decline and plant death). Fifteen symptomatic and five asymptomatic maize plants in vegetative development stage Vn were randomly selected and collected. Symptoms were recorded for each symptomatic plant, and its incidence was obtained in five points within the field by counting the number of diseased plants in a hundred consecutive plants at each location. The capture and counting of 50 adult

members of the species *Dalbulus maidis* were carried out at the same time as the maize collection and taxonomic identification was carried out with a stereo microscope using dichotomous keys (Dietrich, 2005). Total DNA was isolated separately from 100 mg of leaf and 50 insects using the method described by Dellaporta *et al.* (1983) and stored at -20°C. DNA samples extracted from maize and insect tissues were used as templates for PCR.

Direct and nested polymerase chain reaction (PCR) for phytoplasma

PCR was performed in a programmable thermocycler (Applied Biosystems, Foster City, California, EUA) with the following parameters: i) for direct PCR primer pair P1/Tint (Smart *et al.*, 1996): 95 °C for 5 min, 35 cycles of 94 °C for 1 min, 56 °C for 2 min and 72 °C for 2 min, and a final extension cycle of 72 °C for 5 min; ii) for nested PCR primer pair R16F2n/R16R2 (Gundersen and Lee, 1996): 95 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min, and a final extension cycle of 72 °C for 5 min. PCR products were analyzed by electrophoresis on a 1 % agarose gel and visualized by staining with ethidium bromide and UV illumination.

Cloning of PCR products, RFLP analysis, and sequencing of DNA

The 1.25 kb amplicons obtained from positive samples from plants and insects were ligated in the pGEM-T Easy vector (Promega, Madison, Wisconsin, EUA) as indicated by the supplier, and *Escherichia coli* Top-10 calcium competent cells were transformed by the heat shock method. Positive clones were assessed by restriction fragment length polymorphism (RFLP) analysis using *Eco*RI and *Hinf*I enzymes. The digestion products were separated by electrophoresis on 2 % agarose gels, and PCR clones exhibiting different restriction fragment patterns were identified and sequenced at the National Laboratory of Agricultural, Medical and Environmental Biotechnology (LANBAMA) in San Luis Potosi, Mexico.

Phylogenetic and virtual RFLP analysis

A phylogenetic tree showing the 16S rDNA sequence of the phytoplasma isolated from insect and symptomatic maize plants, along with the 16S rDNA gene of 18 known phytoplasmas and *Acholeplasma brassicae* (used as outgroup) was constructed. The analysis of the nucleotide sequences obtained from this study was performed by comparison with available sequences at the NCBI database using the BLASTN software, and phylogeny analysis was performed using the ClustalV method of MegAling (DNASTAR software, Lasergene, London, UK). In addition,

bootstrapping was performed 1000 times to estimate the stability and support of the branches.

Computer-simulated RFLP analysis of the 16S rDNA gene was performed on phytoplasma sequence isolated in this study. The aligned sequences were exported to the virtual RFLP analysis programs of the iPhyClassifier website (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) for computer-simulated restriction digestion and virtual gel plotting. Each aligned DNA fragment was digested *in silico* with 17 distinct restriction enzymes (*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I) that have been routinely used for RFLP analysis of the 16S phytoplasma rDNA gene (Reveles-Torres *et al.*, 2018). After *in silico* restriction digestion, a virtual 3.0 % agarose gel electrophoresis image was plotted and captured as a device-independent pdf file. The RFLP analyses of phytoplasma sequence obtained from insects and maize plants were automatically compared with each 16S group and subgroup of reference phytoplasma strains.

RESULTS AND DISCUSSION

By evaluating the incidence of symptoms related to phytoplasmas infection, information on the risk for maize production could be obtained. In this study, the incidence of maize phytoplasmic disease (15 %) was lower than that reported for maize by *Ca*. Phytoplasma solani (16SrXII) affecting maize in Bulgaria (Genov *et al.*, 2014) and Serbia (Jović *et al.*, 2007) with a range from 30 to 85 % of incidence, while *Ca*. Phytoplasma asteris (16SrI) was reported in maize with an incidence range from 8 to 60 % in Mexico (Pérez-López *et al.*, 2016) and 40 % in Brazil (Galvão *et al.*, 2021). Reports of high incidence of plants with phytoplasmas refer to mature maize plants, whereas the 15 % incidence observed in this research could be due to maize plants being at an early stage of vegetative growth; furthermore, the range of incidence of symptomatic plants may increase later because at that time the interaction of *Dalbulus maidis* with maize crop was in the initial stage of development of the crop.

The identification of *Dalbulus maidis* individuals was based on their morphological characteristics. The adult male was 3.5-4 mm, and the female was 4.1 mm in length; the female has a dark ovipositor under the abdomen, adults are straw-yellow with two round black spots on the vertex of the head, and their rear wings are translucently extending beyond the tip of the abdomen. The identification of *Dalbulus maidis* was confirmed according to the taxonomic keys provided by Dietrich (2005). The presence of *Dalbulus maidis* is related to the presence of Maize bushy stunt, a disease caused by *Ca*. Phytoplasma asteris

from Southern United States to Argentina (De Oliveira *et al.*, 2011; Moya, 2001).

Typical phytoplasma DNA amplicons of 1227 bp were obtained by nested PCR using total DNA extract from the *Dalbullus maidis* mixture and, at the same time, in 14 out of 15 symptomatic DNA samples from maize plants. No amplification was observed in asymptomatic maize plants. BLAST analysis of the 16S rDNA sequences revealed that Phytoplasma from *Dalbulus maidis* and symptomatic maize plants shared 100 % sequence identity with each other and 99.7 % sequence identity with those in group 16SrI, strains of *Candidatus* Phytoplasma asteris. The Maize/*Dalbulus* phytoplasma sequence (GenBank ON314873) was compared with those in the Iphyclassifier database (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi), and the maximum identity was found with isolates of *Candidatus* Phytoplasma asteris.

The virtual RFLP pattern derived from the Maize/*Dalbulus* sequence was more similar to the reference pattern of group 16SrI and subgroup B (GenBank NC005303), with a pattern similarity coefficient of 1.0 (Figure 2). A phylogenetic tree was constructed with the Maize/*Dalbulus* 16S rDNA sequence, with 18 other phytoplasmas representing distinct groups of of this pathogen (Figure 3). The Maize/*Dalbulus* phytoplasma strain was grouped with the *Ca*. Phytoplasma asteris and belongs to the 16SrI group according to the neighbor-joining method. Results show that *Ca*. Phytoplasma asteris is the phytoplasma present in the *Dalbulus maidis* population and is also related to the symptoms described in maize plants in this study.

Phytoplasmas belonging to the 16SrI group are recognized as causal agents of maize bushy stunt disease in Mexico (Pérez-López *et al.*, 2016), Iran (Zibadoost *et al.*, 2016), India (Rao *et al.*, 2017) and Peru (Gamarra *et*
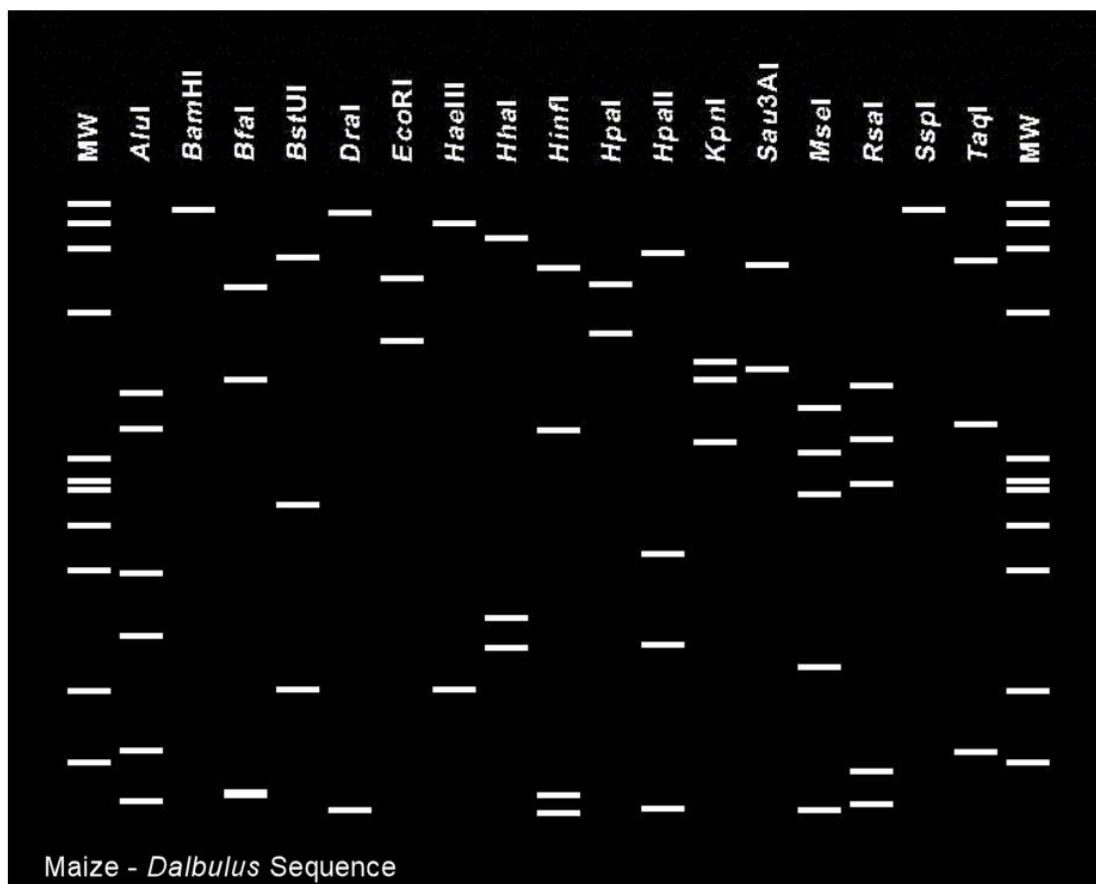

Figure 2. Virtual restriction fragment length polymorphism (RFLP) patterns derived from *in silico* digestions of 16S rDNA gene fragment from Maize/Dalbulus phytoplasma sequence. Image of virtual gel of RFLP patterns of Maize/*Dalbulus* phytoplasma sequence with recognition sites for the following 17 restriction enzymes for simulated digestions: *Alu*I, *BamH*I, *Bfa*I, *BstU*I, *Dra*I, *EcoR*I, *HaeII*I, *Hha*I, *Hinf*I, *Hpa*I, *HpaI*I, *Kpn*I, *Sau3A*I, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I. MW: molecular weight standards from φX174 DNA digested with *Hae*III.
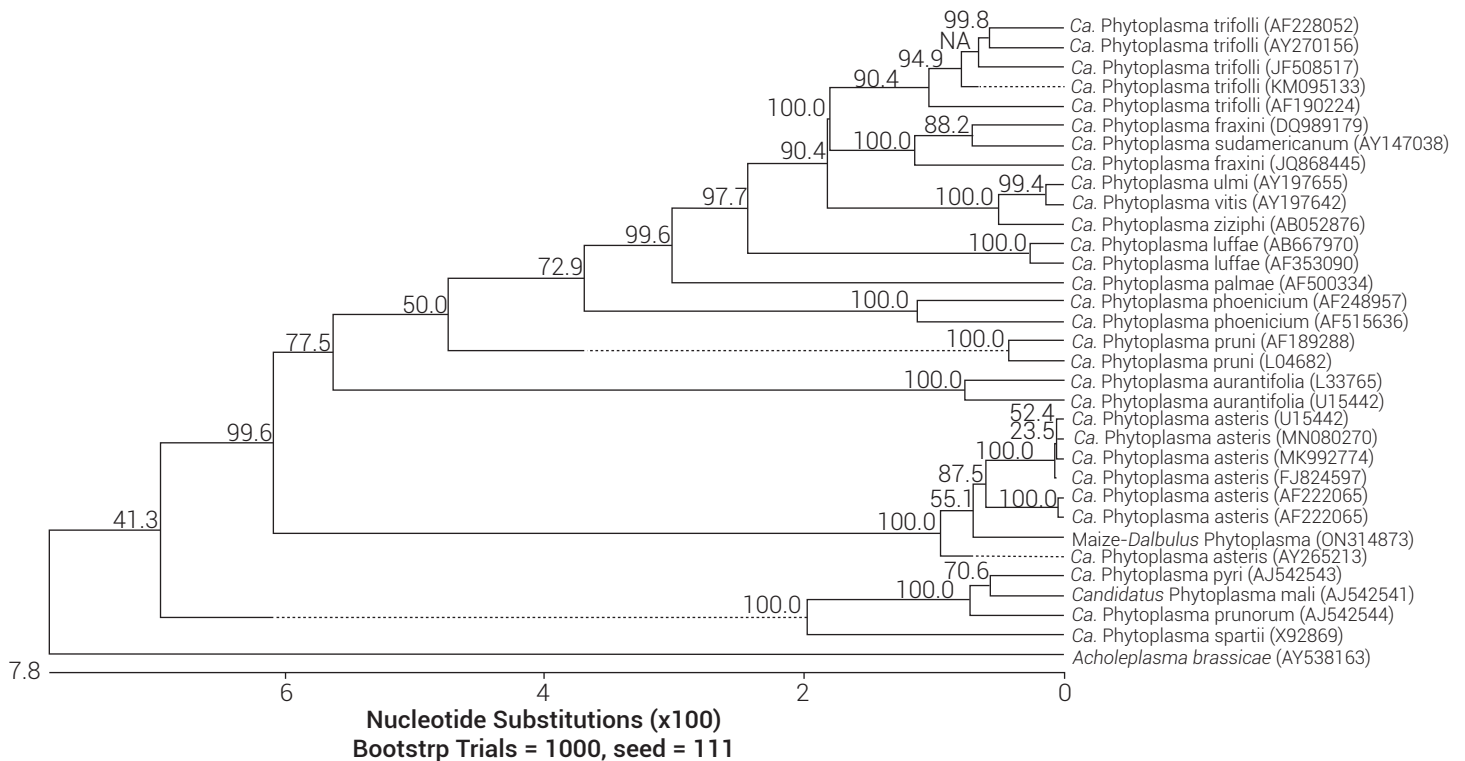
Figure 3. Phylogenetic distance tree constructed using the Clustal V alignment method (DNASTAR), which compares the 16S rDNA gene sequence from Maize/*Dalbulus* phytoplasma with sequences of 18 *Candidatus* Phytoplasma species. *Acholeplasma brassicae* was used as outgroup for the phylogenetic tree reconstruction. GenBank accession numbers for sequence are given in parentheses.

al., 2022), all of them induce symptoms such as reddish leaves, leaves with yellow or chlorotic stripes, stunting and chlorotic spots. Nevertheless, in this case the maize plants showed a new symptomatology characterized by dwarfism, stunting, and white leaves with green stripes and brown edges; except for the leaves with brown edges, similar symptoms are related to *Ca*. Phytoplasma asteris in wild grass belonging to the Poaceae family, as reported by Pérez-López and Dumonceaux (2017) in Mexico.

The presence of phytoplasmas belonging to the 16SrI group associated with diseases in maize could seriously constrain important economic crops in this region of Mexico; detection and identification of *Ca*. Phytoplasma asteris, as a potential pathogenic agent associated with maize crops in Mexico, facilitates the study of the epidemiology of the disease and contributes to the knowledge of the genetic diversity of phytoplasmas present in Mexico.

CONCLUSIONS

This is the first report on the molecular identification of the 16SrI group of phytoplasma associated with a new disease in maize plants and its presence in the *Dalbulus maidis* population in the North-Central region of Mexico.

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