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Sesame phyllody associated with a 16SrI-B phytoplasma, a 'Candidatus Phytoplasma asteris'-related strain, in Paraguay

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ABSTRACT: Sesame (*Sesamum indicum* L.) plants exhibiting symptoms of phyllody disease were observed in commercial fields in Paraguay. The symptoms were indicative of infection by phytoplasmas. Thus, the present study investigated the association between affected plants and phytoplasma, which was later analyzed using molecular and phylogenetic methods. Total DNA was extracted from symptomatic and asymptomatic plants and used in nested PCR assays using primers R16SN910601/R16SN011119 and R16F2n/16R2. Amplified products of 1.2 Kb revealed the presence of phytoplasma in all diseased plants, and electron microscopy confirmed the presence of phytoplasmas within phloem vessels. Nucleotide sequences from sesame phytoplasma shared 99 % similarity with phytoplasmas belonging to group 16SrI. Computer-simulated RFLP indicated that the detected phytoplasma is representative of the 16SrI-B, therefore, a '*Candidatus* Phytoplasma asteris'-related strain. Phylogenetic analysis was in agreement with virtual RFLP. Our findings expand the current knowledge regarding distribution of representatives of the aster yellows group in a new agroecosystem and implicate sesame as a new host of 16SrI-B phytoplasma in Latin America.

Keywords: *Sesamum indicum* (L.), *Mollicutes*, yellows, phloem bacteria

Introduction

Phytoplasmas are cell wall-less bacteria that inhabit the phloem, are naturally transmitted by sucking insects, and are associated with diseases occurring in numerous crops (Lee et al., 2000). Phytoplasma taxonomy has been based mainly on the sequencing of the 16S rRNA gene and they are currently classified in groups and subgroups characterized by distinctive molecular and phylogenetic features (Lee et al., 2010).

Phytoplasmas have been associated with a serious disease in sesame known as phyllody, which is present in several countries, primarily located in the Middle East, Africa, and Asia (Akhtar et al., 2013). Phyllody was first reported in a province of Pakistan in 1908 (Vasudeva and Sahambi, 1955) and for many years, it was regarded as a viral disease (Weiss et al., 1983; Turkmenoglu and Ari, 1959). However, later investigations (Akhtar et al., 2008) recognized that phytoplasmas were associated with phyllody disease. Currently, the disease is a significant threat to production in the most important sesame-producing regions of the world, including India (Nabi et al., 2015), Taiwan (Tseng et al., 2014), Turkey (Ikten et al., 2014), Pakistan (Akhtar et al., 2008), Iran (Salehi et al., 2017), Thailand (Nakashima et al., 1995), South Korea (Rao et al., 2015), and Myanmar (Win et al., 2010). Sesame phyllody has been reported as the major disease in sesame, causing a yield loss of up to 80 % yield (Salehi et al., 2017; Akhtar et al., 2013). Symptoms of sesame phyllody include shoot proliferation, virescence, foliar yellowing, shortened internodes, smaller leaves, abnormal floral organs, generalized stunting, phloem necrosis, and plant decline (Akhtar et al., 2009).

In Latin America, several countries have explored this promising crop, including Mexico, Guatemala and Paraguay, which are the largest producers in the Americas (FAO, 2015). In Brazil, in recent years, the industrial demand for oil and seeds has been increasing and sesame planting has undergone significant expansion due to its productive potential, tolerance of drought and poor soils, and use in crop rotation or in association with other crops (Perin et al., 2010).

Recently, sesame plants displaying typical phytoplasma-induced symptoms were observed in fields located in the municipality of Philadelphia (22°21′12″ S 60°2′0.9″ W, 140 m.a.s.l.), which is the capital of the Boquerón Department in the Gran Chaco of Western Paraguay. The incidence level of diseased plants ranged from 1 to 5 % in the field, and prevalent symptoms included phyllody, virescence, mild leaf chlorosis, intense shoot proliferation, and production of numerous small, deformed leaves (Figure 1).

The present study was undertaken to i) demonstrate the association of a phytoplasma with symptomatic sesame plants and ii) characterize the molecular phylogenetics of the pathogen.

Materials and Methods

Leaves were collected from 15 symptomatic plants and six asymptomatic plants grown in three commercial fields. Segments of leaf veins were prepared for examination by transmission electron microscopy, as previously described (Maunsbach and Afzelius, 1998). Total DNA was extracted for PCR assays using the DNeasy Plant Mini Kit. Detection of phytoplasmas was conducted by nested PCR with universal primers specific to phytoplasmas R16SN910601/R16SN011119 (Namba et

al., 1993; Jung et al., 2003) in the first reaction, followed by R16F2n/R16R2 (Gundersen and Lee, 1996) in the second reaction. Preliminary identification of the phytoplasma was performed using product generated by primers R16SN910601/R16SN011119 as a template for further PCR assays conducted with the group-specific primers R16(I)F1/R16(I)R1 (Lee et al., 1994), which are specific for identification of phytoplasmas belonging to group 16SrI. Extracts from maize (*Zea mays* L.) plants harboring bushy stunt phytoplasma (16SrI group) were used as a positive control, whereas an extract from asymptomatic sesame plants served as a negative control. The products amplified with universal primers were cloned in *Escherichia coli* DH5alpha strain, using the pGEM Easy Vector System I. Subsequently, the fragments were amplified with primers R16F2n/R16R2 and sequenced using the primer pair SP6/T7 (Malembic-Maher et al., 2008). Each phytoplasma identified in each symptomatic sesame plant was considered a strain. Five strains from five different plants were sequenced (three clones of each strain) and a major consensus sequence was selected to represent each strain. Sequences were analyzed using computer programs for construction and sequence analysis (Bioedit, Phred phrap and Multiple Sequence Alignment – CLUSTALW).

A computer-simulated RFLP analysis (Wei et al., 2007) was performed using DNA sequences representative of the phytoplasma found in sesame samples and 16S rRNA sequences representative of strains affiliated with different subgroups within the 16SrI group. Thereafter, the sequences were aligned, cut, and analyzed on virtual gel by the pDRAW32 program, described by AcaClone Software, according to Wei et al. (2007). After *in silico* digestion, an agarose gel of 3 % was plotted and captured as a separate file in PDF format for future comparisons of the profiles. The restriction patterns were compared between themselves and the similarity coefficient (F) was calculated for each pair of the phytoplasma strains as previously published (Wei et al., 2007). A phylogenetic tree was generated using the nucleotide sequences of strains identified in sesame plants and other sequences from representatives of distinct groups and subgroups, using the MEGA program, and the Neighbor-Joining method.

Figure 1 – Sesame phyllody: Left plate: symptomatic (left) and asymptomatic (right) plants; Right plate: characteristic foliar distortion and proliferation in symptomatic plant.

Results and Discussion

The association of phytoplasmas with diseased plants was consistently revealed by amplified products from nested PCR with universal phytoplasma primers, which yielded DNA fragments of approximately 1.2 Kb from all symptomatic sesame samples. Amplicons of 1.1 Kb were generated from all positive samples when PCR assays were performed with group-specific primers. Total DNA extracts obtained from maize plants were phytoplasma-positive for both types of nested PCR. No amplification occurred from DNA extracted from asymptomatic sesame plants. Molecular detection was confirmed using transmission electron microscopy by visualizing small pleomorphic bodies, typical of phytoplasmas, located in the interior of phloem vessels of positive samples (Figure 2).

Sequences representative of each of the five strains were compared; based on the absence of polymorphism, a majority consensus sequence was chosen to represent the phytoplasma associated with the symptomatic sesame plants. This selected sequence was designated SePhy-Br01 (Sesame Phyllody-Brazil 01), and deposited in GenBank under accession number KY933669.

The DNA sequences from SePhy-Br01 showed 99 % similarity to the sequence of the reference phytoplasma of the subgroup 16SrI-B (AY265213), a *Candidatus* Phytoplasma asteris-related strain (Lee et al., 2004; Lee et al., 1998). Computer-simulated RFLP analysis revealed that restriction profiles produced by SePhy-Br01 strain were indistinguishable from those generated by the 16SrI-B phytoplasma. Consequently, the value of similarity coefficient (F) calculated for this pair of strains was equal to 1.0 (Table 1), revealing these strains were identical. Furthermore, the branching pattern of the phylogenetic tree indicated that both phytoplasmas were closely related, since they emerged from the same branch (Figure 3).

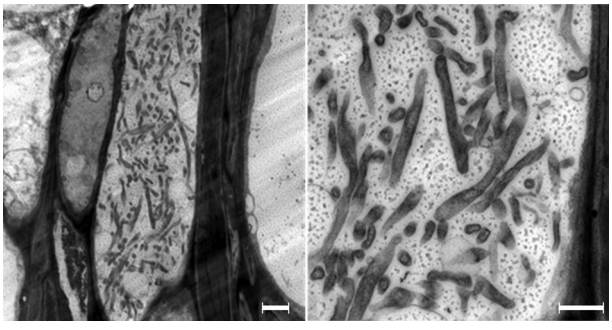

Figure 2 – Presence of pleomorphic bodies of the phytoplasma associated with sesame phyllody in the phloem vessel, observed by transmission electron microscopy. Left plate: scale bar = 1.0 µm; Right plate: scale bar = 0.5 µm.

Table 1 – Similarity coefficients (F) based on restriction patterns derived from virtual RFLP analysis involving the phytoplasma associated with sesame phyllody (SePhy-Br01) and diverse representatives of subgroups belonging to 16SrI group.

Subgroups	A	B	C	D	E	F	L	M	O	P	Q	S	T	U	V	SePhyBr01
16SrI-A	1															
16SrI-B	0.92	1														
16SrI-C	0.91	0.94	1													
16SrI-D	0.94	0.97	0.90	1												
16SrI-E	0.92	0.93	0.92	0.91	1											
16SrI-F	0.87	0.87	0.89	0.86	0.87	1										
16SrI-L	0.89	0.97	0.90	0.94	0.90	0.86	1									
16SrI-M	0.88	0.96	0.93	0.96	0.89	0.84	0.93	1								
16SrI-O	0.87	0.87	0.80	0.84	0.84	0.75	0.84	0.83	1							
16SrI-P	0.92	0.94	0.93	0.91	0.93	0.94	0.91	0.90	0.81	1						
16SrI-Q	0.84	0.92	0.89	0.89	0.89	0.84	0.89	0.92	0.81	0.86	1					
16SrI-S	0.90	0.92	0.93	0.89	0.91	0.88	0.89	0.88	0.81	0.94	0.84	1				
16SrI-T	0.84	0.92	0.85	0.89	0.85	0.80	0.89	0.90	0.83	0.86	0.90	0.84	1			
16SrI-U	0.87	0.93	0.88	0.90	0.87	0.81	0.90	0.90	0.85	0.87	0.87	0.87	0.88	1		
16SrI-V	0.85	0.93	0.87	0.90	0.87	0.81	0.92	0.90	0.83	0.87	0.87	0.85	0.88	0.94	1	
SePhy-Br01	0.94	1	0.95	0.97	0.93	0.87	0.97	0.95	0.89	0.95	0.94	0.92	0.92	0.93	0.93	1

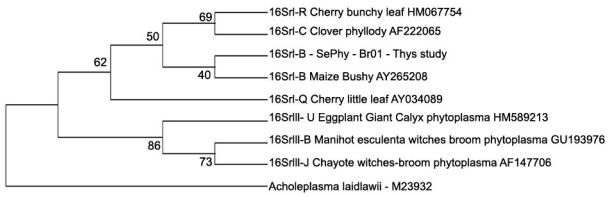

Figure 3 – Phylogenetic tree constructed with nucleotide sequence representative from the phytoplasma associated with sesame phyllody (SePhy-Br01) and distinct sequences from phytoplasmas affiliated with the 16SrI and 16SrIII groups deposited in GenBank. Bootstrapping was conducted 1000 times and *Acholeplasma laidlawii* (M23932) was included as outgroup. Numbers on branches indicate confidence values associated with the bootstrap analysis.

The preliminary diagnosis based on the symptoms exhibited by sesame plants was subsequently confirmed by visualizing phytoplasmas in affected plants. This finding supported the initial assumption that the symptoms of phyllody, chlorosis, proliferation, deformed leaves, and virescence were associated with phytoplasmas. The diversity of symptoms observed in plants used in this study was coincident with those described in other countries that cultivate this host species. Although the symptoms are similar, distinct phytoplasmas have been identified in association with the disease. In a report about genetic diversity of phytoplasmas associated with sesame phyllody, Salehi et al. (2017) indicated that representatives of the groups 16SrI-B, 16SrI-M, 16SrII-C and 16SrI-D are present in India; Myanmar and South Korea report a strain affiliated with group 16SrI-B; Iran has phytoplasmas belonging to groups 16SrII and 16SrIX; Oman and Pakistan have a member of 16SrII-D; Taiwan and Thailand have a strain of group 16SrII-A; and Turkey reported representatives of 16SrII-D, 16SrVI-A, and 16SrIX-C.

Currently, phytoplasmas of the 16SrI group are distributed in various countries of Latin America and the majority of them are affiliated with the 16SrI-B subgroup (Pérez-López et al., 2016). According to these authors, phytoplasmas of this subgroup are associated with numerous diseases present in various cultivated and non-cultivated species. In Bolivia, strains were identified in alfalfa and potato; in Brazil, they were found in sugarcane, broccoli and bougainvillea; in Chile, the group was identified in grapevine; in Colombia, representatives were associated with *Pittosporum undulatum*, *Fraxinus uhdei* and *Populus nigra*; in Costa Rica, it was found in common bean; in Cuba, strains were detected in basil, broad beans, sweet pepper, carrot, cabbage, beetroot, common bean, strawberry, macadamia nut and papaya; and in Mexico, phytoplasmas were reported in amaranth, marigold, maize, and potato. In addition, specifically in Brazil, a 16SrI-B phytoplasma is the causal agent of a serious disease designated maize bushy stunt that causes significant loss in grain production in corn crops (Bedendo et al., 2000). These reports indicate that representatives of subgroup 16SrI-B possess low specificity, since they have been identified in a diversity of host species in distinct geographical areas.

In Paraguay, phytoplasmas belonging to groups 16SrIII and 16SrXIII have been found in association with Chine-tree plants (*Melia azedarach*) showing symptoms of decline (Arneodo et al., 2005) and a representative of group 16SrIII was identified in cassava exhibiting frogskin disease (Cardozo Tellez et al., 2016). However, phytoplasmas of subgroup 16SrI-B have not yet been reported from Paraguay. Our findings revealed the occurrence of this strain in Paraguay, extending the community's knowledge regarding genetic diversity and distribution of phytoplasmas in a new agroecosystem. In addition, the results implicate sesame as being

a new host of 16SrI-B phytoplasma in Latin America. Since representatives of this subgroup have shown low specificity in relation to hosts, our study suggests that the phytoplasma could be associated with other species cultivated in Paraguay. Furthermore, our report should alert other sesame-producing countries in Latin America to scout for the presence of phyllody.

Authors' Contributions

Conceptualization: Segnana, L.G., Bedendo, I.P. Data acquisition: Ganem Júnior, E.J., Segnana, L.G., Kitajima, E.W. Data Analysis: Ganem Júnior, E.J., Kitajima, E.W., Bedendo, I.P. Design of Methodology: Ganem Júnior, E.J., Bedendo, I.P. Software Development: Ganem Júnior, E.J. Writing and Editing: Bedendo, I.P.

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