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Research Article

Phytoplasma of 16SrVII-B subgroup associated to shoot proliferation in *Physalis peruviana* plants

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ABSTRACT: Physalis is an herbaceous species native to the Andes region. Currently, it is cultivated in various Brazilian states due to the economic interest of growers for this new fruit. Physalis plants grown in the field showed symptoms of shoot proliferation, leaf malformation, and chlorosis. Since these symptoms are commonly induced by phytoplasmas, this study investigated to confirm the presence of these prokaryotes in symptomatic plants. After DNA extraction from symptomatic and asymptomatic plants, phytoplasmas were found in all affected plants through the nested PCR. Examination by transmission electron microscopy (TEM) using appropriately prepared segments of leaf veins allowed the visualization of typical pleomorphic cells of phytoplasmas in the phloem of symptomatic plants. The computer-simulated RFLP patterns and the phylogenetic analysis allowed identifying the detected phytoplasmas as a '*Candidatus* Phytoplasma fraxini'-related strain belonging to the 16SrVII-B subgroup. Moreover, physalis was identified as an additional host species for phytoplasmas in the 16SrVII group, expanding the current knowledge on the host range of phytoplasmas in this group.

Keywords: Mollicutes, phloem bacteria, cape-gooseberry, yellows

Introduction

Physalis (*Physalis peruviana* L.) is a species native to the Andes region belonging to the family Solanaceae. It has over a dozen common names worldwide, including cape-gooseberry and goldenberry. The plants are herbaceous at approximately 40-70 cm high and reproduce by seed with an annual life cycle (Lorenzi and Matos, 2008). Morphologically, physalis presents alternating and pubescent leaves with various shapes (Silva and Agra, 2005). In addition, the plants present solitary flowers and berry-type fruits that are small, orange in color, and protected by sepals with the shape of a balloon. Compounds of this plant have medicinal effects and its extract is indicated to treat numerous diseases in humans (Mahalaksmi and Nidavani, 2014). Industrially, the fruits are used to make ice cream, juices, jellies, yogurts, and cakes (Muniz et al., 2014). The roots and leaves are used for pharmaceutical purposes and the sepal and calix are used for artistic purposes. Physalis began to be grown commercially in the state of São Paulo at the end of the nineties and cultivation started to expand to other Brazilian states due to the economic interest of growers 10 years later (Muniz, 2010).

Phytoplasmas are cell wall-less bacteria inhabiting phloem sieve tubes, transmitted by sap sucking insects, and associated to numerous diseases in cultivated and wild plant species (Bertaccini and Duduk, 2009). Molecular and phylogenetic features based on sequence variability of the 16S rRNA gene have been used for classification of these prokaryotes within ribosomal groups and subgroups (Lee et al., 1998; Zhao et al., 2009). Symptoms, such as phyllody, shoot proliferation, leaf abnormality, chlorosis, and dwarfism are frequently associated to the presence of phytoplasmas in plants (Bertaccini et al., 2014). This kind of symptomatology results from interference of these pathogens with hormonal, nutritional, and stress signaling pathways (Dermastia, 2019).

This study investigated physalis plants displaying leaf malformation, chlorosis, and shoot proliferation. Since these symptoms are commonly induced by phytoplasmas, this study aimed to confirm the presence of phytoplasmas in symptomatic physalis plants and identify them.

Materials and Methods

Disease incidence was estimated considering the percentage of symptomatic plants in the fields in the municipality of Piracicaba (22°43′30″ S, 47°38′51″ W, altitude 547 m), São Paulo State, Brazil. Leaves were collected from five symptomatic (Figure 1) and two asymptomatic plants. Segments of leaf veins were prepared for examination by transmission electron microscopy (TEM) according to methods previously described (Maunsbach and Afzelius, 1998). The total nucleic acids were extracted according to a Doyle and Doyle protocol (1990). The nested PCR assays primed by R16mF2/mR1 and R16F2n/R2 were performed to detect phytoplasmas (Gundersen and Lee, 1996). Extracts from *Erigeron bonariensis* infected with a phytoplasma representative of the 16SrVII group (GenBank ErWB-BR01- KJ831066 – Flôres et al., 2015) and asymptomatic physalis plants represented the positive and negative controls, respectively. The no-template control was represented by sterile water.

The products derived from the nested PCR were cloned in the *Escherichia coli* DH5α strain using the pGEM Easy Vector System I and sequenced subsequently. Each phytoplasma detected in each physalis plant was considered a strain and three clones were sequenced for each strain. The contigs were assembled by Embrapa Electropherogram quality analysis website and the BIOEDIT program was used to edit and align the sequences.

The computer-simulated RFLP analysis (Wei et al., 2008) was conducted with 16S rRNA gene sequences of the physalis phytoplasma and sequences from phytoplasmas of diverse subgroups of the 16SrVII group. The restriction profiles were compared with each other and a similarity coefficient (F) was calculated for each pair of phytoplasma sequences (Wei et al., 2008). In addition, the DNA sequence corresponding to the phytoplasma in physalis plants was also analyzed by *i*PhyClassifier (Zhao et al., 2009). A phylogenetic tree including the nucleotide sequence representative of the strain molecularly characterized from physalis (1.25 kb), sequences of phytoplasmas in the 16SrVII subgroups, and other sequences belonging to distinct groups was generated using MEGA 6.0 (Tamura et al., 2013) with the neighbor-joining method and 1,000 bootstraps. The sequence of *Acholeplasma palmae* (GenBank NR 029152) was used as the root of the phylogenetic tree.

Figure 1 – Symptomatic shoots of *Physalis peruviana* showing chlorosis, leaf malformation, and shoot proliferation.

Results and Discussion

Disease incidence was 1-2 % and the occurrence of phytoplasmas was consistently proven in all samples analyzed, confirming the association of symptomatic physalis plants with the presence of this bacterium. The phytoplasma was detected by the nested PCR, which yielded DNA fragments of approximately 1.2 kb. Amplifications were also observed for DNA extracted from the positive control; however, no amplification occurred from extracts obtained from asymptomatic physalis plants and water. These findings were confirmed through TEM by visualizing pleomorphic cells in phloem tubes of PCR-positive plants (Figure 2).

Based on the absence of polymorphism among the clone sequences, a single sequence was selected as a representative of the phytoplasma detected in physalis samples. This sequence was designated as *Physalis peruviana* yellow (Ppy-Br01) and deposited in GenBank under accession number MT218429. This sequence shared identity values higher than 99 % with other members of the 16SrVII group. The computer-simulated RFLP analysis allowed the classification of the physalis phytoplasma in the 16SrVII-B subgroup since the collective restriction patterns were indistinguishable from those of the phytoplasmas of this subgroup (Figures 3A and 3B). The identity between both phytoplasmas was also confirmed by the value of the similarity coefficient (F), which was equal to 1.0 (Table 1). Furthermore, the phylogenetic tree showed that the physalis phytoplasma clustered in a monophyletic branch with other representatives of the 16SrVII group, emerging from the same branch as a strain of the 16SrVII-B subgroup (Figure 4).

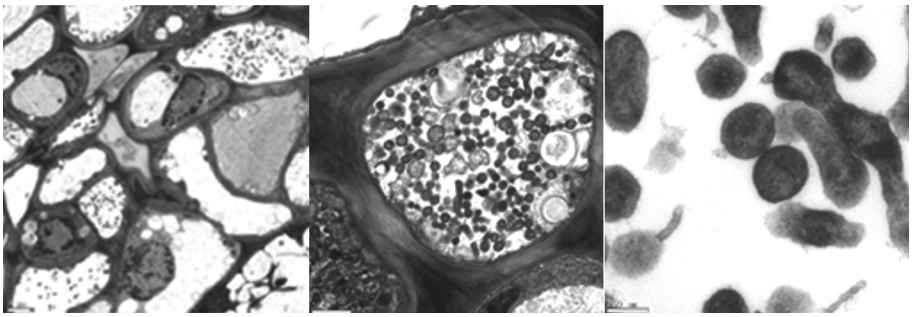

Figure 2 – Phytoplasma cells in the inner of phloem vessels of physalis plants visualized by transmission electron microscopy. The values of magnification bars are 2 µm, 1 µm, 200 nm from left to right.

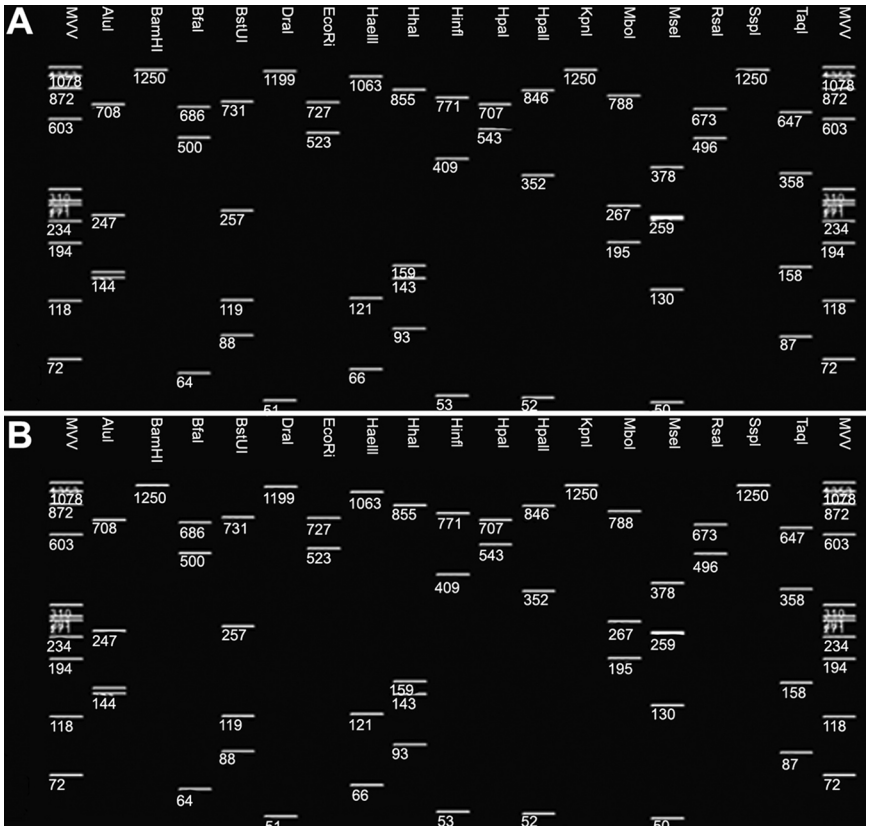

Figure 3 – Virtual RFLP gel generated by in silico digestion of the DNA sequence corresponding to the 16Sr rDNA region of the phytoplasma in *Physalis peruviana* (A) and the reference phytoplasma for subgroup 16SrVII-B (B).

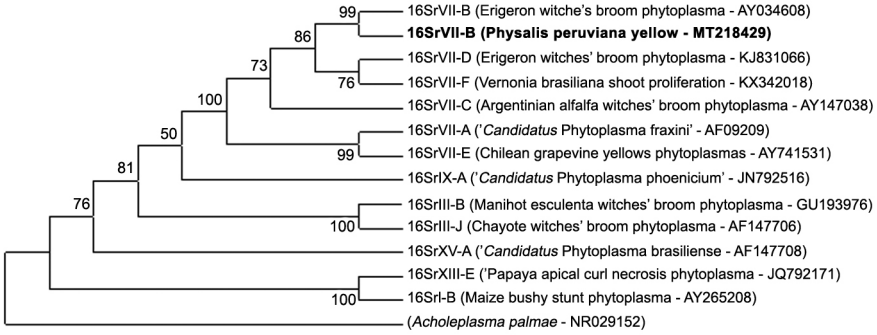

Figure 4 – Phylogenetic tree constructed with nucleotide sequences of phytoplasmas from different groups detected in Brazil and the sequence representative of phytoplasma associated with diseased physalis plants. Bootstrapping was performed with 1,000 times and *Acholeplasma palmae* was included as outgroup.

Table 1 – Similarity coefficients (F) based on collective restriction patterns produced from virtual RFLP analysis, including the phytoplasma identified in physalis plants (Ppy-Br01) and distinct representatives of subgroups within the 16Sr VII group.

Subgroups	16SrVII-A	16SrVII-B	16SrVII-C	16SrVII-D	16SrVII-E	16SrVII-F	**Ppy-Br01**
16SrVII-A	1						
16SrVII-B	0.92	1					
16SrVII-C	0.86	0.89	1				
16SrVII-D	0.87	0.94	0.83	1			
16SrVII-E	0.64	0.61	0.55	0.62	1		
16SrVII-F	0.91	0.96	0.88	0.80	0.60	1	
Ppy-Br01	**0.93**	**1**	**0.89**	**0.95**	**0.59**	**0.93**	**1**

The diagnosis based on the symptoms observed in physalis plants was confirmed by phytoplasma detection and identification in all symptomatic plants. Molecular detection was reinforced by TEM observations, which showed typical pleomorphic cells in the phloem of diseased plants but not in asymptomatic samples. The computer-simulated RFLP analysis with *i*PhyClassifier identified the phytoplasma as a member of the 16SrVII-B subgroup.

The geographic distribution of phytoplasmas in the 16SrVII group seems to be mainly restricted to the American continent. Thus, members of 16SrVII-A were mainly found in the United States and Canada (Griffiths et al., 1999; Zunnoon-Khan et al., 2010). The 16SrVII-B phytoplasma was first identified in Brazil in *Catharanthus roseus* and *Erigeron* sp. (Barros et al., 2002) and then in Argentina in erigeron plants (Meneguzzi et al., 2008). The 16Sr VII-C subgroup was initially described in Argentina and associated with anomalies in alfalfa crops (Conci et al., 2005). Later, a phytoplasma affiliated with this subgroup was reported in Brazil inducing shoot proliferation in sunn hemp (Flôres et al., 2013). The 16SrVII-D subgroup was also first identified in Brazil in erigeron plants (Flôres et al., 2015). In Chile, the phytoplasma now attributed to subgroup 16SrVII-E was identified in grapevine belonging to 16SrVII-A subgroup (Gajardo et al., 2009; Pérez-López et al., 2016). More recently, a phytoplasma of subgroup 16SrVII-F was detected in *Vernonia brasiliana* in Brazil (Fugita et al., 2017).

This study reports physalis as a new host of a 16SrVII-B strain. In addition, to the best of our knowledge, this is the first report of phytoplasmas associated to the genus *Physalis*. Since the disease has occurred with low incidence, surveys involving epidemiological aspects and management practices have not been performed.

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Authors' Contributions

Conceptualization: Ferreira, J.; Fariña, A.; Bedendo, I.P. **Data acquisition:** Oliveira, F.F.; Fariña, A.; Ferreira, J. **Data analysis:** Ferreira, J.; Oliveira, F.F.; Almeida, C.A.; Kitajima, E.W.; Bedendo, I.P. **Design of methodology:** Ferreira, J.; Kitajima, E.W.; Bedendo, I.P. **Software development:** Ferreira, J.; Oliveira, F.F. **Writing and editing:** Ferreira, J.; Bedendo, I.P.

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