

A 'Candidatus Phytoplasma prunorum' related strain, 16SrX-B subgroup, is associated with Vitis vinifera cv. Syrah in Brazil

Antonia Holanda

antoniafabianaph@usp.br

University of Sao Paulo: Universidade de Sao Paulo

Jacson Ferreira

University of Sao Paulo: Universidade de Sao Paulo

Marcel Bellato Sposito

University of Sao Paulo: Universidade de Sao Paulo

João Paulo Rodrigues Marques

University of Sao Paulo: Universidade de Sao Paulo

Elliot Watanable Kitajima

University of Sao Paulo: Universidade de Sao Paulo

Ivan Paulo Bedendo

University of Sao Paulo: Universidade de Sao Paulo

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Abstract

Grapevine plants showing symptoms typically induced by phytoplasmas infection, characterized by leaf reddening or yellowing, reduce vigour, delayed growth and shoot proliferation with reduced leaf size, were observed in the state of São Paulo, Brazil. This disorder causes significant damage to grape production by delaying the onset of fruit bearing and reducing yield. Similar symptoms have been reported in grapevines grown in other countries revealing the presence of phytoplasmas in diseased plants. Based on the described symptoms, this study was conducted in order to demonstrate the association between phytoplasma and symptomatic grapevine plants, as well as characterize molecularly the detected pathogen. Molecular analysis was performed using PCR and RFLP assays, followed by phylogenetic analysis. Amplifications of genomic fragments of 1.2 evidenced the presence of phytoplasma in tissue of symptomatic samples confirming the preliminary diagnosis based on the symptoms. Molecular characterisation identified the phytoplasma as a 'Ca Phytoplasma prunorum' strain, belonging to the 16SrX-B subgroup. This is the first report of a phytoplasma belonging to the 16SrX-B group infecting grapevine plants in Brazil.

Introduction

The grapevine yellows complex occurs in several viticultural regions worldwide, including the Americas, Africa, Australia, Asia, and Europe. A diversity of cultivars are susceptible, especially those belonging to the European species *Vitis vinifera*, whereas rootstocks and American grapevines (*Vitis labrusca*) rarely exhibit symptoms (Constable, 2010). Diseases within this complex can cause severe yield losses and significant economic impact on grape production (Caudwell, 1964; Magarey & Wachtel, 1986b). Despite the similarity of symptoms, the phytoplasmas associated with this complex belong to different ribosomal groups and subgroups, varying according to the region and host. Typical symptoms include irregular yellowing in white cultivars or reddening in red cultivars, leaf rolling, shortened internodes, leaf blade necrosis, shoot dieback, poor lignification, floral abortion, and premature drying of grape clusters (Dermatisia et al., 2017).

Among the diseases comprised in this complex, flavescence dorée (FD) is considered the most economically important and has been reported in various European countries, including France, Italy, Spain, Serbia, Slovenia, and Switzerland (OEPP/EPPPO, 2007). The rapid spread of FD within vineyards is attributed to transmission by the leafhopper *Scaphoideus titanus*. The phytoplasmas associated with FD are classified in the 'Ca Phytoplasma ulmi' species, representing the 16SrV-C and 16SrV-D subgroups (Davis & Dally, 2001). Specifically, the strain 16SrV-D is predominant in France, Spain, and northern Italy (Angelini et al., 2001; Torres et al., 2003; Bianco et al., 2003). In contrast, representatives of the 16SrV-C subgroup occur in regions of Serbia, where mixed infections involving phytoplasmas of the 16SrX-B and 16SrXII-A subgroups have also been reported (Duduk et al., 2004; Zambon et al., 2018).

Another disease within the grapevine yellows complex is 'bois noir', which was initially described in eastern France (Caudwell, 1961) and later reported in Germany under the name "yellow disease"

(Caudwell, 1988). Both diseases differ from FD in several epidemiological aspects, including the absence of the vector *S. tитanus*, differences in varietal susceptibility, and association with phytoplasmas belonging to the 16SrXII-A group (Daire et al., 1997). In Australia, Australian grapevine yellows involves phytoplasmas of the 16SrI, 16SrII group, and 16SrXII-B subgroup (Davis et al., 1997), with restricted spread and probable vector transmission not yet confirmed (Constable et al., 2003). In the United States, phytoplasmas belonging to the 16SrI-A and 16SrIII-I subgroups have been associated with symptomatic grapevines (Davis et al., 1998), which have also been reported in Israel (Orenstein et al., 2001). In Italy, phytoplasmas of the 16SrI-B subgroup have been identified in single and mixed infections (Alma et al., 1996). In Chile, strains of the subgroups 16SrI-B, 16SrI-C, 16SrVII-A, and 16SrXII-A were also detected in plants infected by phytoplasmas (Fiori et al., 2007).

In 2024, in a commercial vineyard located in São Paulo State, Brazil, grapevine plants exhibiting leaf reddening, yellowing, slow growth, and shoot proliferation with reduced leaf size were observed (Fig. 1). Although pruning resulted temporary recovery of affected plants, symptoms reappeared after a short period. Similar symptoms have been reported in vineyards established in countries across Europe, the Americas, and Asia. In these regions, the disease is known as a complex referred to as grapevine yellows, which is associated with phytoplasmas and disseminated mainly by insect vectors and vegetative propagation material used in grapevine nursery production. The resulting losses are substantial, as the disease delays plant development, postpones the onset of production, and significantly reduces grape yield.

Material and methods

Sample collection

Samples consisting of leaves and new branches of symptomatic and asymptomatic *V. vinifera* cv. Syrah plants were collected in commercial grapevine in state of São Paulo, Brazil.

Total DNA extraction and PCR detection

The total DNA was extracted using the kit Dneasy plant mini kit (Qiagen) according to the manufacturer's instructions. Detection tests were conducted by nested PCR. In the first reaction the universal primer pair R16S P1 / R16S P6 (Deng & Hiruki, 1991) was used and in the second reaction the pair R16F2n / R2 (Gundersen and Lee, 1996). The positive control for the reactions was the DNA extracted from *Solidago microglossa* (Ferreira et al., 2022), carrier of phytoplasma, and the negative patterns were represented by samples of healthy grapevine plants proven by PCR test e water. The products generated in the PCR reactions were analyzed using electrophoresis in 1% agarose gel, containing the Sybr safe® 1% dye. The amplified fragments were visualized using the ultraviolet light transilluminator.

Cloning and sequencing of the phytoplasma 16S rRNA gene

The purified and quantified nested PCR products were cloned in *Escherichia coli*, strain DH5 α , using the Pgem-T Easy Vector System I cloning kit (Promega), according to the manufacturer's instructions. For the transformation reaction, the MAX Efficiency DH5 α Competent Cells kit (Promega™) was used, following the manufacturer's instructions. The plasmid was extracted using the Wizard® Plus SV Minipreps DNA Purification System Kit (Promega), according to the manufacturer's instructions. Seven plasmids were sequenced and of each plasmid three clones were sequenced.

Phytoplasma identification by virtual RFLP

The sequences of the 16S rRNA genomic fragment were submitted to Embrapa's Electropherogram quality analysis to obtain the contigs. Editing and alignment were performed using the BIOEDIT program. The pDraw32 V.1.1.121 program (AcaClone Software) was used to analyze putative phytoplasma sequence restriction site maps, simulating virtual digestion with the 17 enzymes proposed by Wei et al. (2007) for the 16S rDNA gene. The enzymes used were *AluI*, *BamHI*, *Bfal*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI*, *MseI*, *RsaI*, *SspI* and *TaqI*. After in silico digestion, a simulated image of 3% agarose gel electrophoresis was generated in the pDRAW32 program. The restriction patterns obtained were compared with phytoplasma patterns belonging to the 16Sr groups. The calculation of similarity coefficients (F) was performed according to the methodology described by Nei & Li (1979).

Phylogenetic analysis

A phylogenetic tree was constructed using the nucleotide sequences of the 16S rRNA gene of the phytoplasma under study and of representatives of phytoplasmas belonging to the groups 16Sr. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1252.70251465 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of base differences per sequence. The analysis involved 33 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1184 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). As an outgroup, the mollicute *Acholeplasma palmae* strain J233 (GenBank accession number: NR 029152) was used.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

Midrib and petiole samples from symptomatic plants and controls were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2–7.4) and paraformaldehyde at 4 °C for 24–48 h, washed in buffer, and stored in 70% glycerol for 24 h. Samples were then cryofractured in liquid nitrogen, dehydrated through a graded ethanol series (30–100%), and critical-point dried with CO₂ (EM CPD300, Leica). The fragments were mounted on stubs using carbon tape, sputter-coated with gold (MED 010 Balzers Union), and examined using a JSM-IT300 scanning electron microscope (JEOL) operated at 20 kV (Horridge &

Tamm, 1969). For TEM analysis, samples were fixed in 2–4% glutaraldehyde in 0.1 M cacodylate buffer for 2–4 h at 4 °C, followed by post-fixation in 1% osmium tetroxide for 1–2 h. After dehydration through a graded ethanol or acetone series, the material was infiltrated with epoxy resin (Araldite/Epon) and polymerized at 60–70 °C for 24–48 h. The blocks were sectioned using an ultramicrotome to obtain ultrathin sections (~70 nm), contrasted with uranyl acetate and lead citrate, and finally examined using a transmission electron microscope operated at 80–120 kV.

Light Microscopy and Confocal Analysis

Petioles and midribs of grapevine leaves were fixed and dried according to the procedure described above, with the critical-point drying step omitted. After drying, the tissues were subjected to gradual resin infiltration (2:1, 1:1, and 100% resin), maintained under vacuum for 15 min and at 4 °C for 24 h at each step. Embedding was performed using resin and hardener at a 15:1 ratio. Sections were stained for 3–5 min with 50 µL of Dienes' stain (5 µL mL⁻¹) and rinsed with distilled water. The presence of phytoplasmas, observed as dark blue aggregates in the phloem, was confirmed by confocal epifluorescence microscopy (Leica TCS SP2/MP, Leica Microsystems, Bensheim, Germany).

Results

Phytoplasma detection

The presence of phytoplasmas in tissues of symptomatic grapevine plants was demonstrated by the amplification of the genomic fragments corresponding to the 16S rRNA. The amplifications, obtained by the use of nested PCR, were confirmed by the visualization of bands of approximately 1.2kb. Twenty plants were analyzed (each plant represented a sample), of these, 11 revealed the presence of phytoplasma. All samples positive for the pathogen showed symptoms characteristic of the disease. The 1.2kb bands were also observed in the positive control and no amplification occurred in the negative control.

Phytoplasma identification by virtual RFLP analysis

Seven samples were sequenced, as the sequences of all seven were identical, one of them was used to create the consensus sequence. This nucleotide sequence was called "Grapevine Leaf Reddening Phytoplasma – GLRPBr-01" (GenBank accession number: PZ098738). The alignment carried out between the GLRPBr-01 sequence of this study and representative sequences of all groups of phytoplasmas deposited in Genbank, showed that the phytoplasma detected in the grapevine plants of this study shows 99.84% identity with sequences of the 16S rRNA gene of phytoplasmas belonging to the 16SrX group. Using the interactive online tool *l*phyclasser to check which species this phytoplasma belongs to, the result obtained was 99.52% similar to the '*Candidatus* Phytoplasma prunorum' species. The virtual RFLP analysis showed that the phytoplasma GLRPBr-01 belongs to the subgroup 16SrX-B, with the value of the similarity coefficient equal to 1.00 when compared to the "Phytoplasma sp." (GenBank accession number: X68374) (Fig. 2 and 6). In addition, the percent identity of the sequence of

the phytoplasma identified in this study was 99.84% when this sequence was compared to the “Phytoplasma sp.”

Phylogenetic analysis

The phylogenetic study showed that the phytoplasma identified in the present study shares the same monophyletic clade as the reference phytoplasma of the 16SrX-B subgroup, showing a close evolutionary relationship between both (Fig. 6).

Observation in Scanning Electron Microscopy (SEM), Transmission Electronic Microscopy (TEM) and Light Microscopy and Confocal Analysis

Pleomorphic bodies with diameters ranging from 362 to 1,140 nm were observed within the sieve tubes of the phloem (Fig. 3), providing evidence of phytoplasma presence in the symptomatic samples. Starch crystals with diameters of 3,603 to 4,636 nm were also observed (Fig. 3). As well as the observation of pleomorphic bodies in phloem cells by transmission electron microscopy (Fig. 4). Under light microscopy, dark blue–stained punctate structures were detected in the phloem cells, whereas no such structures were observed in plants known to be healthy (Fig. 5).

Discussion

The symptomatology observed in the field, including interveinal reddening, chlorosis, leaf blade reduction, stunting, and excessive shoot proliferation, is consistent with typical manifestations of grapevine yellows previously described in major viticultural regions worldwide (Dermatisia et al., 2017; Constable et al., 2010). The progression to severe foliar senescence and plant death in several monitored vines underscores the pathogenic potential of phytoplasma infection under the environmental conditions of the study area (Angelini et al., 2001; Constable et al., 2003).

The detection of a phytoplasma belonging to the 16SrX-B subgroup in grapevine is particularly noteworthy, as this subgroup is rarely associated with grapevine yellows, in contrast to the predominance of 16SrV-C/D phytoplasmas responsible for the flavescente dorée disease and 16SrXII-A phytoplasmas associated with ‘*bois noir*’ in most affected regions. Although 16SrX-B members have been documented in mixed infections in Serbia and northeastern Italy (Duduk et al., 2004; Zambon et al., 2018), no significant outbreaks have been linked to this subgroup. Its occurrence in Brazilian vineyards therefore raises important epidemiological questions regarding introduction pathways, inoculum sources, and local adaptation. The absence of the principal vectors of FD (*S. tianus*) and ‘*bois noir*’ (*Hyalesthes obsoletus*), in Brazilian vineyards highlights a major knowledge gap concerning potential native or introduced vectors involved in transmission.

The 16SrX-B subgroup largely corresponds to ‘*Candidatus* Phytoplasma prunorum’, the causal agent of European Stone Fruit Yellows (ESFY), which is primarily associated with *Prunus* spp. and transmitted by *Cacopsylla* spp. (Seemüller & Schneider, 2004; Bertaccini & Duduk, 2010). Its detection in grapevine

suggests either unusual host colonization or the presence of local ecological interactions that favour cross-host transmission. Although rare, the presence of this phytoplasma in grapevine has been previously reported, with the most robust evidence provided by Contaldo et al. (2018), reinforcing that its presence, while rare, cannot be dismissed in diagnostic surveys.

Molecular diversity studies have shown that vineyards can harbour atypical or low-prevalence phytoplasmas (Zambon et al., 2018), and EPPO (2020) recommendations emphasize the inclusion of 'Ca. P. prunorum' in surveillance panels due to its regulatory importance. The successful detection of 16SrX-B in the present study using nested PCR and sequencing highlights the sensitivity of molecular diagnostics for low-titre infections and supports the need for broad-spectrum phytoplasma screening in regions where multiple groups may coexist.

Microscopy analyses provided additional evidence for the involvement of phytoplasmas, with pleomorphic bodies (≈ 362 nm) consistent with reported dimensions of these pathogens (Jelmini et al., 2021; Wang et al., 2024). The frequent association of starch crystal accumulation with phytoplasma-like bodies supports previous findings that altered carbohydrate metabolism and callose deposition contribute to phloem dysfunction and yellowing symptoms (Musetti et al., 2013; Wei et al., 2022; Ahmed et al., 2022). Histochemical detection using Dienes' stain further corroborated molecular results, demonstrating high diagnostic reliability when pathogen titres are adequate.

The persistence of phytoplasma-like structures in post-pruning regrowth for up to 20 days, even in the absence of functional roots, indicates systemic survival within remnant tissues. This finding is consistent with the strictly phloem-limited nature of phytoplasmas and their movement via assimilate flow (Wang et al., 2024). Previous studies have shown that latent buds and secondary phloem may act as internal reservoirs (Chkhaidze et al., 2016; Jelmini et al., 2021), explaining the inefficiency of pruning alone as a sanitation measure.

The heterogeneous distribution of the pathogen across tissues, also documented by qPCR quantification in other studies (Roggia et al., 2014), reinforces that management strategies relying solely on pruning are unlikely to eliminate infected vines. Instead, integrated approaches that combine removal of infected plants and vector management are required to limit spread. The unexpected detection of 16SrX-B in grapevine strengthens the need for enhanced surveillance and a broader diagnostic framework to prevent establishment of new phytoplasma complexes in viticultural regions.

The detection of a phytoplasma belonging to the 16SrX-B subgroup in symptomatic grapevines in São Paulo represents a significant and unprecedented finding for Brazilian viticulture. The combined evidence obtained in this study, nested PCR amplification of the 16S rRNA region, ultrastructural visualization of pleomorphic bodies in phloem sieve elements, and histological detection of dark-blue aggregates in stained sections, provides robust support for the association of a 16SrX-B phytoplasma with symptomatic *V. vinifera* cv. Syrah plants. To our knowledge, this is the first report of a phytoplasma of the 16SrX group in grapevine in Brazil.

Declarations

The authors declare that they have no conflict of interest.

A sample of the dataset used in this study is available upon request from the authors.

AI technology was not used to paraphrase any of the original content.

Authors' Contributions

Conceptualization: Sposito MB, Bedendo IP. **Data curation:** Sposito MB, Bedendo IP, Holanda AFP, Ferreira J. **Formal analysis:** Sposito MB, Bedendo IP, Holanda AFP, Ferreira J. **Funding acquisition:** Sposito MB, Bedendo IP. **Investigation:** Sposito MB, Bedendo IP, Holanda AFP, Ferreira J. **Methodology:** Holanda AFP, Ferreira J, Marques JPR, Kitajima EW. **Project administration:** Sposito MB, Bedendo IP. **Resources:** Sposito MB, Bedendo IP. **Supervision:** Sposito MB, Bedendo IP. **Writing - original draft:** Sposito MB, Bedendo IP, Holanda AFP, Ferreira J. **Writing - review & editing:** Sposito MB, Bedendo IP, Holanda AFP, Ferreira J.

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Figures



Figure 1

Foliar symptoms associated with grapevine yellows at different severity levels. Leaves exhibit chlorotic mosaic, interveinal reddening and blade reduction and shoot proliferation. Irregular discoloration reflects the progression of the physiological disorder typical of phytoplasma infections in grapevine.

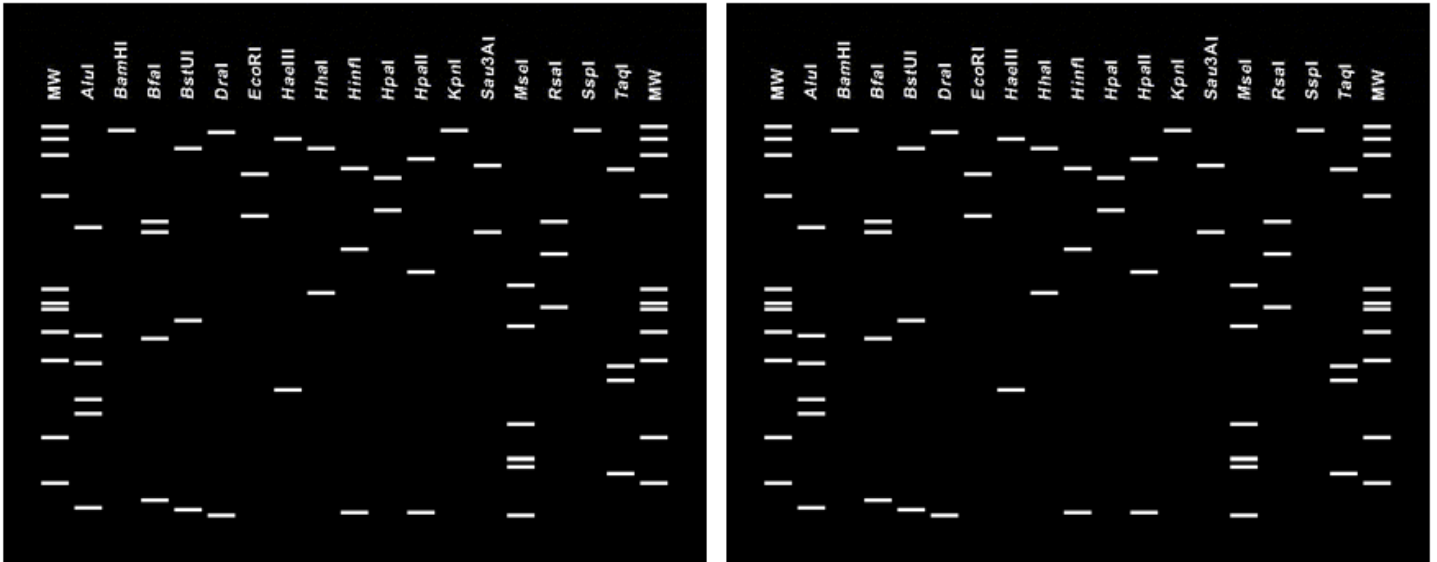


Figure 2

Profiles generated by enzymatic digestion. Left, the profile generated by GLRP-Br01 represents the phytoplasma found in Grapevine. Right, the profile produced by the Phytoplasma sp. (GenBank accession number: X68374), the reference phytoplasma of the subgroup 16SrI-B.

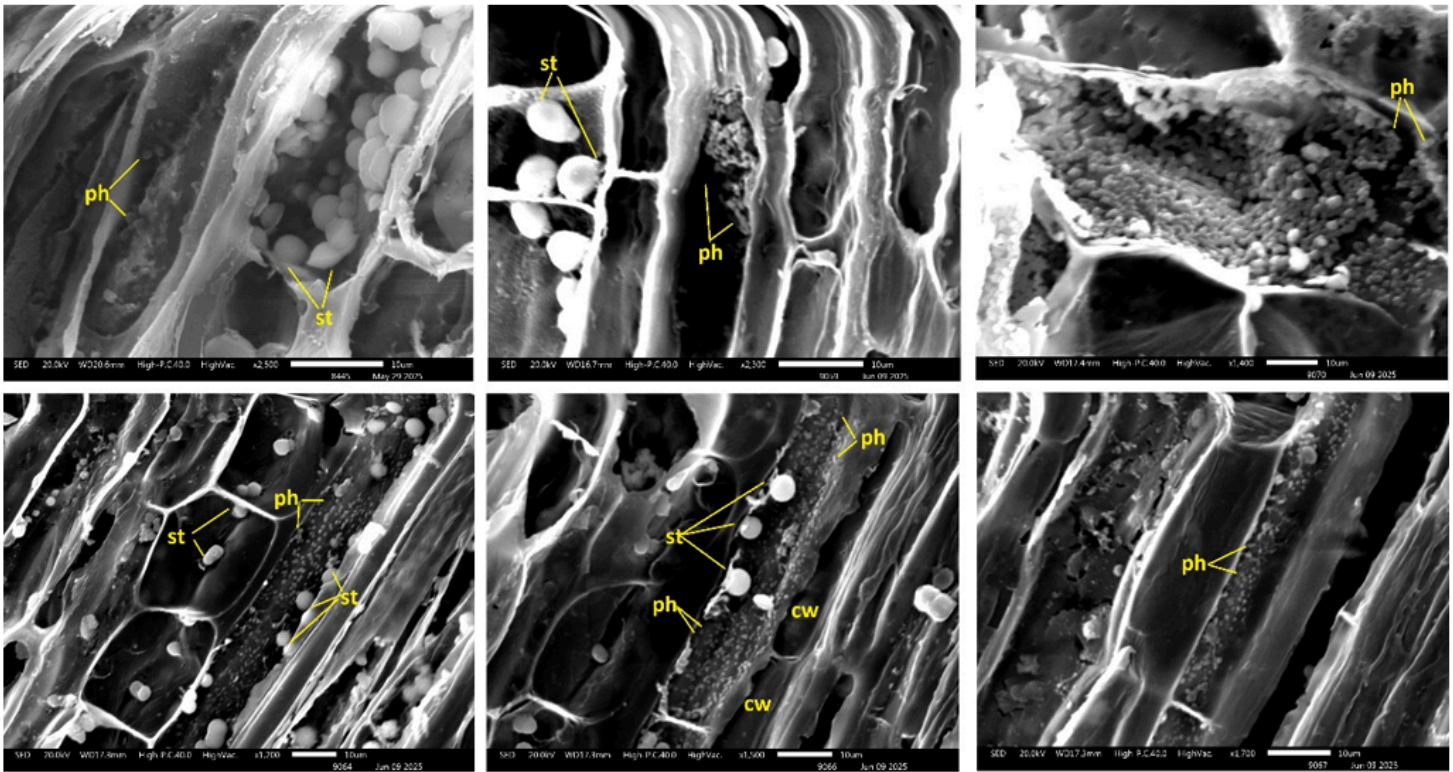


Figure 3

Ultrastructural visualization of phytoplasma-like bodies in leaf tissues. Scanning electron micrograph showing pleomorphic, spherical bodies of phytoplasma (ph) distributed along phloem-associated cells

and starch granules (st) in phloem cell (cw = cell wall) and parenchyma cell (scale bar = 10 μm).

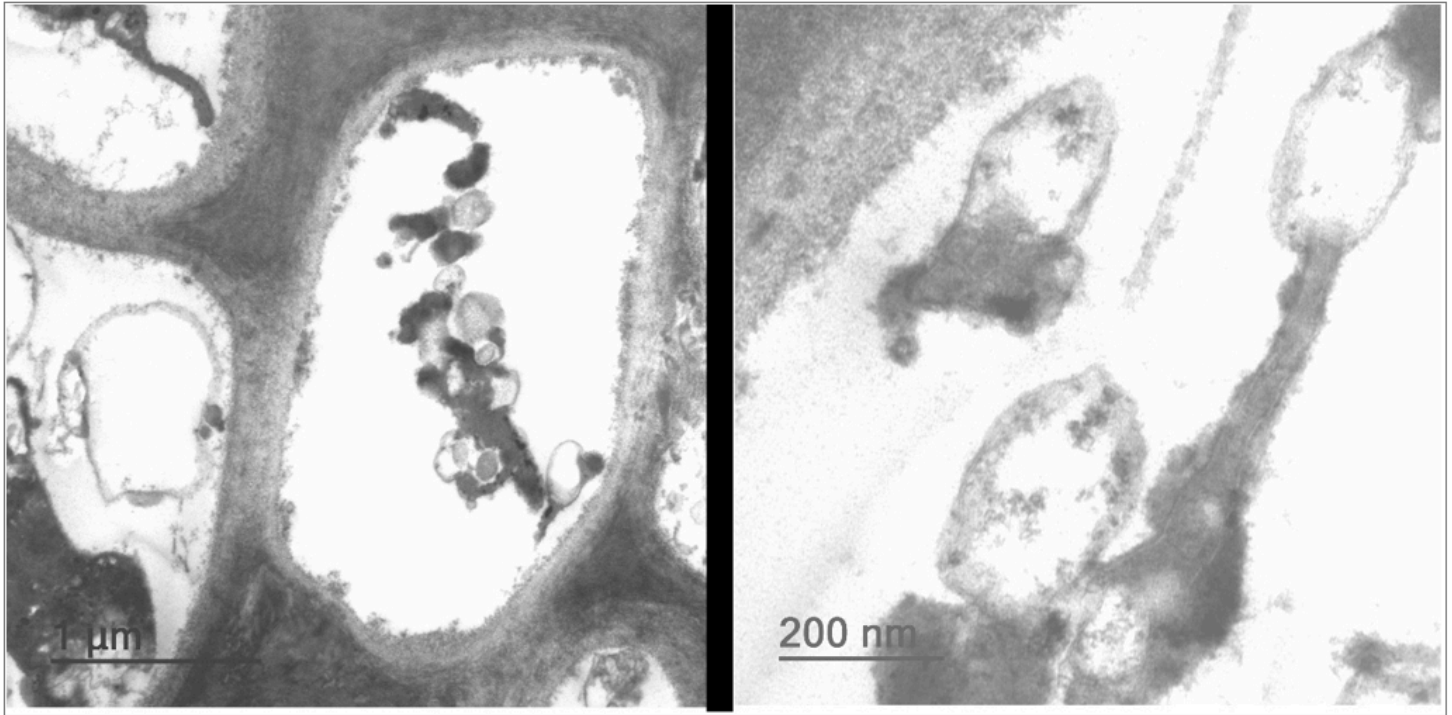


Figure 4

Transmission electron micrographs revealing phytoplasma-like structures within sieve elements (scale bars = 1 μm in B and 200 nm in C). Images illustrate the presence of intracellular, membrane-bound bodies consistent with phytoplasma infection.

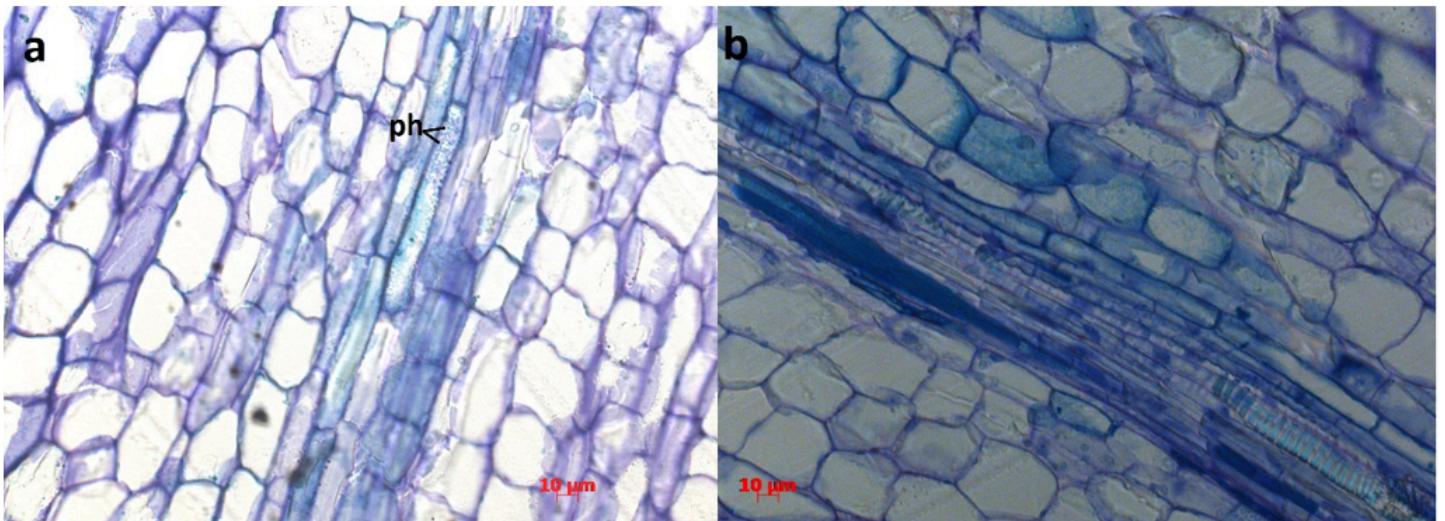


Figure 5

Longitudinal sections of stem tissues stained with Diene's Stain solution. On left, a microsection of a phloem vessel from a plant known to be infected shows the presence of dark-blue punctuations. On the

right, a microsection of the phloem from a plant know to be healthy shows the absence of dark-blue punctuations. Scale bar = 10 µm.

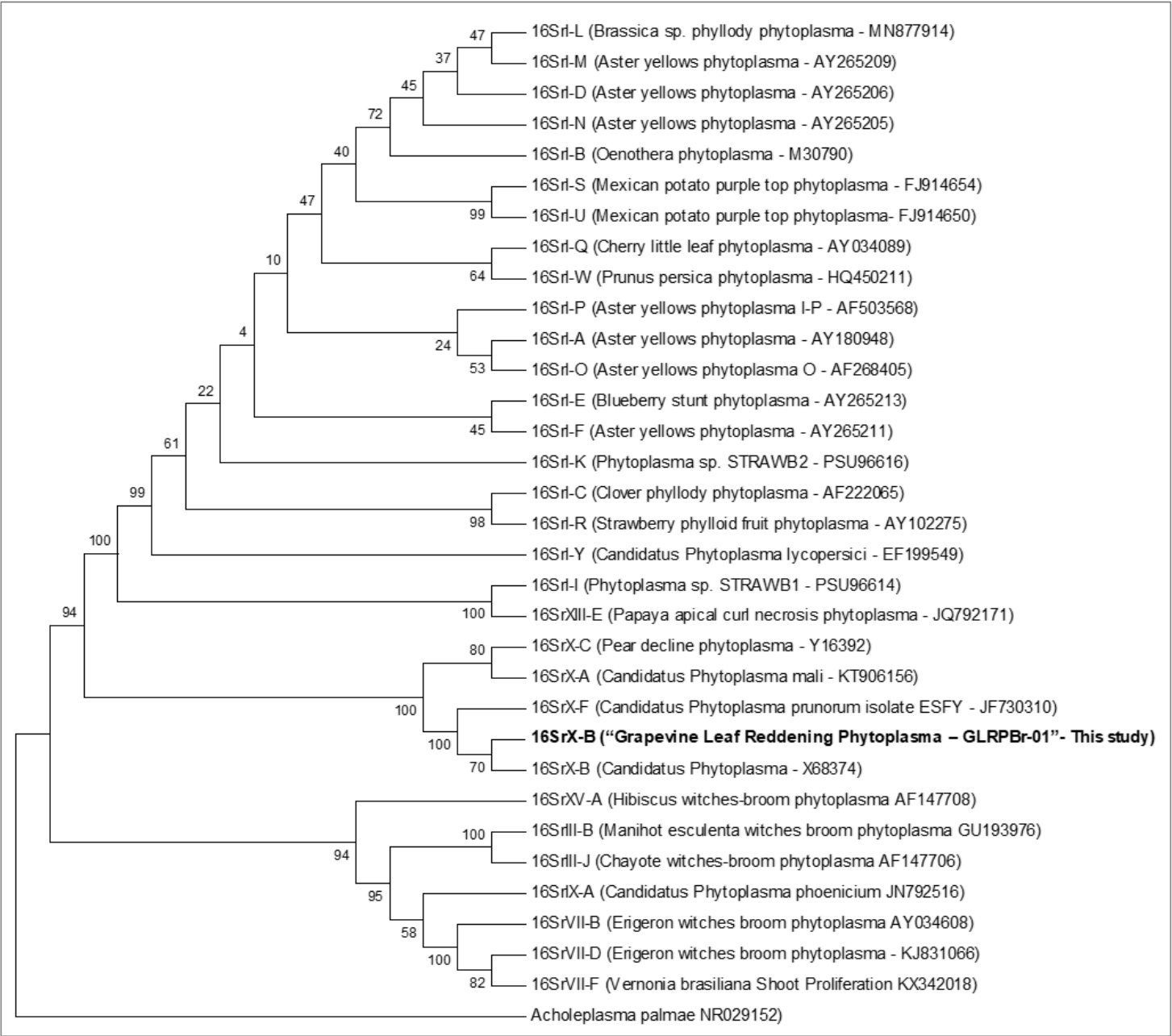


Figure 6

Phylogenetic tree constructed with the nucleotide sequences of the phytoplasma identified in the grapevine plants and phytoplasmas belonging to diverse 16Sr groups.