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Review Article

Phytoplasmas associated with grapevine yellows in Georgia, South Caucasus

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Abstract

Georgia is one of the primary centers of *Vitis vinifera* domestication and maintains a rich and diverse grapevine germplasm comprising over 500 autochthonous cultivars. Recent investigations have revealed distinct genetic identity and potential tolerance to major grapevine pathogens of this gene pool. Among these, grapevine yellows (GY) diseases, principally "flavescence dorée" and "bois noir" (BN), represent severe threats to viticulture worldwide. Surveys conducted in Georgian vineyards documented for the first time the presence of 'Candidatus Phytoplasma solani' (BN-associated phytoplasma) in both grapevines and *Convolvulus arvensis*, whereas the "flavescence dorée" phytoplasma was not detected. Molecular characterization revealed high genetic diversity among 'Ca. P. solani' strains, mainly linked to the bindweed-related pathosystem, and indicated that Georgian grapevine varieties display lower susceptibility to BN compared to international cultivars, which exhibited severe symptoms and yield loss. Subsequent large-scale surveys identified phytoplasmas belonging to the 16SrV group in Georgian vineyards, likely introduced through imported propagation material. Complementary field trials in Piedmont (northwestern Italy), a region heavily infected by "flavescence dorée", demonstrated that several Georgian cultivars infected by the "flavescence dorée" phytoplasma (genotype M54) exhibited mild or no symptoms and preserved stable berry and wine quality traits, indicating tolerance to the infection. The unique genetic diversity and reduced susceptibility of Georgian *V. vinifera* varieties to GY make this germplasm a valuable genetic resource for breeding programs aimed at enhancing phytoplasma tolerance while maintaining high oenological quality. The implementation of mandatory certification of grapevine planting materials produced in Georgia (since 2024) and the application of sensitive molecular diagnostic tools will be crucial to prevent phytoplasma spread and ensure the production of healthy nursery stock.

Introduction

Georgia is an ancient center of viticulture and winemaking, and one of the primary homelands of the cultivated grapevine (*Vitis vinifera* L.). Its geographical location determines the diversity of its natural conditions, flora, and fauna, creating a unique environment for the development of original

viticulture and winemaking (McGovern *et al.*, 2017). Over the past decade, Georgian grapevine cultivars and have been the focus of intensive scientific and viticultural investigations (Chkhartishvili and Maghradze, 2012; Maghradze *et al.*, 2009). The native Georgian germplasm comprises more than 500 cultivars, of which only a fraction is currently cultivated and vinified (Chkhartishvili and Maghradze, 2012). Among these, the prominent cultivars Saperavi (red) and Rkatsiteli (white) are internationally recognized and cultivated across Eastern Europe and Central Asia.

Genetic studies have demonstrated that Georgian grapevine germplasm represents a unique genetic pool, clearly distinct from both European (Riaz *et al.*, 2018, Imazio *et al.*, 2013) and Central Asian (Bacilieri *et al.*, 2013) populations. This genetic specificity, together with observed relationships between Georgian wild and cultivated grapevines, aligns with archaeological evidence attesting to the antiquity and originality of Georgian viticulture. Phenotypically, these genetic features are reflected in wide variability in grapevine morphology, phenology, and agronomic and enological traits, which differentiate Georgian germplasm from both neighboring and geographically distant grapevine populations (Maghradze *et al.*, 2009). Furthermore, recent studies have indicated that grapevine varieties selected in domestication centers of grapevine such as Georgia show tolerance or resistance to plant pathogens like *Plasmopara viticola* (Berk & MA Curtis) Berl & De Toni, which causes downy mildew (Bitsadze *et al.*, 2015; Toffolatti *et al.*, 2018) and *Erysiphe necator* Sch, which causes powdery mildew (Possamai *et al.*, 2021). Moreover, some resistant genotypes against *Plasmopara viticola* were also observed among accessions of European wild grapevine *V. vinifera sylvestis* Gmel originated from Georgia (Bitsadze *et al.*, 2024) and making Georgian grapevine gene pool more attractive for future research and breeding purposes.

Phytoplasmas are cell-wall less bacteria of the Mollicutes class associated with diseases in many crops (Bertaccini, 2007). In grapevine the diseases associated with phytoplasmas are grouped within the grapevine yellows (GY) complex. The two main GY diseases are "flavescence dorée" (FD), associated with the grapevine "flavescence dorée" phytoplasma (subgroups 16SrV-C and -D) (Martini *et al.*, 1999), and "bois noir" (BN), associated with '*Candidatus* Phytoplasma solani' (subgroup 16SrXII-A) (Quaglino *et al.*, 2013). Although FD- and BN-associated phytoplasmas are genetically and biologically distinct, infected grapevines show undistinguishable symptoms of chromatic alterations of the leaf lamina frequently accompanied by rolling or down curling of the leaf margins; incomplete lignification of shoots and progressive bunch dehydration and desiccation during June-July, culminating in withering during August-September (Belli *et al.*, 2010).

In Europe, FD is the only epidemic and the most economically damaging disease within GY. Grapevine "flavescence dorée" phytoplasma is transmitted from grapevine to grapevine by the leafhopper *Scaphoideus titanus* Ball (Homoptera: Cicadellidae), a grapevine-feeding leafhopper of American origin, which completes its life cycle exclusively on grapevines (Gonella *et al.*, 2024). Owing to its high epidemic potential, it is classified as a quarantine pathogen by European Union, with control measures including insecticide treatments targeting *S. titanus* and the removal of symptomatic vines (EFSA 2020, 2025). Recent studies revealed that FD epidemiology extends beyond the classical grapevine-*S. titanus* system, involving multiple vectors (*Dictyophara europaea*, *Orientus ishidae*, *Allygus mixtus*) and reservoir hosts (*Alnus glutinosa*, *Clematis vitalba*, *Ailanthus altissima*, *Corylus avellana*). While these alternative vectors may not sustain grapevine-to-grapevine transmission, they likely acquire this phytoplasma from reservoir hosts and transmit to grapevine, facilitating the spread by *S. titanus* (Casati *et al.*, 2017; Malembic-Maher *et al.*, 2020; Krstic *et al.*, 2022; Rigamonti *et al.*, 2023). The withdrawal of broad-spectrum insecticides used against leafhoppers may further contribute to recent FD recrudescence, underscoring the increasing complexity of FD epidemiology.

BN phytoplasma is present in Europe, South America, and Asia (Gajardo *et al.*, 2009; Quaglino *et al.*,

2013; Duduk *et al.*, 2010, Pierro *et al.*, 2019). This '*Ca.* P. solani' is occasionally transmitted to grapevines by *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) (Maixner, 1994), a polyphagous planthopper living preferentially on nettle (*Urtica dioica* L.), bindweed (*Convolvulus arvensis* L.), and chaste tree (*Vitex agnus-castus* L.) (Langer and Maixner, 2004; Kosovac *et al.*, 2016).

Recent studies show that the cixiid *Reptalus panzeri* is a vector of BN phytoplasma in Serbian vineyards (Cvrkovic *et al.*, 2014), other insects are alternative vectors of BN phytoplasmas to grapevines in northern Italy (Quaglino *et al.*, 2019), and several weeds in the vineyard agroecosystem are involved in BN phytoplasma epidemiology (Kosovac *et al.*, 2019; Moussa *et al.*, 2019; Quaglino *et al.*, 2021). Due to the ineffectiveness of insecticide treatments against the main insect vector, BN incidence in vineyards can reach 80% (Laimer *et al.*, 2009). It was reported that the quality of wine produced from grapes harvested from BN phytoplasma infected plants is significantly reduced, due to the unbalanced content of pectin, the reduction of sugar content, and the acidity increase (Ember *et al.*, 2018).

An important approach to managing GY diseases is the identification of resistant or tolerant plant varieties, either cultivated or wild, that can serve as sources of resistance genes for breeding programs. However, to date, no *Vitis* species or *V. vinifera* cultivars have exhibited immunity or strong resistance to the phytoplasmas associated with GY (Laimer *et al.*, 2009).

The aim of this paper is to make overview of the distribution of the GY diseases in Georgia as well as reaction of the Georgian autochthonous cultivars against these pathogens in both countries Georgia and Italy, where the Georgian cultivars were investigated in comparison to Italian and French cultivars.

Grapevine yellows in Georgia

Despite the importance and impact of GY diseases on grapevine productivity in Europe, investigation about GY diseases in Georgia have been carried out only recently. The first survey conducted in vineyards of the Kakheti region in September 2013 revealed *V.*

vinifera cv. Chardonnay plants exhibiting typical GY symptoms. In the same vineyards, bindweed plants showing shoot proliferation and leaf yellowing were also observed, suggesting the possible involvement of phytoplasmas in the disease. Molecular analyses revealed the presence of '*Ca.* P. solani' (associated with BN) in both symptomatic grapevines and bindweeds (Quaglino *et al.*, 2014). Consequently, in 2014 large surveys on GY symptoms were carried out in vineyards and *V. vinifera* germplasm collections in Khaketi and Shida (Inner) Kartli regions in eastern Georgia, including four western European or international varieties and 37 native Georgian varieties. Obtained data highlighted that most Georgian grapevine varieties showed moderate (color alterations and curling of the leaves, partial irregular ripening of wood, and mild berry shrivel; partially reduced production) and mild (color alterations and curling of the leaves; normal production) symptoms, while international cultivars exhibited severe symptoms (berry shrivel, desiccation of inflorescences, color alterations and curling of the leaves, reduction of growth, and irregular ripening of wood; complete loss of production) (Figure 1). '*Ca.* P. solani' was detected by polymerase chain reaction-based amplification of 16S rRNA gene in symptomatic grapevines. Further molecular characterization by multiple gene typing analysis (*vmp1* and *stamp* genes) revealed the presence of 11 distinct '*Ca.* P. solani' types. Only the type Vm53/St15 was identical to a type previously reported in periwinkle from Lebanon; the other '*Ca.* P. solani' types were described for the first time and found uniquely in Georgia. Phylogenetic analyses showed that '*Ca.* P. solani' types in Georgia are associated mainly with the bindweed-related BN host system. Moreover, the homogeneous distribution of '*Ca.* P. solani' strains in plants showing different symptom intensity suggests different susceptibility of grapevine varieties to BN, suggesting Georgian varieties as less susceptible to BN (Quaglino *et al.*, 2016). Interestingly, other study carried out in the same years evidenced that BN was spreading in Azerbaijan (South Caucasus region), where some '*Ca.* P. solani' strains, identified both in symptomatic grapevines and in *H. obsoletus*, were genetically similar

Figure 1. Mild or moderate symptoms shown by Georgian grapevine varieties. Odjaleshi (A), Shavkapito (B), Aladasturi (C), Tchvitiluri (D), Saperavi Atenis (E), Paneshi (F).
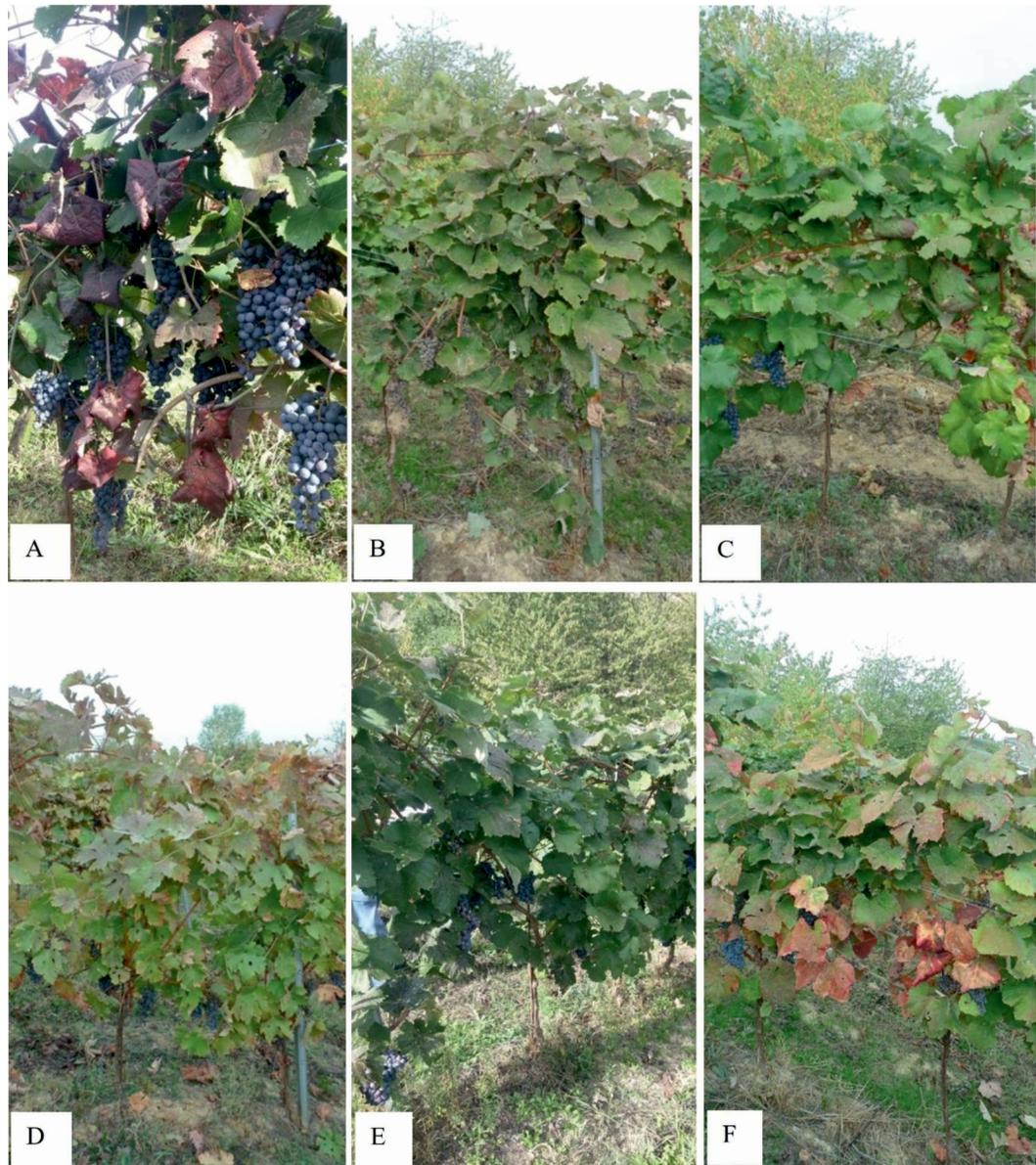
to '*Ca*. P. solani' type St15 (prevalent in Georgia) (Balakishiyeva *et al.*, 2018). This evidence highlighted that BN epidemiology in South Caucasus region could be associated mainly with the *H. obsoletus*-bindweed-grapevine pathosystem, prevalent in several Countries in Europe and Middle East (Passera *et al.*, 2020; Abu Alloush *et al.*, 2023; Plavec *et al.*, 2024).

More recently, the Shota Rustaveli National Science Foundation of Georgia funded a research project (NFR-18-874) to extensively survey the status of grapevine yellows in mother stocks, nursery collection vineyards, and commercial vineyards in eastern Georgia, including 15,000 grapevine plants of 16 varieties (4 Georgian and 12 international) (Megrelishvili *et al.*, 2022). Based on the observation of typical or suspicious GY symptoms such as leaf color alterations (yellowing or reddening) and rolling, the average GY incidence was around 2%. As reported in Quaglino *et al.* (2016), most of the symptomatic grapevine plants of Georgian cultivars showed mild symptoms of GY with no alterations on the berries. Even if examined grapevines exhibited typical or suspicious GY

symptoms, only 35% of such plants were found phytoplasma-infected by triplex quantitative PCR assays (Angelini *et al.*, 2007; Pelletier *et al.*, 2009). As expected from previous studies reporting the wide presence of BN in Georgian vineyards (Quaglino *et al.*, 2016), 'Ca. P. solani' was identified in most phytoplasma-infected plants (47.6%), with the highest infection rate in Chardonnay, one of the most susceptible varieties exhibiting unambiguously typical GY symptoms (Eveillard *et al.*, 2016). Phytoplasmas in group 16SrV, were never reported before in Georgia and in the South Caucasus region but were erratically found in Turkey (Ertunc *et al.*, 2015) but were detected in 45.6% of the grapevines phytoplasma-infected. Further molecular typing of 16SrV phytoplasmas, conducted by sequence analysis of marker genes (*map*, *vmp1*) (Malembic-Maher *et al.*, 2020), have been planned to further clarify the identity of these phytoplasmas. It is important to notice that international grapevine cultivars Chardonnay and Cabernet Sauvignon, found to be infected by 16SrV phytoplasmas, are cultivated in Georgia in commercial vineyards (Sargolzaei *et al.*, 2021). Thus, it is reasonable to hypothesize that the import in Georgia from abroad of international grapevine cultivar planting materials could lead to the risk of emerging of other phytoplasma infectious diseases, such as FD. Due to the effect and impact of climate change on plant health and agriculture, including the entrance and adaptation of plant pathogens and pests in agroecosystems (Skendzic *et al.*, 2021; Gullino *et al.*, 2022; Lahlali *et al.*, 2024), it is reasonable to hypothesize that FD phytoplasmas and their vector(s) could adapt to Georgian climatic conditions.

Among GY, FD phytoplasma is an EPPO quarantine pathogen which requires permanent field and laboratory monitoring. Testing rootstock and grafting stock plants for phytoplasma diseases, choosing phytoplasma free plants and removing infected stocks, are useful steps to reduce the spreading of FD and BN. Thus, the knowledge of typical GY symptoms and the utilization of accurate diagnostic tools are crucial for preventing pathogen spread and producing healthy planting material (Megrelishvili *et al.*, 2016; Quiroga *et al.*, 2020; Nair *et al.*, 2021; Elbakidze *et al.*, 2022).

Performance and adaptation of Georgian varieties in Europe

As the abovementioned studies showed that Georgian *V. vinifera* varieties have low susceptibility to BN, recent research investigated the performance of white (Goruli Mtsvane, Mtsvane Kakhuri, Rkatsiteli, Sapena, and Tchvitiluri) and red (Aladasturi, Odjaleshi, Paneshi, Saperavi, and Shavakapito) Georgian grapevine varieties in a highly FD-infected area in Piedmont (northwestern Italy), exploring their susceptibility to FD and testing their oenological potential through berry and wine quality analyses (Portaccio *et al.*, 2025). Field surveys, conducted in a case-study vineyard containing central-western European, Georgian, and PIWI (fungus-resistant grapevine varieties) varieties, evidenced that mortality of young plants and infection percentage index were significantly higher in Georgian and central-western European varieties, respectively. All Georgian varieties exhibited none or mild symptoms without a reduction of the number of symptomless berries (Figure 1). Molecular analyses identified only the FD phytoplasma genotype M54 in infected grapevines, suggesting that differences in symptom severity were related to variety-specific response to infection. Despite infection, Georgian varieties maintained stable berry and wine quality parameters, showing no significant changes in acidity, sugar content, and flavor profile. Thus, Georgian *V. vinifera* varieties examined in this study could represent a valuable resource for viticulture in Piedmont (and, probably, in other viticultural regions of Italy and Europe as well) by offering a sustainable solution to reduce the FD economic impact and to contribute to the diversification and resilience of local vineyards. Georgian varieties had great oenological potential and responded well to both FD phytoplasma infection and local agroecosystem conditions of Piedmont.

Summarized data on the reaction of Georgian autochthonous varieties to BN and FD are presented in Table 1, showing a genotype-selective reaction of the

Table 1. Symptom severity observed in Georgian grapevine varieties in Caucasus Region and Italian environmental conditions. In bold are provided the varieties having infection of both phytoplasmas ("bois noir" and "flavescence dorée"), having mild or moderate symptom severity.

Georgian varieties	GY survey		Symptom severity
	Georgia (BN)	Italy (FD)	
Adznizhi	yes	no	mild
Aladasturi	no	yes	mild
Alexandrouli	no	yes	mild
Amlakhy	yes	no	mild
Asuretuli Shavi	yes	no	mild
Chinuri	**yes**	**yes**	**mild**
Chkhaverii	yes	no	mild
Chuberi	yes	no	mild
Grdzelmtevana	yes	no	mild
Khikhvi	yes	no	mild
Khikhvi variation	yes	no	mild
Kikhvi Loladzis	yes	no	mild
Kistauri Saghvine	yes	no	mild
Korkaula	yes	no	mild
Manavis Mtsvane	yes	no	mild
Mtredisphekha	yes	no	mild
Mtsvane Kakhuri	**yes**	**yes**	**mild**
Mujuretuli	yes	no	mild
Odjaleshi	no	yes	mild
Okhtoura	no	yes	mild
Paneshi	no	yes	mild
Qistauruli sagvine	yes	no	mild
Rkatsiteli	**yes**	**yes**	**mild**
Sapena	no	yes	mild
Saperavi Atenis	no	yes	mild
Saperavi Budeshuri	yes	no	mild
Shavkapito	no	yes	mild
Tavkveni Saperaviseburi	yes	no	mild
Tavkveri	yes	no	mild
Tchvitiluri	no	yes	mild
Tshnoris Tetri	yes	no	mild
Tsitska	yes	no	mild
Tsolikouri	yes	no	mild
Usakhelouri	yes	no	mild
Buera	yes	no	moderate
Goruli Mtsvane	**yes**	**yes**	**moderate**
Saperavi	**yes**	**yes**	**moderate**
Saperavi Pachkha	yes	no	moderate
Kisi	yes	no	severe

varieties in general, with 34 varieties demonstrating mild symptoms, 4 varieties with moderate, and only 1 (Kisi) with severe symptoms.

Conclusions and perspectives

The less susceptibility to BN and FD observed in the majority of Georgian *V. vinifera* varieties in Georgia (BN) and Italy (FD) could be attributed to host-pathogen interaction dynamics or physiological mechanisms. The practical application of these varieties in BN- and/or FD-affected regions could reduce the economic impact of these diseases. Moreover, in the case of FD, for which grapevine is the main phytoplasma acquisition source for the vector *S. titanus*, the tolerance of Georgian varieties could also limit the pathogen propagation and vector acquisition efficiency. Phytoplasma quantification and controlled inoculation studies coupled with transcriptomic analyses are necessary to elucidate the tolerance mechanisms. Moreover, Georgian *V. vinifera* germplasm could provide genetic traits for introgression into susceptible European varieties through conventional or marker-assisted breeding programs facilitating the development of new varieties combining FD tolerance with desirable enological and agronomic traits. Furthermore, vineyard diversification through the introduction of Georgian varieties may be useful in adapting to climate change and maintaining viticultural productivity and economic viability across wine-growing regions of Europe.

In Georgia, the certification of grapevine planting material, produced by the local nurseries, became mandatory starting in 2024. The information provided in this review, including studies describing phytoplasma disease symptoms and tolerant varieties (Quaglino *et al.*, 2016; Megrelishvili *et al.*, 2022; Portaccio *et al.*, 2025) and reliable and sensitive detection protocols based on molecular biology techniques (Megrelishvili *et al.*, 2022), will help the control operations in nursery vineyards, the production of certified nursery stocks, and the categorization of Georgian grapevine varieties based on their susceptibility to FD and BN diseases.

Acknowledgement

References

Abu Alloush AH, Bianco PA, Busato E, Alkhawaldeh Y, Alma A, Tedeschi R and Quaglino F 2023. Grapevine yellows in Jordan: associated phytoplasmas, putative insect vectors and reservoir plants. *Plant Pathology*, 72(8): 1380-1392.

Angelini E, Bianchi G, Filippin L, Morassutti C and Borgo M 2007. A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. *Journal of Microbiological Methods*, 68: 613-622.

Bacilieri R, Lacombe T, Le Cunff L, Di Vecchi-Staraz M, Lacouu V, Genna B, Péros J-P, This P and Boursiquot J-M 2013. Genetic structure in cultivated grapevines is linked to geography and human selection. *BMC Plant Biology*, 13: 25.

Balakishiyeva G, Bayramova J, Mammadov A, Salar P, Danet J-L, Ember I, Verdin E, Foissac X and Huseynova I 2018. Important genetic diversity of 'Candidatus Phytoplasma solani' related strains associated with bois noir grapevine yellows and planthoppers in Azerbaijan. *European Journal of Plant Pathology*, 151: 937-946.

Belli G, Bianco PA and Conti M 2010. Grapevine yellows: past, present and future. *Journal of Plant Pathology*, 92: 303-326.

Bertaccini A 2007. Phytoplasmas: diversity, taxonomy, and epidemiology. *Frontiers in Bioscience*, 12: 673-689.

Bitsadze N, Aznarashvili MA, Vercesi A, Chipashvili R, Failla O and Maghradze D 2015. Screening of Georgian grape germplasm for susceptibility to downy mildew. *Vitis*, 54: 193-196.

Bitsadze N, Kikilashvili Sh, Chipashvili R, Mamasakhlisashvili L, Maghradze T, Kikvadze M, Toffolatti SL, De Lorenzis G, Failla O, Ocete Rubio R and Maghradze D 2024. Resistance to downy mildew in wildly growing Eurasian *Vitis vinifera* L. grapevines. *Journal of Plant Pathology*, 106: 1759-1771.

Casati P, Jermini M, Quaglino F, Corbani G, Schaerer S, Passera A, Bianco PA and Rigamonti IE 2017. New insights on "flavescence dorée" phytoplasma ecology in the vineyard agro-ecosystem in southern Switzerland. *Annals of Applied Biology*, 171: 37-51.

Chkhartishvili N and Maghradze D 2012. Viticulture and winemaking in Georgia. Caucasus and Northern Black Sea Region Ampelography. *Vitis*, Special Issue: 169-176.

Cvrkovic T, Jovic J, Mitrovic M, Krstic O and Tosevski I 2014. Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of "bois noir". *Plant Pathology*, 63: 42-53.

Duduk B, Tian JB, Contaldo N, Fan XP, Paltrinieri S, Chen QF, Zhao QF and Bertaccini A 2010. Occurrence of phytoplasmas related to "stolbur" and to 'Candidatus Phytoplasma japonicum' in woody host plants in China. *Journal of Phytopathology*, 158(2): 100-104.

EFSA, European Food Safety Authority, Tramontini S, Delbianco A and Vos S 2020. Pest survey card on "flavescence dorée" phytoplasma and its vector *Scaphoideus titanus*. *EFSA Supporting Publications*, 17(8).

EFSA, European Food Safety Authority, Nougadère A, Makowski D, Rzepecka D, Scala M, Sánchez B, Baldassarre F, Tramontini S, Vos S, Ferretti L, Costantini E and Gentili A 2025. Grapevine "flavescence dorée" phytoplasma pest report to support the ranking of EU candidate priority pests. *EFSA Supporting Publications*, 22(7).

Elbakidze T, Gaganidze D, Aznarashvili M and Badalashvili K 2022. A survey for two major grapevine viruses in Georgian vineyards, Caucasus region. *International Journal of Agriculture and Biology*, 28: 113-117.

Ember I, Bodor P, Zsófi Z, Pálfi Z, Ladányi M, Pásti G, Deák T, Nyitrainé DS, Bálo B, Szekeres A, Bencsik O, Foissac X, Palkovics L, Hunter JJ and Bisztray GD 2018. "Bois noir" affects the yield and wine quality of *Vitis vinifera* L. cv. Chardonnay. *European Journal of Plant Pathology*, 152: 185-197.

Ertunc F, Orel DC, Bayram S, Paltrinieri S, Bertaccini A, Topkaya S and Soylemezoglu G 2015. Occurrence and identification of grapevine phytoplasmas in main viticultural regions of Turkey. *Phytoparasitica*, 43: 303-310.

Eveillard S, Jollard C, Labroussaa F, Khalil D, Perrin M, Desqué D, Salar P, Razan F, Hevin C, Bordenave L, Foissac X, Masson J and Malembic-Maher S 2016. Contrasting susceptibilities to "flavescence dorée" in *Vitis vinifera*, rootstocks and wild *Vitis* species. *Frontiers in Plant Science*, 7: 1762.

Gajardo A, Fiore N, Prodan S, Paltrinieri S, Botti S, Pino AM, Zamorano A, Montealegre J and Bertaccini A 2009. Phytoplasmas associated with grapevine yellows disease in Chile. *Plant Disease*, 93: 789-796.

Gonella E, Benelli G, Arricau-Bouvery N, Bosco D, Duso C, Dietrich CH, Galetto L, Rizzoli A, Jovic J, Mazzoni V, Mori N, Nieri R, Roversi PF, Strauss G, Thiéry D, Trivellone V, Virant-Doberlet M, Lucchi A and Alma A 2024. *Scaphoideus titanus* up-to-the-minute: biology, ecology, and role as a vector. *Entomologia Generalis*, 44: 481-496.

Gullino ML, Albajes R, Al-Jboory I, Angelotti F, Chakraborty S, Garrett KA, Hurley BP, Juroszek P, Lopian R, Makkouk K, Pan X, Pugliese M and Stephenson T 2022. Climate change and pathways used by pests as challenges to plant health in agriculture and forestry. *Sustainability*, 14(19): 12421.

Kosovac A, Jakovljevic M, Krstic O, Cvrkovic T, Mitrovic M, Tosevski I and Jovic J 2019. Role of plant-specialized *Hyalesthes obsoletus* associated with *Convolvulus arvensis* and *Crepis foetida* in the transmission of 'Candidatus Phytoplasma solani'- inflicted "bois noir" disease of grapevine in Serbia. *European Journal of Plant Pathology*, 153: 183-195.

Kosovac A, Radonjic S, Hrncic S, Krstic O, Tosevski I and Jovic J 2016. Molecular tracing of the transmission routes of "bois noir" in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus* (Cixiidae). *Plant Pathology*, 65: 285-298.

Krstic O, Cvrkovic T, Marinkovic S, Jakovljevic M, Mitrovic M, Tosevski I and Jovic J 2022. Genetic diversity of "flavescence

dorée" phytoplasmas in vineyards of Serbia: from the widespread occurrence of autochthonous Map-M51 to the emergence of endemic map-FD2 (vectotype II) and new map-FD3 (vectotype III) epidemic genotypes. *Agronomy*, 12: 448.

Imazio S, Maghradze D, De Lorenzis G, Bacilieri R, Laucou V, This P, Scienza A and Failla O 2013. From the cradle of grapevine domestication: molecular overview and description of Georgian grapevine (*Vitis vinifera* L.) germplasm. *Tree Genetics & Genomes*, 9: 641-658.

Lahlali R, Taoussi M, Laasli SE, Gachara G, Ezzouggari R, Belabess Z, Aberkani K, Assouguem A, Meddich A, Jarroudi ME and Barka EA 2024. Effects of climate change on plant pathogens and host-pathogen interactions. *Crop and Environment*, 3(3):159-170.

Laimer M, Lemaire O, Herrbach E, Goldschmidt V, Minafra A, Bianco P and Wetzel T 2009. Resistance to viruses, phytoplasmas and their vectors in the grapevine in Europe: a review. *Journal of Plant Pathology*, 91: 7-23.

Langer M and Maixner M 2004. Molecular characterisation of grapevine yellows associated phytoplasmas of the "stolbur" group based on RFLP-analysis of non-ribosomal DNA. *Vitis,* 43: 191-199.

Maghradze D, Rossoni M, Imazio S, Maitti C, Failla O, Del Zan F, Chkhartishvili N and Scienza A 2009. Genetic and phenetic exploration of Georgian grapevine germplasm. *Acta Horticolturae*, 827: 107-114.

Maixner M 1994. Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). *Vitis,* 33: 103-104.

Malembic-Maher S, Desqué D, KhaliI, D, Salar P, Bergey B, Danet J-L, Duret S, Dubrana-Ourabah M-P, Beven L, Ember I, Acs Z, Della Bartola M, Materazzi A, Filippin L, Krnjajic S, Krstic O, Tosevski I, Lang F, Jarausch B, Kolber M, Jovic J, Angelini E, Arricau-Bouvery N, Maixner M and Foissac X 2020. When a Palearctic bacterium meets a Nearctic insect vector: Genetic and ecological insights into the emergence of the grapevine "flavescence dorée" epidemics in Europe. *Plos Pathogens*, 16: e1007967.

Martini M, Murari E, Mori N and Bertaccini A 1999. Identification and epidemic distribution of two "flavescence dorée" related phytoplasmas in Veneto (Italy). *Plant Disease*, 83(10): 925-930.

McGovern P, Jalabadze M, Batiuk S, Callahan MP, Smith KE, Hall GR., Kvavadze E, Maghradze D, Rusishvili N, Bouby L, Failla O, Cola G, Mariani L, Boaretto E, Bacilieri R, This P, Wales N and Lordkipanidze D 2017. Early Neolithic wine of Georgia in the South Caucasus. *Proceedings of the National Academy of Sciences of the United States of America*, 114(48): E10309-E10318.

Megrelishvili I, Khidesheli Z, Ujmajuridze L and Chiqovani N 2016. The study of viral diseases in Georgian vine grafted nurseries. *International Journal of Development Research*, 6(7): 8299-8302.

Megrelishvili I, Khidesheli Z, Elbakidze T, Ujmajuridze L, Quaglino F and Maziashvili N 2022. Survey on phytoplasmas associated with grapevine yellows in Eastern Georgia, Caucasus region. *Journal of Plant Protection Research*, 62(3): 231-237.

Moussa A, Mori N, Faccincani M, Pavan F, Bianco PA and Quaglino F 2019. *Vitex agnus-castus* cannot be used as trap plant for the vector *Hyalesthes obsoletus* to prevent infections by 'Candidatus Phytoplasma solani' in northern Italian vineyards: experimental evidence. *Annals of Applied Biology*, 175: 302-312.

Nair S and Manimekalai R 2021. Phytoplasma diseases of plants: molecular diagnostics and way forward. *World Journal of Microbiology and Biotechnology*, 37: 102.

Passera A, Zhao Y, Murolo S, Pierro R, Arsov E, Mori N, Moussa A, Silletti MR, Casati P, Panattoni A, Wei W, Mitrev S, Materazzi A, Luvisi A, Romanazzi G, Bianco PA, Davis RE and Quaglino F 2020. Multilocus genotyping reveals new molecular markers for differentiating distinct genetic lineages among 'Candidatus Phytoplasma solani' strains associated with grapevine "bois noir". *Pathogens*, 9: 970.

Pelletier C, Salar P, Gillet J, Cloquwmin G, Very P, Foissac X and Malembic-Maher S 2009. Triplex real-time PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. *Vitis*, 48: 87-95.

Pierro R, Semeraro T, Luvisi A, Garg H, Vergine M, De Bellis L and Gill HK 2019. The distribution of phytoplasmas in South and East Asia: an emerging threat to grapevine cultivation. *Frontiers in Plant Science*, 10: 1108.

Plavec J, Ivancan G, Skoric D, Foissac X and Seruga Music M 2024. Genetically divergent 'Candidatus Phytoplasma solani' isolates in Croatian vineyard pathosystems suggest complex epidemiological networks. *Phytopathology Research*, 6: 46.

Portaccio L, Paissoni MA, Giacosa S, Passera A, Barbieri C, Maghradze D, Rolle L, Gerbi V, Failla O, Bianco PA and Quaglino F 2025. Performance of Georgian grapevine varieties in a vineyard infected by "flavescence dorée" phytoplasma in Piedmont, Northwestern Italy. *Agriculture*, 15(18): 1988.

Possamai T, Wiedemann-Merdinoglu S, Merdinoglu D, Migliaro D, De Mori G and Cipriani G 2021. Construction of a high-density genetic map and detection of a major QTL of resistance to powdery mildew (*Erysiphe necator* Sch.) in Caucasian grapes (*Vitis vinifera* L.). *BMC Plant Biology*, 21: 528.

Riaz S, De Lorenzis G, Velasco D, Koehmstedt A, Maghradze D, Bobokashvili Z, Musayev M, Zdunic G, Walker MA, Failla O, Preece JE, Aradhya M and Arroyo-Garcia R 2018. Analysis of the genetic diversity and structure of cultivated and wild grapevine (*Vitis vinifera* L.) accessions around the Mediterranean basin and Central Asia regions. *BMC Plant Biology*, 18(1): 137.

Quaglino F, Zhao Y, Casati P, Bulgari D, Bianco PA, Wei W and Davis RE 2013. 'Candidatus Phytoplasma solani', a novel taxon associated with "stolbur" and "bois noir" related diseases of plants. International Journal of Systemic and Evolutionary Microbiology, 63: 2879-2894.

Quaglino F, Maghradze D, Chkhaidze N, Failla O, Casati P and Bianco PA 2014. First report of 'Candidatus Phytoplasma solani' and 'Candidatus Phytoplasma convolvuli' associated with grapevine "bois noir" and bindweed yellows, respectively, in Georgia. Plant Disease, 98: 1151.

Quaglino F, Maghradze D, Casati P, Chkhaidze N, Lobjanidze M, Ravasio A, Passera A, Venturini G, Failla O and Bianco PA 2016. Identification and characterization of new 'Candidatus Phytoplasma solani' strains associated with "bois noir" disease in Vitis vinifera L. cultivars showing a range of symptoms severity in Georgia, the Caucasus Region. Plant Disease, 100: 904-915.

Quaglino F, Sanna F, Moussa A, Faccincani M, Passera A, Casati P, Bianco PA and Mori N 2019. Identification and ecology of alternative insect vectors of 'Candidatus Phytoplasma solani'. Scientific Reports, 9: 19522.

Quaglino F, Passera A, Faccincani M, Moussa A, Pozzebon A, Sanna F. Casati P, Bianco PA and Mori N 2021. Molecular and spatial analyses reveal new insights on "bois noir" epidemiology in Franciacorta vineyards. Annals of Applied Biology, 179: 151-168.

Quiroga N, Gamboa C, Soto D, Pino AM, Zamorano A, Campodonico J and Fiore N 2020. Update and new epidemiological aspects about grapevine yellows in Chile. Pathogens, 9(11): 933.

Rigamonti IE, Salvetti M, Girgenti P, Bianco PA and Quaglino F 2023. Investigation on "flavescence dorée" in north-western Italy identifies map-M54 (16SrV-D/map-FD2) as the only phytoplasma genotype in Vitis vinifera L. and reveals the presence of new putative reservoir plants. Biology, 12: 1216.

Sargolzaei M, Rustioni L, Cola G, Ricciardi V, Bianco PA, Maghradze D, Failla O, Quaglino F, Toffolatti SL and De Lorenzis G 2021. Georgian grapevine cultivars: ancient biodiversity for future viticulture. Frontiers in Plant Science, 12: 94.

Skendzic S, Zovko M, Zivkovic IP, Lesic V and Lemic D 2021. The impact of climate change on agricultural insect pests. Insects, 12(5): 440.

Toffolatti SL, De Lorenzis G, Costa A, Maddalena G, Passera A, Bonza MC, Pindo M, Stefani E, Cestaro A, Casati P, Failla O, Bianco PA, Maghradze D and Quaglino F 2018. Unique resistance traits against downy mildew from the center of origin of grapevine (Vitis vinifera). Scientific Reports, 8: 12523.

Review Article

Citrus stubborn disease (*Spiroplasma citri*) in Türkiye: the past, the present and the future

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Abstract

Citrus stubborn disease (CSD) was originally discovered in California in 1915 and it has been one of the most important problems which affects the citrus industry in the East Mediterranean region of Türkiye since 1950's. The first studies began in 1965 and were remained limited to survey and biological indexing studies until 1980s. *Spiroplasma citri* as the causal agent of stubborn disease was cultured for the first time from symptomatic citrus plants and other hosts in 1987 in Türkiye. Then polyclonal antisera was produced against local *S. citri* isolates, one dimensional gel-electrophoresis was used for further characterization and spiroplasmas were observed under scanning and transmission electron microscopes both in infected citrus tissues and also in culture. *S. citri* was also isolated from the *Catharanthus roseus* placed in citrus orchards where stubborn disease was detected and *Exitianus capicola* STAL, collected from the same plots indicating the first evidence of natural possible insect transmission of stubborn disease. In the following years the epidemiological aspects of CSD were investigated in more details and new leafhopper and herbaceous hosts of *S. citri* were reported. Because of CSD diagnosis is difficult due to low and variable concentrations of *S. citri* in diseased trees and the random distribution of the pathogen, recently molecular detection techniques like PCR has been used both for diagnostic and genetic diversity studies of local *S. citri* isolates. The future studies will be focued on functional genomics in host-pathogen interaction using transcriptomic and proteomic approaches to learn more about the full mechanisms of the S. citri pathogenicity to develop new diagnostic methods and plant protection strategies to control citrus stubborn disease.

Keywords: insect transmitted bacteria, host-pathogen interaction, citrus disease, overview

Introduction

Türkiye has great potential for citrus growing, with a total production of 7,877,982 tons, ranking 5th in the world (FAO, 2023). Citrus stubborn disease (CSD) is a worldwide citrus disease that reduces the productivity and growth of infected trees. It was initially observed in California in 1915 and it was later proven as a graft-transmissible disease in North Africa, Middle East and most of the Mediterranean countries (EFSA, 2014). It has been one of the most important problems which affects the citrus industry especially in the East Mediterranean region of Türkiye since 1950's. CSD was first detected on Washington Navel oranges grown in Dörtyol and Mersin provinces in 1959 (Chapot, 1959). After that, the first survey studies by Turkish agronomists were conducted in the Eastern

Mediterranean Region and the incidence of the disease was found about 75-89% in Navel oranges whereas it was very low in local orange cultivars (Cengiz, 1965). Further studies in the Aegean Region in the same year showed that CSD was also present in that region but much lower incidence (Ozalp and Azeri, 1967). While studies on CSD were limited to survey and biological indexing until the 1980s, studies on the diagnosis and characterization of the disease agent, *Spiroplasma citri*, were carried out in the following years. In frame of these studies, *S. citri*, was first time cultured, characterized by microscopic, serological and molecular techniques (Çaglayan, 1987). Then detection of possible insect vectors and experimental transmission studies were performed (Çaglayan and Markham, 1994; Çinar and Çaglayan, 1988). Between 1990 and 2000, *S. citri*-vector relationships and shoot tip grafting studies to elucidate the epidemiological aspects of the disease and to obtain clean propagation materials were conducted (Baspnar *et al.*, 1993; Kersting *et al.*, 1992; Sas-Sertkaya, 1999). Recent studies have applied molecular techniques to detect, characterize, and compare *S. citri* isolates from citrus, herbaceous hosts and possible insect vectors. PCR targeting specific genes such as housekeeping, membrane protein, plasmid-associated markers, or pathogenicity related genes has provided higher sensitivity compared to serological assays and has allowed consistent detection of *S. citri* across diverse hosts and environments. To elucidate the phylogenetic relationships of Turkish *S. citri* isolates with global strains, historical isolates preserved for approximately 40 years, together with recently obtained isolates, are under investigation by whole-genome sequencing using High-Throughput Sequencing (HTS) and Oxford Nanopore Technology (ONT) (Kara *et al.*, unpublished data).

CSD seems to be no longer a major problem in Türkiye following the use of *S. citri*-free budwoods in new plantings, the implementation of indexing initiatives and successful eradication programs. This overview summarizes the past and present state of knowledge about CSD in Türkiye, as well as future research directions.

Survey and indexing studies

The first big survey studies by Turkish agronomists were conducted in Eastern Mediterranean and Aegean Regions to study prevalence and economical importance of CSD (Cengiz, 1965; Ozalp and Azeri, 1967). Although stubborn symptoms were observed in all local and foreign orange varieties, grapefruit, mandarin, shaddock and kumquats, the most severe symptoms were observed in Washington and Thomson Navel oranges in Eastern Mediterranean Region. The incidence of the disease in local oranges was 12% while it was 75% on Thomson Navel and 89% on Washington Navel oranges. Yield losses caused by CSD were found to be 13% in local oranges, 17% in Washington Navel and 25% in Thomson Navel oranges. In addition, the ratio of discarded fruits collected from CSD infected oranges were was 5% in local cultivars but 44% and 45% in Thomson and Washington Navels (Cengiz, 1965). In the same years another big survey was conducted in Türkiye in another important citrus growing region, Aegean Region, (Ozalp and Azeri, 1967). This study showed that CSD was also present in this region but comparing to the Mediterranean part of Türkiye, disease prevalence was much lower. After these symptomological observations the first indexing studies were performed by using Madam Vinous oranges by Calavan during in 1967 and the first symptoms were observed two months after inoculation confirming the presence of CSD in the country. Further survey studies were conducted in Eastern Mediterranean Region between 1980-1985 to update the status of stubborn disease and the most severe symptoms werw observed in Washington Navel oranges followed by Valencia and Jaffa oranges, grapefruits, mandarins and tangelos (Çaglayan, 1987). The prevelance of the disease was detected from 39% to 67% depending on the province where different citrus species were grown (Çaglayan and Cinar, 1990). In a recent survey study conducted in Eastern Mediterranean Region, 141 citrus, 8 sesame, 2 periwinkle (*Catharanthus roseus*), and 918 potential insect vector samples belonging to the *Cicadellidae* family were collected and tested by PCR analyses using

primers targeting the P58 and P89 gene regions, along with nine newly designed primer pairs specific to secretion system and fructose metabolism genes. The results showed that *S. citri* was detected in 38 citrus, two sesame and one periwinkle plants while all tested insect samples were found negative (Kara *et al.*, unpublished). Comparing the results of the current study with those conducted approximately 35 years ago in the same region, the prevalence rate of the disease is quite low (7.8%). This is attributed to the successful eradication program and the selection of new citrus varieties, replacing the Navel group oranges, known to be most susceptible to stubborn disease, with new citrus varieties.

Detection and characterization of *Spiroplasma citri*

Isolation and culture. Spiroplasma citri was first isolated and cultured from symptomatic Washington Navel, Skags Bonanza Navel, Valencia, and local sweet oranges, as well as from Fremont and Encore mandarins and periwinkle placed in stubborn-infected citrus orchards to assess natural CDS transmission in Adana Province, Eastern Mediterranean Region (Çaglayan, 1987). Subsequent isolations from stubborn-infected citrus demonstrated that *S. citri* could be cultivated all the year-round. The presence of *S. citri* was confirmed by helical cell structures observed with dark-field and electron microscopy. Characteristic fried-egg and satellite colonies were additionally observed on solid media (Figure 1) (Çaglayan, 1987; Çaglayan and Çinar, 1990).

Serological and molecular diagnosis. The polyclonal antisera raised against *S. citri* cultures obtained from stubborn infected Washington Navel orange (isolate SPT-WN) proved to be a reliable tool for *S. citri* detection (Çaglayan, 1987; Çaglayan and Çinar, 1990). *S. citri* antisera was found specific for their respective homologous antigens. After IgG was purified and conjugate was prepared, these reagents were used for optimization studies to develop routine ELISA tests by using different *S. citri* cultures to adapt it to field tests. After this optimization studies, ELISA reagents developed against Turkish *S. citri* isolates were successfully used for routine DAS-ELISA analysis to confirm the presence of *S. citri* in different citrus species (Çaglayan, 1987; Çaglayan and Çinar, 1990).

The first molecular characterization of *S. citri* isolates obtained from different citrus species exhibiting stubborn symptoms in Türkiye was conducted using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to examine differences in protein patterns and the mobility of major proteins among nine Turkish isolates (Çaglayan, 1987). An Israeli isolate of *S. citri* (SPA) and a honeybee spiroplasma (*S. melliferum*) isolate (BC3) were included for comparison. Results indicated that all Turkish isolates displayed highly similar protein patterns, with approximately 40 bands detected. No significant differences were observed in the mobility of the major protein, spiralin (about 26 kDa), between the Turkish isolates and the SPA isolate, although patterns differed slightly for honeybee spiroplasma. One-dimensional SDS-PAGE has previously been used successfully to compare *S. citri* strains, confirming that most strains exhibit similar protein profiles (Daniels *et al.*, 1980). Later studies demonstrated that *S. citri* isolates could be separated into groups and subgroups based on the mobility of major proteins with molecular weights of 23–25 kDa (Foissac *et al.*, 1996). Spiralin, the major and one of the most thoroughly characterized *S. citri* membrane proteins, shows 83.5%, 85.1%, and 88.9% sequence identity with spiralins of *S. kunkelii*, *S. phoeniceum*, and *S. melliferum*, respectively (Chevalier *et al.*, 1990; Foissac *et al.*, 1997).

Because symptom-based diagnosis of CSD can be misleading, and alternative methods such as culture or

Figure 1. *Spiroplasma citri* cells observed under dark-field microscopy (A) and transmission electron microscopy at ×28,000 magnification (B). Fried-egg colonies of *S. citri* stained with Dienes stain at ×500 magnification (C).
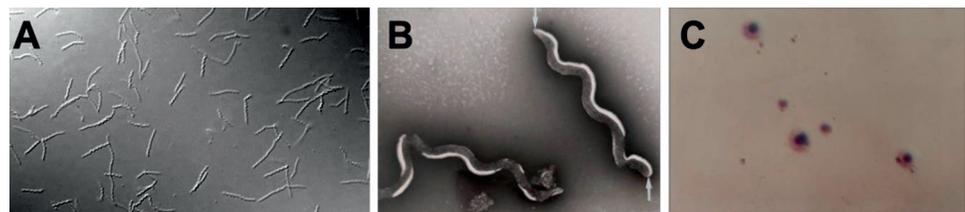
serological assays are time-consuming and costly, molecular assays based on PCR have been developed to overcome these limitations. PCR sensitivity is 100 to 1000-fold higher than that of ELISA or culture-based assays (Saillard and Bové, 1983). It has been reported that spiralin primers lack sufficient sensitivity for detecting the pathogen in citrus trees under field conditions. Therefore, new primers targeting multicopy membrane protein genes, which provide more sensitive results for the definitive diagnosis of *S. citri*, have been developed (Yokomi *et al.*, 2008). Among these genes, P89 is assumed to be an adhesin protein gene located both on the *S. citri* genome and on a plasmid, while P58 is considered an adhesin multigene present in multiple copies in the *S. citri* genome due to multiple viral insertions.

The only study focusing on molecular characterization of Turkish *S. citri* isolates was conducted on seven citrus samples showing stubborn disease symptoms collected from Adana Province. In this study, three different primer pairs (spiralin-f/r, P89-r/f, and P58-6f/4r) were used for *S. citri* detection by PCR analysis and while no positive results were obtained with the spiralin-f/r primer pair in the limited number of tested samples, all seven samples tested positive with the P89-r/f and P58-6f/4r primer pairs (Çaglar *et al.*, 2020). A more detailed study was recently performed using both symptomatic and symptomless citrus materials collected from Eastern Mediterranean Region (Kara *et al.*, unpublished).

To characterize different *S. citri* isolates, PCR-based detection technique was performed by focusing on *spiralin*, P58 putative adhesin-like multigene, and P89 putative adhesin genes of *S. citri*. DNA extracted from 141 citrus, 8 sesame, 2 periwinkle, and 918 potential insect vector samples belonging to the *Cicadellidae* family and were used as a template for amplification of products of 675 bp, 450 bp and 707 bp using Spiralin-f/r, P89-r/f, and P58-6f/4r primer pairs by PCR, respectively. The results showed that *S. citri* was detected in 38 citrus, 2 sesame and 1 periwinkle plants while all tested insect samples were found negative. Furthermore, the amplicons were subjected to Sanger sequencing and the obtained isolates were characterized molecularly by sequence analysis showing 95.06% identity with *S. citri* GII3-3X strain which was originally isolated from the leafhopper *Circulifer haematoceps*, collected in Morocco and 99.05% identity with BLH-MB strain which was originally isolated from a Navel orange tree in Riverside, California, USA (Kara *et al.*, unpublished). In addition, in this new study, novel primers have been designed that can be used for the identification of *S. citri*, for distinguishing strain differences, and for providing further insights into pathogenicity among strains. For the design of these primers, secretion system genes and fructose operon genes, which are closely associated with pathogenicity, were targeted.

S. citri lacks type II and III secretion systems and relies on the Sec secretion system, a *sec* operon-dependent pathway associated with bacterial conjugation that delivers substrates to target cells and contributes to pathogenicity (Cascales and Christie, 2003). Sec-dependent genes (*secY*, *secA*, *secE*, *ftsY*, *ffh*, *yidC*) are conserved across isolates. Additionally, fructose operon genes (*fruR*, *fruA*, *fruK*) are major virulence factors enabling fructose utilization for plant pathogenicity (Dubrana *et al.*, 2016; Gaurivaud *et al.*, 2000). These nine genes represent potential targets for the design of new primers for identification of Turkish *S. citri* isolates.

To date, complete genome sequences of *S. citri* from 11 different strains have been published; however, strain-to-strain variations have not been fully analyzed (Davis *et al.*, 2017; Khanchezar *et al.*, 2022; Rattner *et al.*, 2021; Saillard *et al.*, 2008; Yokomi *et al.*, 2020). Numerous spiroplasma species still lack genome sequence data. Previous reports have demonstrated that *Spiroplasma* genomes are highly variable among strains, making it difficult to interpret spiroplasma biology solely based on the genome sequences. Therefore, whole-genome sequencing of *S. citri* isolates from different geographic regions and their deposition in GenBank is of great importance for a complete understanding of the spiroplasma genome. However, assembling complete spiroplasma genomes remains challenging due to their relatively small size (570–1000 kbp) and the presence of numerous plasmids. In

addition, they contain a large number of repeats and mobile units covering significant portions of their genomes, indicating a high degree of genomic plasticity. With the advent of Illumina sequencing technology, many draft genome sequences of *Spiroplasmas* have been reported using High-Throughput Sequencing (HTS). Although draft genomes provide insights into spiroplasma biology, their fragmented nature severely limits the scope of comparative genomic analyses. In the current comprehensive study, a hybrid sequencing approach, which combines short-read data generated by HTS with long-read data generated by Oxford Nanopore Technologies (ONT), is being used to assemble complete circular and annotated genome sequences. The genomic features and full genome analysis of *S. citri* by using HTS and ONT technologies in naturally infected citrus, sesame and periwinkle plant materials are still under investigation (Kara *et al.*, unpublished).

Transmission and epidemiology

CSD can be transmitted by insect vectors as well as through the use of infected buds collected from diseased plants. The transmission efficiency of *S. citri* is strongly influenced by temperature and increases under warm conditions (Calavan *et al.*, 1975). Leafhoppers have been shown to transfer the mollicute to and from a wide range of weeds and vegetable hosts (Oldfield *et al.*, 1984). Transmission primarily occurs from infected weeds to citrus, with less frequent transmission from infected citrus to citrus (Bové *et al.*, 1988). The first evidence for natural transmission of *S. citri* was obtained by culturing the pathogen from healthy periwinkle plants placed in infected citrus orchards, with confirmation via symptom observation,

DAS-ELISA, and transmission electron microscopy (TEM) (Çaglayan, 1987) (Figure 2).

In subsequent years, the natural vectors of *S. citri* and its potential hosts, beyond *Citrus* spp., were investigated in the Çukurova Region (Kersting *et al.*, 1992). The only known vector of *S. citri* in Mediterranean countries, *Circulifer opacipennis*, was not detected in citrus orchards but was abundant on sesame, which was also highly infected by *S. citri*. These findings indicated that sesame and its associated *C. opacipennis* population play an important role in the epidemiology of CSD in the Çukurova Region. The experimental transmission and pathogenicity of different *S. citri* isolates were further studied by Çaglayan and Markham (1994). Late-instar nymphs and young adults of *C. tenellus* were anesthetized with carbon dioxide, and microinjection was performed by hand under a stereoscopic microscope using *S. citri* cultures. The microinjected cicadellids were then caged in groups of 20–30 on healthy Madam Vinous orange, broad bean, periwinkle, red clover, and cabbage plants for an incubation period of 14 days. *S. citri* was successfully transmitted to all tested herbaceous and woody hosts except red clover, and the pathogen was subsequently reisolated, fulfilling Koch's postulates (Figure 3).

In the following years the epidemiological aspects of CSD were investigated in more details and *Circulifer opacipennis, Balclutha hebe, Cicadulina bipunctella* and *Orosius orientalis* were detected as potential vectors for *S. citri*. However, transmission experiments with field collected leafhoppers showed that only *C. opacipennis* is able to transmit *S. citri* to *C. roseus* in Turkish conditions (Kersting *et al.*, 1992). Recent studies have shown that although *S. citri* was detected at low

Figure 2. Yellowing symptoms observed in a periwinkle plant placed in citrus orchards infected with *Spiroplasma citri* and healthy periwinkle (right). *S. citri* cells in phloem of infected periwinkle plant (left). 5,000-fold magnification.

Figure 3. Left: young *Circulifer tenellus* adults ready for microinjection by using *Spiroplasma citri* culture (center). Symptoms of *S. citri* on inoculated broad bean (on the left) for an incubation period of 14 days and healthy broad bean (on the right).

rates in citrus and its herbaceous hosts, sesame and periwinkle, *S. citri* was not detected in any of the 918 possible insect vector belonging to the family *Cicadellidae* collected from infected orchards and their surroundings (Kara *et al.*, unpublished data).

Conclusion and future directions

Because CSD diagnosis is difficult due to low and variable concentrations of *S. citri* in diseased trees and the random distribution of the pathogen, recently molecular detection techniques have been used both for a diagnostic approach and genetic diversity of local *S. citri* isolates. The molecular characterization of *S. citri* isolates in Türkiye has provided important insights into the diversity and detection of this pathogen. Initial SDS-PAGE analyses during 1980s demonstrated that Turkish isolates share highly similar protein profiles, particularly in the major membrane protein spiralin, consistent with prior studies indicating conserved protein patterns among *S. citri* strains. Slight differences observed with *S. melliferum* emphasize the specificity of protein profiles among spiroplasma species, reinforcing the utility of SDS-PAGE as a preliminary comparative tool. However, one-dimensional SDS-PAGE alone lacks sufficient resolution to distinguish closely related strains, highlighting the need for complementary molecular methods. PCR-based approaches have markedly improved *S. citri* detection and strain differentiation in Türkiye. Primers targeting multicopy membrane protein genes (*p58*, *p89*) outperformed spiralin-based primers in sensitivity, enabling the detection of *S. citri* in both symptomatic and asymptomatic citrus tissues. Detection rates were notably higher in fruit columella than in leaf midribs, suggesting tissue-specific

pathogen distribution that may influence sampling strategies. The negative results in insect samples indicate that potential vectors in the studied regions do not either carry detectable levels of *S. citri* or that transmission occurs at low frequencies, warranting further entomological investigation. The development of novel primers targeting Sec secretion system genes (*secA*, *secE*, *secY*, *ffh*, *ftsY*, *yidC*) and fructose operon genes (*fruR*, *fruA*, *fruK*) represents a significant advance for both detection and pathogenicity studies. The Sec system is essential for protein export and virulence in *S. citri*, while fructose operon genes are key determinants of host colonization and virulence. The multi-gene PCR approach provided higher accuracy in detection and allowed preliminary phylogenetic differentiation among isolates, demonstrating its potential as a robust tool for molecular epidemiology.

Despite the advances in PCR-based detection, comprehensive understanding of genomic diversity among Turkish *S. citri* isolates remains limited. Published genome sequences from other regions indicate high variability, with numerous plasmids, mobile elements, and repetitive regions complicating genome assembly. The ongoing use of hybrid sequencing approaches combining HTS and ONT data promises to overcome these challenges, enabling the generation of complete, circular, and annotated genomes from naturally infected plants. Such data will be critical for comparative genomics, identification of strain-specific virulence factors, and understanding adaptation mechanisms in different hosts. Overall, the integration of protein-based, PCR-based, and genome-level analyses provides a comprehensive framework for studying *S. citri* in Türkiye. These approaches complement each other: SDS-PAGE offers initial protein

profiling, multi-gene PCR provides sensitive detection and strain differentiation, and genome sequencing allows detailed insights into genetic variability and pathogenic potential.

Future research should integrate high-resolution genomic data with multi-gene PCR approaches to achieve strain-level differentiation, functional characterization of virulence factors, and improved epidemiological surveillance. Functional genomics, including transcriptomic and proteomic studies, will be essential to uncover the full mechanisms of *S. citri* pathogenicity and support the development of new diagnostic tools and plant protection strategies for controlling CSD. Although CSD incidence has declined due to the use of *S. citri*-free buds, certification programs, and indexing initiatives, continued research is needed to better understand pathogen biology, host adaptation, and to safeguard sustainable citrus production in Türkiye.

Acknowledgments

References

Baspinar H, Kersting U, Sengonca Ç and Uygun N 1993. Studies on taxonomy, distribution and host plants of Turkish species of *Circulifer zakhvatkin* (Homoptera: Cicadellidae). . *Türkish Entomology Derg*, 17(3): 11.

Bové J-M, Fos A, Lallemand J, Raie A, Ali Y, Ahmed N, Saillard C and Vignault J 1988. Epidemiology of *Spiroplasma citri* in the old world. *International Organization of Citrus Virologists Conference Proceedings (1957-2010)*, 10(10): 295-299.

Çaglar BK, Satar G, Baloglu S, Drais MI and Djelouah K 2020. Detection of *Spiroplasma citri* from citrus trees in Turkey by molecular techniques. *Mediterranean Agricultural Sciences*, 33(1): 37-42.

Çaglayan K 1987. Turunçgil yediverenlesme (stubborn = palamutlasma) hastaligi etmeni Spiroplasma citri'nin izolasyonu, tans ve patojen-vektör iliskilerinin arastirilmas Çukurova Üniversitesi, Fen Bilimleri Enstitüsügrazie. Adana, Turkey.

Çaglayan K and Çinar A 1990. Dogu Akdeniz bölgesinde turunçgil agaçlarinda gözlenen stubborn hastaligi etmeni *Spiroplasma citri*'nin simptomoloji ve tanilanmasi üzerinde arastirmalar. *Doga Türk Tarim ve Ormancilik Dergisi*, 14(2): 12.

Çaglayan K and Markham P 1994. A study on the experimental transmission possiblities of some of Turkish isolates of *Spiroplasma citri. Turkish Phytopathology*, 23(2): 551-553.

Calavan E, Blue R, Harjung M, Cartia G, Granett A, Rana G and Gumpf D 1975. Seasonal and geographical variations in natural spread of citrus stubborn disease. *Proceedings of the Seventh International Organization of Citrus Virologists Conference Proceedings* (1957–2010), Athens, Greece, 7(7): 7-9.

Cascales E and Christie PJ 2003. The versatile bacterial type IV secretion systems. *Nature Reviews Microbiology*, 1(2): 137-149.

Cengiz A 1965. Turunçgillerde zarar yapan Palamutlasma (stubborn) hastaliginin taninmasi ve mücadele tedbirleri. *Ziraî Mücadele Enstitüsü yayn. Adana*, 15.

Chapot H 1959. First studies on the stubborn disease of citrus in some Mediterranean countries. *International Organization of Citrus Virologists: Riverside, CA, USA*, 109-117.

Chevalier C, Saillard Cand Bové J-M 1990. Spiralins of *Spiroplasma citri* and *Spiroplasma melliferum*: amino acid sequences and putative organization in the cell membrane. *Journal of Bacteriology*, 172(10): 6090-6097.

Çinar A and Çaglayan K 1988. First report of natural transmission of *Spiroplasma citri, c*ausal agent of citrus stubborn disease, in Eastern Mediterranean Region of Turkey. *Journal of Turkish Phytopathology*, 17(3): 102.

Daniels MJ, Archer DB, Stephens MA, Townsend R, Longland JM and Best J 1980. Comparison of spiroplasmas by polyacrylamide gel electrophoresis of cell proteins. *Current Microbiology*, 4(6): 377-380.

Davis RE, Shao J, Zhao Y, Gasparich GE, Gaynor BJ and Donofrio N 2017. Complete genome sequence of *Spiroplasma citri* strain R8-A2T, causal agent of stubborn disease in Citrus species. *Genome Announcements*, 5(16): 10.1128/genomea.00206-17.

Dubrana M-P, Béven L, Arricau-Bouvery N, Duret S, Claverol S, Renaudin J and Saillard C 2016. Differential expression of *Spiroplasma citri* surface protein genes in the plant and insect hosts. *BMC Microbiology*, 16(1): 53.

EFSA Panel on Plant Health 2014. Scientific opinion on the pest categorisation of *Spiroplasma citri. EFSA Journal*, 12(12): 3925.

FAO 2023. World Citrus Production FAOSTAT. FAO. Food And Agriculture Organizations of the United Nations 2023. Available from: http://www.fao.org/faostat/en/#data.

Foissac X, Saillard C, Gandar J, Zreik L and Bové J-M 1996. Spiralin polymorphism in strains of *Spiroplasma citri* is not due to differences in posttranslational palmitoylation. *Journal of Bacteriology*, 178(10): 2934-2940.

Foissac X, Bové J-M and Saillard C 1997. Sequence analysis of *Spiroplasma phoeniceum* and *Spiroplasma kunkelii* spiralin genes and comparison with other spiralin genes. *Current Microbiology*, 35(4): 240-243.

Gaurivaud P, Danet J-L, Laigret F, Garnier M and Bové J-M 2000. Fructose utilization and phytopathogenicity of *Spiroplasma citri*. *Molecular Plant-Microbe Interactions*, 13(10): 1145-1155.

Kersting U, Sengonca Ç and Cinar A 1992. Detection of *Spiroplasma citri* in non-citrus host plants and their associated leafhopper vectors in southern Turkey. *FAO Plant Protection Bulletein*, 40(3): 89-94.

Khanchezar A, Izadpanah K, Taghavi M and Béven L 2022. Genetic diversity of strains of *Spiroplasma citri* isolated in southern Iran. *European Journal of Plant Pathology*, 163(2): 381-392.

Oldfield G, Sullivan D and Calavan E 1984. Inoculativity of leafhopper vectors of stubborn disease in California. *International Organization of Citrus Virologists Conference Proceedings* (1957-2010), 9(9): 125-130.

Ozalp M and Azeri T 1967. Virus disease survey on citrus in Aegean region. *Plant Protection Bulletin*, 7(4): 167-187

Rattner R, Thapa SP, Dang T, Osman F, Selvaraj V, Maheshwari Y, Pagliaccia D, Espindola AS, Hajeri S and Chen J 2021. Genome analysis of *Spiroplasma citri* strains from different host plants and its leafhopper vectors. *BMC Genomics*, 22(1): 373.

Saillard C and Bové J-M 1983. Application of ELISA to spiroplasma detection and classification. In: *Methods in Mycoplasmology*. Eds S Razin and JG Tully, Academic Press, New York, USA, 471-476,

Saillard C, Carle P, Duret-Nurbel S, Henri R, Killiny N, Carrère S, Gouzy J, Bové J-M, Renaudin J and Foissac X 2008. The abundant extrachromosomal DNA content of the *Spiroplasma citri* GII3-3X genome. *BMC Genomics*, 9(1): 195.

Sas-Sertkaya G 1999. Transmission of *Spiroplasma citri* and sesame phyllody to test plants by grafting, dodder and insect vector. *Journal of Plant Pathology*, 82(1): 75.

Yokomi R, Rattner R, Osman F, Maheshwari Y, Selvaraj V, Pagliaccia D, Chen J and Vidalakis G 2020. Whole genome sequence of five strains of *Spiroplasma citri* isolated from different host plants and its leafhopper vector. *BMC Research Notes*, 13(1): 320.

Yokomi RK, Mello AF, Saponari M and Fletcher J 2008. Polymerase chain reaction-based detection of *Spiroplasma citri* associated with citrus stubborn disease. *Plant Disease*, 92(2): 253-260.

Research Article

Differential acquisition of Brazilian maize bushy stunt phytoplasma strains by the corn leafhopper vector, *Dalbulus maidis* (Hemiptera: Cicadellidae)

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Abstract

Maize bushy stunt phytoplasma is a pathogenic bacterium transmitted by the corn leafhopper, *Dalbulus maidis* (Hemiptera: Cicadellidae), in a persistent-propagative manner. This phytoplasma is a key component of a re-emerging disease complex that poses a major threat to maize production across the American continent. While some studies have reported genetic variation among maize bushy stunt strains, it remains unclear whether these variations influence transmission efficiency by *D. maidis*. Here, it was assessed the efficiency of acquisition by its insect vector. Six Brazilian strains were obtained from field samples and maintained in a greenhouse through serial transfer using *D. maidis* from a healthy colony. Acquisition efficiency was evaluated by exposing groups of 100 third-instar *D. maidis* nymphs to maize bushy stunt phytoplasma-infected plants. The findings indicate that these Brazilian strains exhibit differential acquisition efficiencies. A higher likelihood of acquisition may be linked to host manipulation strategies that enhance pathogen persistence and dissemination to new niches.

Introduction

Maize, commonly known as corn (*Zea mays* L.), is widely cultivated in Brazil, the third-largest producer globally, and has faced severe epidemics of the corn stunt complex diseases leading to substantial economic losses (Massola *et al.*, 1999; Klein and Luna, 2022; Oliveira and Frizzas, 2022). The corn leafhopper, *Dalbulus maidis* (DeLong and Wolcott) (Hemiptera: Cicadellidae) holds agricultural significance as vector for the pathogens infecting maize crops. These infections are caused by mollicutes (class Mollicutes - kingdom Bacteria), including maize bushy stunt phytoplasma (MBSP) ('*Candidatus* Phytoplasma asteris', 16SrI-B subgroup) and corn stunt spiroplasma (CSS) (*Spiroplasma kunkelii*), as well as maize rayado fino virus (MRFV) and maize striate mosaic virus (MSMV), the latter being the most recent identified member of the *D. maidis*-vectored disease complex (Nault, 1980; Hammond and

Bedendo, 2005; Vilanova *et al.* 2022). Among the two mollicutes, MBSP appears to be the most wide spread in Brazil (Galvão *et al.*, 2021).

MBSP is a phloem-limited bacterium that moves systemically within the plant but accumulates unevenly, concentrating primarily in sieve tubes near the roots and actively growing sink tissues (Kitajima and Costa, 1972; Marcone, 2010; Orlovskis *et al.*, 2015; van Bel and Musetti, 2019). *D. maidis* feeds on plant vascular system, ingesting phloem sap by piercing plant tissues with its mouthparts, making it a conduit for acquisition of the corn stunt pathogens and inoculation to maize plants (Orlovskis *et al.*, 2015; Maluta *et al.*, 2023). MBSP has a persistent-propagative relationship with its corn leafhopper vector, meaning it not only colonizes the insect but also propagates throughout its body (Legrand and Power, 1994; Orlovskis *et al.*, 2015; van Bel and Musetti, 2019). Optimal acquisition of MBSP from an infected maize plant requires a minimum of two hours of continuous phloem feeding (Legrand and Power, 1994).

The differential susceptibility of maize varieties to MBSP is a well-established concern in the commercial sector, particularly for corn breeding companies (Junqueira *et al.* 2004, 2011; Castilhos *et al.*, 2022; Pozebon *et al.*, 2022). However, little is known about the occurrence of MBSP strains (Gomes *et al.*, 2004), and even less about their potential implications in infection and differences in microorganism-plant interactions in infected plants. Orlovskis *et al.* (2017) reported that polymorphisms associated with two effector genes among Brazilian MBSP strains are associated with variations in organ proliferation symptoms of MBSP-infected maize plants. MBSP variability has also been reported in Mexico, with the identification of distinct strains with possible polyphyletic origins (Moya-Raygoza and Nault, 1998; Pérez-López *et al.*, 2016). Different MBSP strains were also reported to show discrepancies in transmission efficiencies by *D. maidis* (Legrand and Power, 1994). To study this further, the efficiency of acquisition of six previously genotyped Brazilian MBSP strains (Orlovskis *et al.*, 2017) by *D. maidis* was assessed.

Material and Methods

Field collection of MBSP strains involved sampling whole maize plants displaying typical symptoms of MBSP from field (Orlovskis *et al.*, 2017). To recover MBSP strains from the field samples, 200-300 non-infective laboratory-reared leafhopper vectors (mostly 3rd-5th instar nymphs) were allowed to feed on the symptomatic leaves and stems of field-collected maize samples for an acquisition access period (AAP) of 48 h, followed by a 25-day latency period on healthy maize seedlings (test plants), and then transferred to new healthy test plants for an inoculation access period (IAP) of 3 days, as described by Oliveira *et al.* (2018). Phytoplasma initially acquired from a single field-collected maize plant and later transmitted to test seedlings in the greenhouse, was considered a distinct strain. Further experiments were performed with the Brazilian strains R4, G2, M3, Bouquet, T14 and E10. R4 was obtained in Piracicaba (São Paulo state - SP), in 2012; M3, T14 and E10 were obtained in Piracicaba, in 2013; G2 and Bouquet were obtained in Guaíra, SP, in 2013. Further details about these strains are published by Orlovskis *et al.* (2017).

The strains were maintained in maize hybrid 2B433PW. Several subculturing (transmission from a diseased plant to a healthy plant for maintenance) of those strains were performed prior to the experiments execution. Maize plants infected with each MBSP strain were kept in separate vector-proof cages to prevent cross-inoculation by leafhoppers casually present in the greenhouse. Cages frames were made with welded 1/4″ iron [0.50 x 0.80 (base) x 1.0 (height) m], covered with fine-mesh fabric (voile). The bench underneath the cage was covered with a fine-mesh nylon screen.

Third instar *D. maidis* nymphs were confined onto MBSP-infected plants with each referred strain plants for 48 h for AAP (Figure 1). For this, plants infected with each strain were individually caged using voile bag cages. After a 3-week latency period on healthy plants, *D. maidis* were transferred to new healthy test plants of the maize hybrid 2B433PW at V3 stage (three visible collared leaves) for an IAP of 3 days (Oliveira *et al.*, 2018). Test plants were individually sown in plastic

Pl0 pots containing soil mix (Topstrato Hortaliças HT, Vida Verde, Mogi Mirim, SP, Brazil) and kept in a leafhopper vector-proof screenhouse, inside protective cages. Each test plant was inoculated using 10 insects. These plants were then used as MBSP-source plants for the acquisition experiment (Figure 1), after symptoms onset for all plants inoculated with the different strains, at 40 days after inoculation. With the purpose of demonstrating differential acquisition of MBSP strains by *D. maidis*, third instar nymphs of *D. maidis* were allowed to feed from the standardized prepared MBSP-source plants for 48 h. Then, leafhoppers were maintained on healthy plants for a week to allow the increase of the bacterial title. After AAP, insects exposed to plants infected with MBSP strains were stored in 70% ethanol before DNA extraction. All the strains mentioned above were included in the experiment. A total of 85 insects were tested for strain G2; 90 insects each for strains E10, M3, Bouquet, and R4; and 98 insects for strain T14 (Figure 1).

For molecular detection of MBPS in *D. maidis*, total DNA was extracted from corn leafhoppers using DNeasy Blood & Tissue Kit (Qiagen). Leafhoppers were macerated individually in 1.5 ml microtubes with pestles in 40 µl of ATL buffer. After that, 4 µl of proteinase K and 200 µl of buffer AL were added. The mixture was vortexed and incubated at 56°C for 12 min. Next, 200 µl of ethanol 100% was added, the mixture was vortexed and transferred to the column in the collection tube. After centrifugation at 8,000 rpm for 2 minutes, the flow through was discarded and DNA was washed with 500 µl of AW1 and AW2 buffer. After centrifugation, the column was transferred to a new 1.5 ml microtube, and DNA was eluted in 50 µl of AE buffer. The specific group 16SrI phytoplasma primer pair P1/AYint was used to amplify MBSP (Deng and Hiruki, 1991; Smart *et al.*, 1996). Reactions were performed in 25 µl, using Thermo Scientific PCR Master Mix (Thermo Fisher Scientific, Walthan, MA, USA) 1X (2X contains 0.05 U/µl Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP), 0.5 µM of each forward and reverse primer and 1 µl of template DNA, at a concentration of approximately 30 ng/µl. PCR settings at Veriti 96 Well Thermal Cycler (Applied Biosystems) were: 2 minutes at 94°C for initial denaturation; 30 cycles of 1 minute at 94°C for

Figure 1. Scheme of the experiment to show differential acquisition of several maize bushy stunt phytoplasma strains by *Dalbulus maidis*. Several strains collected from field are maintained *in vivo* via insect transmission. Then plants infected with each strain were cage-isolated for acquisition using the leafhopper vector. Then, the insects, already infective, were confined to new healthy plants for inoculation, after a latency period. These inoculated plants were then used as source plants of MBSP strains, 40 days after inoculation. Healthy nymphs of the corn leafhopper were confined on each MBSP isolate source plants for a 48-h acquisition and then, after a week saved for phytoplasma titer increase inside the insect, they were stored in microtubes containing alcohol till molecular diagnostics using DNA individually extracted from leafhoppers.
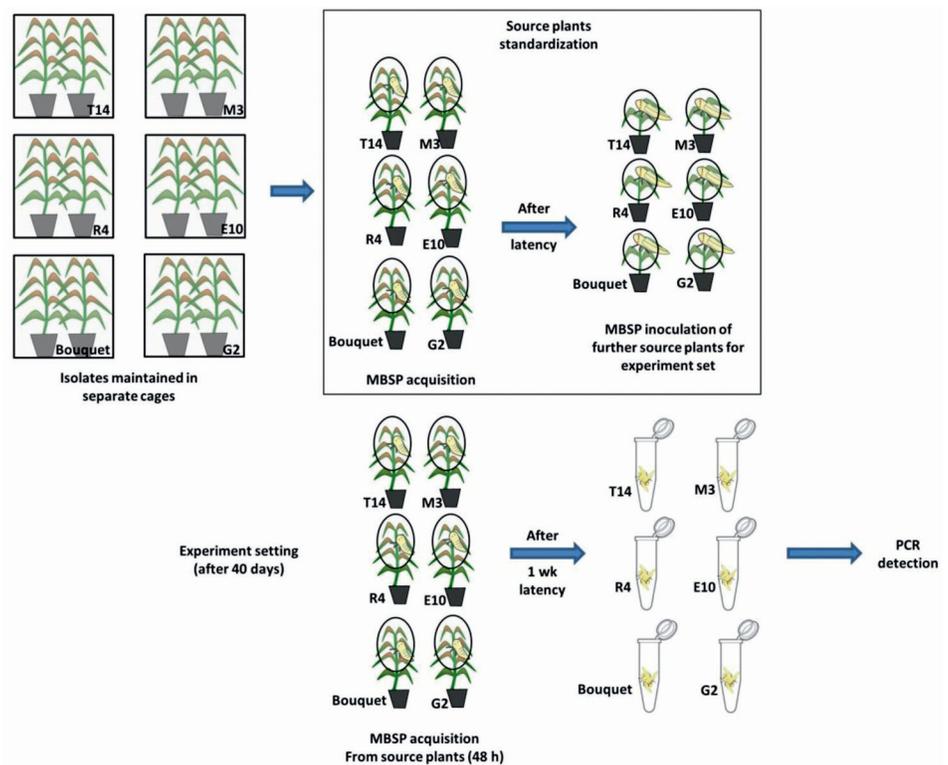
denaturation, 1 minute at 56°C for annealing and 2 minutes at 72°C for extension; followed by 5 minutes at 72°C for final extension. Amplicons were visualized in 1% agarose gel using a UV transilluminator (Bio-Rad UView, USA), and the expected product size was approximately 1.5 kb.

To identify statistically significant differences among MBSP strains regarding acquisition by *D. maidis*, data were submitted to Tukey testing using the R environment (R Core Team, 2021).

Results and Discussion

In the present study, differential acquisition by corn leafhoppers was observed depending on the MBSP strain. The acquisition efficiency of strains G2 and E10 differed significantly from that of strains Bouquet and R4 (Figure 2). This may potentially be attributed to better and more efficient colonization of the plant by the most efficiently acquired phytoplasma strains, which could provide a greater number of bacterial cells available and consequently a higher chance of getting acquired by the insect during feeding. This characteristic may favor strains with propensities to get acquired by the vector, and a consequent greater chance of success to produce more infected plants and thereby perpetuating themselves. As a consequence, particular strains may succeed in a determined location (Moya-Raygoza and Nault, 1998; Pérez-López *et al.*, 2016).

Phytoplasmas are associated with physiological changes in host plants, with a notable impact on the behavior of insect vectors, thus facilitating their own dispersal (Sugio and Hogenhout, 2012; Ramos *et al.*,
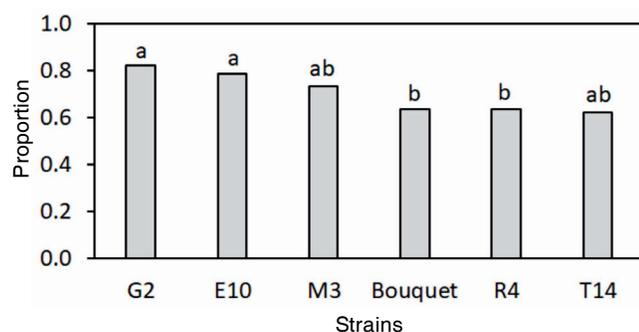

Figure 2. Efficiency of acquisition of strains of maize bushy stunt phytoplasma by 3rd instar nymphs of the corn leafhopper, *Dalbulus maidis*.

2020). The success of plant pathogen dissemination by insect vectors is influenced, among other factors, by the host selection behavior of the vector, which encompasses the flight orientation towards the plant, as well as its acceptance for feeding and/or oviposition. This study shows that Brazilian MBSP strains also have differential chances of acquisition. These characteristics of MBSP strains are related to the manipulation of hosts to ensure the pathogen's perpetuation and its ability to reach new niches.

Transmission studies of the corn stunt complex pathogens by the corn leafhopper vector - comprehending acquisition, latency, inoculation, and retention - should be conducted with Brazilian strains, as well as those from other countries, both for MBSP and for CSS, as well as for the viruses that are part of this complex of diseases. Comparing different isolates and strains is crucial for understanding the potential manipulation mechanisms these microorganisms use to thrive. This may shed light on the host selection behavior of *D. maidis* (Gonzalez *et al.*, 2018) and the potential for increased dissemination of MBSP due to the propensity for acquisition, which is related to the vector manipulation hypothesis (Mayer *et al.*, 2011; Ramos *et al.*, 2020) and the perpetuation of MBSP in nature. Such experiments are challenging, requiring laboratory and greenhouse facilities, molecular assay equipment, an insect rearing colony, and the maintenance of these biotrophic pathogens *in vivo*. Furthermore, obtaining from field and maintaining strains is a labor-intensive process. However, these studies will help to better understand the dynamics of the microorganisms-vector-plant relationships that are part of this complex, mandatory for developing effective management strategies.

Acknowledgements

References

Castilhos RV, Parizotto C, Bermudez FB, Ribeiro LP and Canale MC 2022. Severity of corn stunt disease in the Midwest of the Brazilian State of Santa Catarina. *Revista Brasileira de Milho e Sorgo*, 21: e1278.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods*, 14: 53-61.

Galvão SR, Sabato EO and Bedendo IP 2021. Occurrence and distribution of single or mixed infection of phytoplasma and spiroplasma causing corn stunting in Brazil. *Tropical Plant Pathology*, 46: 152-155.

Gomes EA, Jardim SN, Guimarães CT, Souza IRP and Oliveira E 2004. Genetic variability of Brazilian phytoplasma and spiroplasma isolated from maize plants. *Pesquisa Agropecuária Brasileira*, 39: 61-65.

Gonzalez JG, Jaramillo MG and Lopes JRS 2018. Undetected infection by maize bushy stunt phytoplasma enhances host-plant preference to *Dalbulus maidis* (Hemiptera: Cicadellidae). *Environmental Entomology*, 47: 396-402.

Hammond RW and Bedendo IP 2005. Molecular confirmation of Maize rayado fino virus as the Brazilian corn streak virus. *Scientia Agricola*, 62: 601-603.

Junqueira A, Bedendo I and Pascholati S 2004. Biochemical changes in corn plants infected by the maize bushy stunt phytoplasma. *Physiological and Molecular Plant Pathology*, 65: 181-185.

Junqueira ACB, Bedendo IP and Pascholati SF 2011. Effect of phytoplasma infection on the activity of peroxidase, β-1,3-glucanase and chitinase in corn plants. *Summa Phytopathologica*, 37: 194-198.

Kitajima EW and Costa AS 1972. Microscopia electrônica de microrganismos do tipo micoplasma nos tecidos de milho afetado pelo enfezamento e nos órgãos da cigarrinha vectora portadora. *Bragantia*, 31: 75-82.

Klein HS and Luna FV 2022. The impact of the rise of modern maize production in Brazil and Argentina. *Historia Agraria*, 86: 1-38.

Legrand AI and Power AG 1994. Inoculation and acquisition of maize bushy stunt mycoplasma by its leafhopper vector *Dalbulus maidis*. *Annals of Applied Biology*, 125: 115-122.

Maluta N, Castro T and Lopes JRS 2023. DC-electrical penetration graph waveforms for *Dalbulus maidis* (Hemiptera: Cicadellidae) and the effects of entomopathogenic fungi on its probing behavior. *Scientific Reports*, 13: 22033.

Marcone C 2010. Movement of phytoplasmas and the development of disease in the plant. In *Phytoplasmas: Genomes, Plant Hosts and Vectors*. Eds PG Weintraub and P Jones. Wallingford, Oxfordshire, UK: CAB International, 114-131.

Massola Jr NS, Bedendo IP, Amorim L and Lopes JRS 1999. Quantificação de danos causados pelo enfezamento vermelho e enfezamento pálido do milho em condições de campo. *Fitopatologia Brasileira*, 24: 136-142.

Mayer CJ, Vilcinskas A and Gross J 2011. Chemically mediated multitrophic interactions in a plant-insect vector-phytoplasma system compared with a partially nonvector species. *Agricultural and Forest Entomology*, 13: 25-35.

Moya-Raygoza G and Nault LR 1998. Transmission biology of maize bushy stunt phytoplasma by the corn leafhopper (Homoptera: Cicadellidae). *Annals of the Entomological Society of America*, 91: 668-676.

Nault LR 1980. Maize bushy stunt and corn stunt: a comparison of disease symptoms, pathogen host ranges, and vectors. *Phytopathology*, 70: 659-662.

Oliveira CM and Frizzas MR 2022. Eight decades of *Dalbulus maidis* (DeLong & Wolcott) (Hemiptera, Cicadellidae) in Brazil: what we know and what we need to know. *Neotropical Entomology*, 51: 1-17.

Oliveira CM, Lopes JRS and Querino RB 2018. Técnicas de criação da cigarrinha-do-milho para estudos de transmissão e de controle biológico. Eds. CM Oliveira and EO Sabato. Sete Lagoas: Embrapa Milho e Sorgo, 156-178.

Orlovskis Z, Canale MC, Thole V, Pecher P, Lopes JRS and Hogenhout SA 2015. Insect-borne plant pathogenic bacteria: getting a ride goes beyond physical contact. *Current Opinion in Insect Science*, 9: 16-23.

Orlovskis Z, Canale MC, Haryono M, Lopes JRS, Kuo C-H and Hogenhout SA 2017. A few sequence polymorphisms among isolates of maize bushy stunt phytoplasma associate with organ proliferation symptoms of infected maize plants. *Annals of Botany*, 119: 869-884.

Pérez-López E, Olivier CY, Luna-Rodríguez M, Rodríguez Y, Iglesias LG, Castro-Luna A, Adame-Garcia J and Dumanceaux TJ 2016. Maize bushy stunt phytoplasma affects native corn at high elevations in Southeast Mexico. *European Journal of Plant Pathology*, 145: 963-971.

Pozebon H, Stürmer GR and Arnemann JA 2022. Corn stunt pathosystem and its leafhopper vector in Brazil. *Journal of Economic Entomology*, 115: 1817-1833.

Ramos A, Esteves MB, Cortés MTB and Lopes JRS 2020. Maize bushy stunt phytoplasma favors its spread by changing host preference of the insect vector. *Insects*, 11: 600.

R Core Team 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Smart CD, Schneider B, Blomquist CL, Guerra LJ, Harrison NA, Ahrens U, Lorenz KH, Seemüller E and Kirkpatrick BC 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Applied and Environmental Microbiology Journal*, 62: 2988-2993.

Sugio A and Hogenhout SA. 2012. The genome biology of phytoplasma: modulators of plants and insects. *Current Opinion in Microbiology*, 15(3): 247-254.

van Bel AJE and Musetti R 2019. Sieve element biology provides leads for research on phytoplasma lifestyle in plant hosts. *Journal of Experimental Botany*, 70: 3737-3755.

Vilanova ES, Ramos A, Oliveira MCS, Esteves MB, Gonçalves MC and Lopes JRS 2022. First report of a mastrevirus (Geminiviridae) transmitted by the corn leafhopper. *Plant Disease*, 106: 1330-1333.

Research Article

Effects of disease severity stage and quality of extracted DNA on molecular detection of the Weligama coconut leaf wilt disease agent in Sri Lanka

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Abstract

Weligama coconut leaf wilt disease (WCLWD) is one of the devastating diseases in the Southern province of Sri Lanka, causing drastic yield reductions in coconut. The agent associated with WCLWD is phytoplasma, which is an intra-cellular obligate bacterium. Accurate molecular detection of phytoplasmas is important for effective disease management, especially due to its long latent period, during which the disease could be spread from non-symptomatic palms. Two sampling rounds were conducted using 30 selected WCLWD palms, with 10 each from mild, moderate and severe disease categories and 10 asymptomatic coconut palms as control. Sugarcane white leaf disease infected samples were used as positive control. DNA was extracted from the midrib of milky white emerging bud leaf tissues using CTAB method, and nested PCR was performed to detect phytoplasma. Extracted DNA was purified using Qiagen DNA purification commercial kit to evaluate the effect of DNA purification on PCR detection. PCR results were assessed and compared between pre-purified and purified DNA samples. The highest PCR positivity was observed in the moderate disease severity stage after the DNA purification. However, upon DNA purification PCR positivity increased to 30%, 60% and 30% respectively for mild, moderate and severe palm categories. None of the asymptomatic control samples showed PCR positivity. In conclusion, DNA purification improved the quality of extracted DNA and efficiency of PCR detection, and the moderate disease severity stage was identified to be the best stage for detection of WCLWD phytoplasma. The findings would be useful in WCLWD management programs.

Keywords: disease severity, phytoplasma, polymerase chain reaction, Weligama coconut leaf wilt disease.

Introduction

Weligama coconut leaf wilt disease (WCLWD) is a devastating disease associated with the presence of phytoplasmas confined to the Southern province of Sri Lanka. The disease was first identified from the Weligama area of the country in 2006 and it was then named after this (Perera *et al.*, 2012). Phytoplasmas are wall-less, obligate bacterial pathogens that colonize the

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phloem tissue of plants, they are associated with a wide range of symptoms mainly related to phloem dysfunctions (Bertaccini *et al.*, 2014). Phytoplasmas are primarily transmitted by phloem-feeding insect vectors, such as planthoppers and leafhoppers, which acquire the pathogen while feeding on infected plants and subsequently spread it to healthy plants (Weintraub and Beanland, 2006).

The WCLWD is comparable to lethal yellowing and lethal decline diseases that have had a significant negative impact on coconut plantations in Southeast Asia, the Caribbean, and Africa which have resulted in notable declines in coconut output and palm viability (Bertaccini *et al.*, 2021; 2023). Infected palms lose the angular shape of the leaflets, resulting in a flattened appearance, which is termed flaccidity. The initial symptoms of WCLWD include flaccidity of the leaflets, leading to a drooping appearance. Flaccid leaflets tend to bend downward, giving a ribcage-like appearance. Affected palms exhibit marginal leaf necrosis followed by uneven yellowing of leaflets, where the edges of the leaflets begin to brown (De Silva *et al.*, 2021, 2023). As the disease progresses to the moderate stage, more severe yellowing is observed across the fronds, often starting from the mid whorl and moving towards the younger ones. This stage is also characterized by a reduction in leaf size and a noticeable decline in crown density, which can be indicative of significant physiological distress in the plant. Severely affected palms ultimately prone to other infections like leaf rot that result in the death of the palm (De Silva *et al.*, 2023; Wijesekara *et al.*, 2008).

Due to their obligate intracellular nature, the detection of phytoplasmas relies almost entirely on molecular techniques. Traditional microscopic and serological methods have limited application due to the low concentration of phytoplasmas in infected tissues. Molecular detection using polymerase chain reaction (PCR) and sequence analysis of 16S rRNA gene confirmed the presence of phytoplasma DNA in symptomatic coconut samples, providing evidence for the association between phytoplasma and WCLWD (De Silva *et al.*, 2023). This study aimed at assessing the effect of disease severity stage; mild moderate and severe, on molecular detection of the phytoplasma using polymerase chain reaction (PCR) and investigating possibilities for enhancing PCR accuracy.

Materials and Methods

Site and palm selection

Commercial coconut plantation with a history of WCLWD was selected from Kotawila area in the Matara district in the Southern province of Sri Lanka for this study. A total of 30 coconut palms were selected from mild, moderate and severe stages of the disease using disease severity index from Nugagahawatta estate of Matara ((Nainanayaka *et al.*, 2010). Ten asymptomatic coconut palms were selected from Bandirippuwa estate in Lunuwila from WCLWD free area in the Northwestern province of Sri Lanka.

Table 1. Categorization of WCLWD severity stages using disease severity index.

Disease severity index (DSI)	Severity category
<50	Mild
50 - 65	Moderate
>65	Severe

Sampling

Ten bud leaf samples each were taken separately from WCLWD infected coconut palms in mild, moderate and severe stages (Figure 1). Ten bud leaf samples were taken from asymptomatic coconut palms selected from WCLWD free areas. Sampled tissues were kept in polyethylene zip lock bags separately, transported to the laboratory, and stored in a refrigerator until DNA extraction. Totally, 40 tissue samples were sampled, and sampling was repeated after a two month interval.

Molecular detection of the pathogen

DNA was extracted from milky white emerging bud leaf tissues using a cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1990) with some modifications (Ariyarathna *et al.*, 2007). Pre-autoclaved and oven dried mortar and pestle was kept at -20ºC at least 30 minutes before starting the DNA extracting procedure. Leaves were washed with distilled water and 70% ethanol and wiped to remove

Figure 1. Disease severity stages of WCLWD infected palms.

| Mild | Moderate | Severe |

water and ethanol droplets. Ekel (midrib) was separated from the milky white bud leaves and hard parts were removed with a sterilized pair of scissors cut into small pieces (2-3 mm) mixed and ground to a fine powder using mortar and pestle adding liquid nitrogen. Then 0.5 g of obtained white powder was weighed and transferred into 2 ml microcentrifuge tube. Preheated (65°C) extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 mM NaCl and 1% PVP, pH 8.0, 0.2% β mercaptoethanol) was added into each sample and mixed thoroughly by gentle tapping or inverting tubes and incubated at 65°C for 30 minutes while gentle mixing the content every 10 minutes. After cooling to room temperature, 750 µl of chloroform: isoamyl alcohol (24:1) were added and the samples were mixed for about 1 minute by inverting the tube and centrifuged (Remi, India) at 13,000 rpm for 10 minutes. About 500 µl of top aqueous phase were carefully transferred into a clean 1.5 ml microcentrifuge tube. Again 750 µl of chloroform: isoamyl alcohol (24:1) was added. Samples were mixed for about 1 minute by inverting the tube and centrifuged at 13,000 rpm for 10 minutes. DNA was precipitated by adding 500 µl of ice-cold isopropanol and mixed gently by inverting the tubes. Then, samples were incubated at 4°C overnight and the content was centrifuged at 13,000 rpm for 10 minutes to collect the DNA. The pellet was washed with 500 µl of 70% ethanol, centrifuged at 13,000 rpm for 5 minutes. After removing ethanol, the obtained DNA pellet was air-dried and dissolved in 40 µl of 1x TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and stored at -20°C.

DNA purification was done using the DNeasy Power Clean Pro Cleanup commercial kit (Qiagen, Germany) following the manufacturer's protocol. The eluted DNA was stored at -20°C. The quality and concentration of extracted DNA before and after purification were measured using a Nanodrop system. From each sample 1 µl was used for the absorbance readings at 260 nm and 280 nm. All measurements were performed using TE as the blank. DNA concentration was automatically calculated, based on the assumption that 1 A260 unit corresponds to 50 µg/ ml of double stranded DNA.

PCR was used to detect the presence of phytoplasma DNA in coconut leaf samples. The universal primers specific to phytoplasma 16S rRNA genes P1 /Tint (Deng and Hiruki, 1991; Smart *et al.*, 1996) and FU5/RU3 (Lorenz *et al.*, 1995) were used in nested PCR carried out in a thermal cycler (Takara Bio, Otsu, Japan), The PCR was performed in a 30 µl final reaction mixture containing 1X GoTaq Flexi buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$ 0.5 µM each primer, 150 µM (each) dATP, dCTP, dGTP and dGTP, 4 µl (800 ng) template DNA; and 1 U of GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR conditions were 30 cycles with denaturation for 1 minute at 94°C, annealing for 1 minute at 56°C and extension for 2 minutes at 72°C. 8 µl of the reaction mixture was electrophoresed in a 1% agarose gel. Nested PCR mixture in a 25 µl final reaction mixture contained 1X GoTaq Flexi buffer, 1.5 mM $MgCl_2$ 0.8 µM each primer, 200 µM (each) dATP, dCTP, dGTP and dGTP, 1 µl PCR product; and 1.25 U of GoTaq DNA polymerase. PCR conditions were 35 cycles with denaturation for 30 seconds at 94°C; annealing for 45 seconds at 56°C; and extension for 1 minute at 72°C. Asymptomatic coconut DNA was used as negative control and the DNA extracted from the leaf tissues with symptoms of sugarcane white leaf disease (GenBank accession number OP279594) was used as positive control. To visualize the PCR products, they

were subjected to electrophoresis in 1% agarose gel under 100 V for 1 h (Labnet, Taiwan, China), stained with ethidium bromide and visualized with a UV transilluminator (Cell Biosciences, UK). A 100 bp DNA ladder was used to estimate the size of the PCR amplicons, and the presence of a band at the expected size of 880 bp.

Results

Figure 2 shows the gel image of nested PCR. Clear amplification bands according to the expected 880 bp band size which aligns with the PCR product of sugarcane white leaf diseased DNA sample were observed in mild, moderate and severe disease categories. No amplification was detected in negative controls (nuclease free water), and asymptomatic coconut samples.

Figures 3 to 6 show the quantity (more than 1000 ng/µl) and quality (A260/A280 ratio) of extracted DNA from the bud leaf samples before and after the purification step in the first and second sampling, respectively. Also, it was observed that the A260/A280

ratio exceeded 2.0 in both sampling rounds before DNA purification. After DNA purification, reduction of DNA quantity (50-100 ng/µl) and the improvement of the DNA quality (A260/A280 ratio) closer to 1.8 was observed.

Figures 7 and 8 show the PCR positivity of DNA samples extracted from bud leaf samples before and after the purification. According to Figure 7, the highest number of PCR positive samples was observed in samples from moderate disease severity stage before and after the purification step. The lowest PCR positive samples was observed from the bud leaf samples from severe disease stage before DNA purification and the highest improvement in PCR positivity was observed after the DNA purification in both samplings.

The data showed that DNA purification significantly improved phytoplasma detection rate in all the disease severity stages. Although there was no considerable difference between disease severity and molecular detection, the detection of phytoplasma through PCR was most successful in palms at the

Figure 2. Agarose 1% gel of nested PCR products.
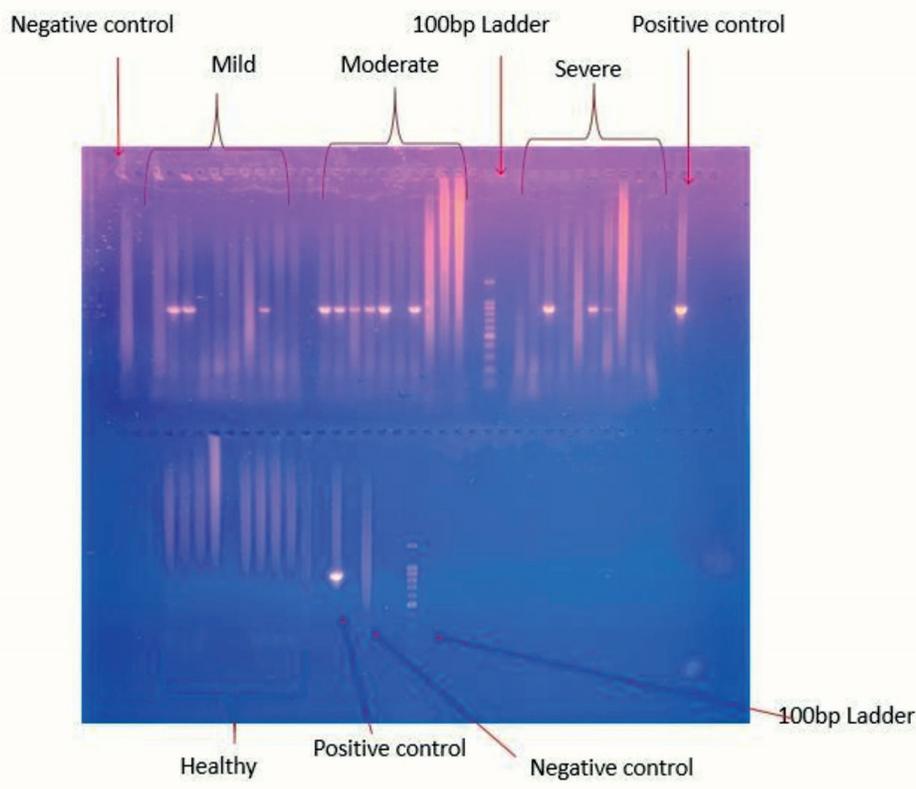
Figure 3. Quantity of DNA before and after purification in 1st sample collection
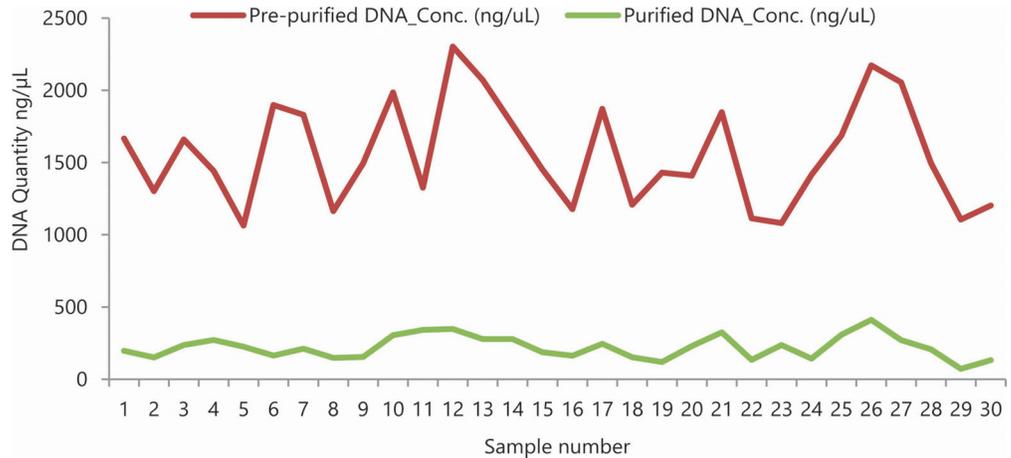
Figure 4. Purity of DNA before and after purification in 1st sample collection.
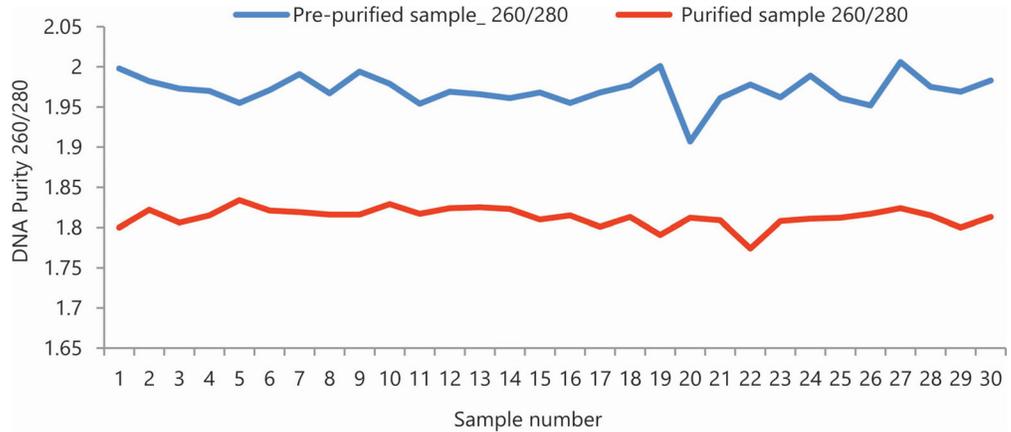
Figure 5. Quantity of DNA before and after purification in 2nd sample collection.
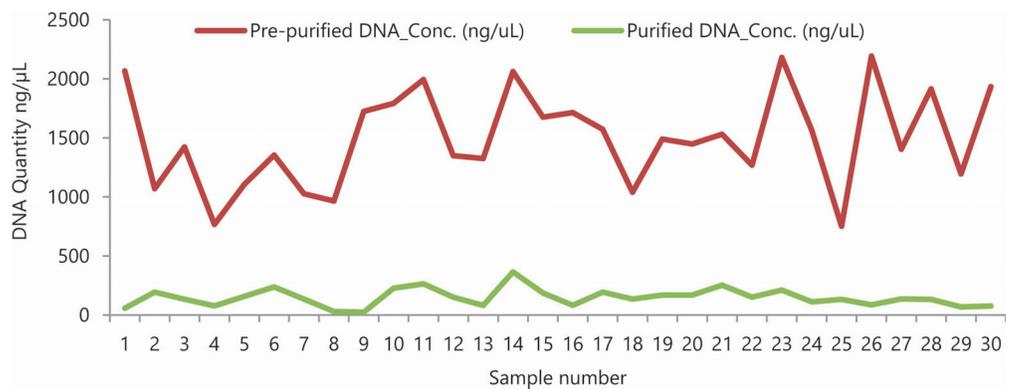
moderate disease severity stage following DNA purification. This results revealed that the moderate disease severity stage is the best stage to take bud leaf samples for the PCR detection of phytoplasma.

Although the symptoms were clearly visible in severe stage palms, they showed the lowest detection rate. This could be attributed to the host tissues degradation and accumulation of PCR inhibitors like polyphenols and polysaccharides with the age of the palm which are common in coconut leaf tissues. Therefore, these inhibitors can interfere with the PCR amplification and lead to false negative results (Pathirana *et al.*, 2018). But after the DNA purification, severe stage palms showed significant improvement in

Figure 6. Purity of DNA before and after purification in 2nd sample collection.
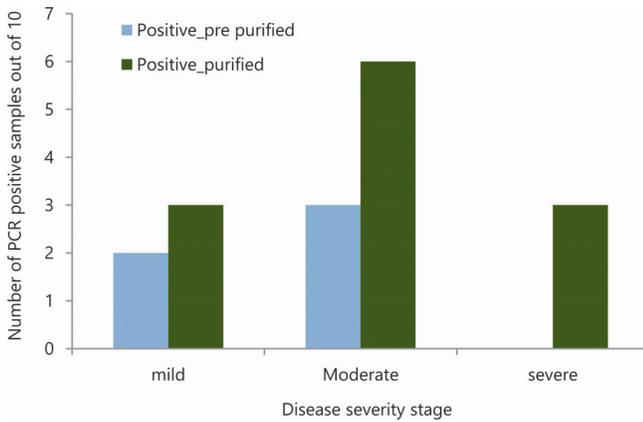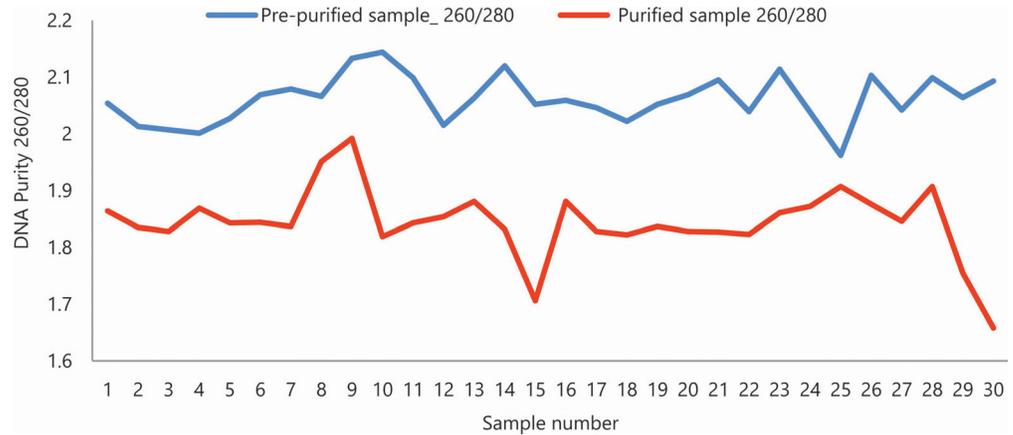

Figure 7. PCR results of DNA from samples collected for the first time from palm samples.
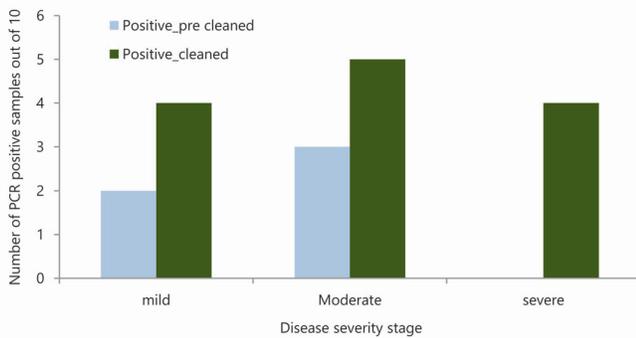
Figure 8. PCR results of DNA from samples collected the second time from palm samples.

PCR detection. Mildly disease affected palms showed the lowest PCR detection rate comparatively even after the DNA purification. At the initial stages of the disease progression the titter of phytoplasma in the palm may be low for the molecular detection by the nested PCR.

Discussion

From these results it was clear that after the optimization of available PCR protocol with the DNA purification using Qiagen plant Pro commercial kit, significant improvement of the molecular detection of phytoplasma from diseased palms could be achievable among all disease severity stages. This may be due to the improvement of quality of the DNA extracted after the purification by removing PCR inhibitors and other polyphenolic compounds. Furthermore, since there were any PCR amplifications observed in asymptomatic control samples proved the specificity of the P1/Tint and RU3/FU5 primer pair which is important to avoid the false positives.

Detecting phytoplasmas, particularly in asymptomatic or early-stage infections, requires extremely sensitive molecular techniques due to their low abundance in plant tissues. Nested PCR was developed to overcome limitations in sensitivity and specificity of PCR, providing a more precise and reliable approach (Lee *et al.*, 1995; De Silva *et al.*, 2023). This method significantly enhances both sensitivity and specificity, making it one of the most effective techniques for diagnosing phytoplasma-associated diseases, including those associated with WCLWD.

The selection of primers is important for the success of nested PCR. The first-round primers (external primers) are typically designed to amplify conserved regions of the 16S rRNA gene, ensuring that multiple phytoplasma strains can be detected. Commonly used external primers which generate a larger amplicon (˜1.8

kb) covering a significant portion of the phytoplasma ribosomal gene region. While this broad amplification is useful, the presence of non-target DNA may interfere with specificity. To address this, the nested (internal) primers are chosen to bind within the initial amplicon, thereby amplifying only phytoplasma-specific sequences. In recent advancements, additional primer pairs such as RU3/FU5 have been developed to further improve phytoplasma detection. These primers have been particularly useful in enhancing the detection of phytoplasmas from different host plants, including coconut palms affected by WCLWD (De Silva *et al.*, 2021). In a recent study, it was identified that milky white emerging bud leaf tissue of WCLWD affected palms was the best tissue type for phytoplasma detection. Also nested PCR using P1/Tint and rU3/fU5 primer pairs showed minimum 88% accuracy and 100% specificity in PCR detection of WCLWD causing phytoplasma (De Silva *et al.*, 2023).

Effective disease management relies on the early detection of infections. In the case of WCLWD, early identification of infected but asymptomatic trees is crucial for limiting the spread of the disease and preventing economic losses. PCR-based diagnostic methods, particularly nested PCR provides the sensitivity needed to detect infections before symptoms develop. This enables to implement control measures while the disease is still in its initial stages and easier to contain (De Silva *et al.*, 2023).

Although the developed PCR protocol is accurate and sensitive for phytoplasma detection, there are inconsistencies in detection depending on the severity stage of the affected palms. Also, the content of polyphenolic compounds and other PCR inhibitors affecting the accuracy of PCR detection may differ with the severity stage of the infected coconut palms among the palms in more or less same age and the same variety. Therefore, cleaning the extracted genomic DNA from milky white emerging bud leaves using a cleaning kit resulted able to improve the quality of DNA and enhanced the accuracy of PCR detection.

Conclusions

Among the three disease severity stages examined, moderate stage samples showed the highest detection rate suggesting that those palms have the most favorable balance between phytoplasma titter and the tissue integrity for successful DNA extraction and PCR amplification. In mild stage, the titer of phytoplasma may be low, while severely affected palms containing PCR inhibitors reduce amplification efficiency.

A key finding of the research was that DNA purification enhanced the detection rate of phytoplasma. The severe stage samples showed the major improvement of detection after the purification, strongly suggesting the presence of PCR inhibitors in extraction. Therefore, this protocol optimization can improve the reliability of phytoplasma detection, particularly in samples infected with WCLWD at severe stages. Therefore, it can be recommended that DNA purification be included as a standard procedure in molecular detection protocol of WCLWD associated phytoplasma.

References

Ariyarathna HACK, Everard JMDT and Karunanayake EH 2007. Diseased sugarcane in Sri Lanka is infected with sugarcane grassy shoot and/or sugarcane white leaf phytoplasma. *Australasian Plant Disease Notes*, 2(1): 123.

Bertaccini A, Duduk B, Paltrinieri S and Contaldo N 2014. Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. *American Journal of Plant Sciences*, 5(12): 1763-1788.

Bertaccini A and the TROPICSAFE consortium 2021. TROPICSAFE project: detection and management of lethal yellowing and grapevine yellows diseases in partner countries. *Phytopathogenic Mollicutes*, 11(1): 9-14.

Bertaccini A, Contaldo N, Feduzi G, Ela Andeme AM, Yankey EN and Rovesti L 2023 Molecular identification of '*Candidatus* Phytoplasma palmicola' associated with coconut lethal yellowing in Equatorial Guinea. *Annals of Applied Biology*, 183(3): 262-270.

De Silva PHPR, Aratchige NS, Ranasinghe CS and Kantha AAFL 2021. Diseased palm removals as a strategy for the successful management of Weligama coconut leaf wilt phytoplasma

disease of coconut in Sri Lanka, *Phytopathogenic Mollicutes*, 11(2): 79-85.

De Silva PR, Perera CN, Bahder BW and Attanayake RN 2023. Nested PCR-based rapid detection of phytoplasma leaf wilt disease of coconut in Sri Lanka and systemic movement of the pathogen. *Pathogens*, 12(2): 294.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods*, 14(1): 53-61.

Doyle JJ and Doyle J 1990. Isolation of plant DNA from fresh tissue. *Focus*, 12(1): 13-15.

Lee I-M, Bertaccini A, Vibio M and Gundersen DE 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology*, 85: 728-735.

Lorenz KH, Schneider B, Ahrens U and Seemüller E 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. *Phytopathology*, 85: 771–776.

Nainanayaka AD, Weerakkody WATL, Wijesekara HTR, Waidyaratne KP and Subhathma WGR 2010. Impact of Weligama coconut leaf wilt disease (WCLWD) on morphological, physiological and yield aspects of coconut palms. *Third Symposium on Plantation Crop Research – Stakeholder Empowerment Through Technological Advances*. Eds RS Dharmakeerthi and AMWK Senevirathna. Rubber Research Institute of Sri Lanka. Dartonfield, Agalawatta, 258: 275.

Perera L, Meegahakumbura MK, Wijesekara HRT, Fernando WBS and Dickinson J 2012. A phytoplasma is associated with the Weligama coconut leaf wilt disease in Sri Lanka. *Journal of Plant Pathology*, 94(1): 205-209.

Pathirana CK, Chandrasekara CHWMRB, Attanayake SRMR, Weerakkody WAP, Eeswara JP and Bandaranayake PCG 2018. Optimization of DNA extraction and PCR protocols for plants with high phenolics: bael, mango, pomegranate as examples. *Ceylon Journal of Science*, 47(1).

Smart CD, Schneider B, Blomquist CL, Guerra LJ, Harrison NA, Ahrens U, Lorenz KH, Seemüller E and Kirkpatrick BC 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Applied and Environmental Microbiology*, 62(8): 2988–2993.

Wijesekara HTR, Perera L, Wickramananda IW, Herath I, Meegahakumbura MK, Fernando WBS and De Silva PHPR 2008. Preliminary investigation on Weligama coconut leaf wilt disease: a new disease in Southern Sri Lanka. *Proceedings of the Second Symposium on Plantation Crop Research—Export competitiveness through quality improvements*. Eds NADP Nainanayake and JMDT Everard. Coconut Research Institute, Lunuwila, Sri Lanka, 336-341.

Weintraub PG and Beanland L 2006. Insect vectors of phytoplasmas. *Annual Review of Entomology*, 51(1): 91-111.

Research Article

A '*Candidatus* Phytoplasma solani' strain associated with cassava witches' broom disease in Kanchanaburi province, Thailand

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Abstract

Cassava witches' broom disease (CWB) is associated with the presence of phytoplasmas. Typical symptoms include witches' broom, shoot proliferation, short internodes and stunting. Severely infected plants decline, resulting in yield losses of up to 80%. CWB has been reported in several cassava growing regions in Thailand. In total, 25 CWB symptomatic cassava plant samples were collected from 9 plots in Kanchanaburi Province in November 2023. Detection of CWB phytoplasma was carried out using nested PCR assay to amplify the 16S rRNA gene using primers P1/P7, followed by R16F2n/R16R2. A target DNA fragment of approximately 1,800 bp was obtained by PCR in 15 samples, and a 1,200 bp fragment from nested PCR was obtained in 24 samples. Sample KRI-M-4 (GenBank accession number PQ333137) was used for molecular identification. It showed 98.65% identity with the reference strain '*Candidatus* Phytoplasma solani' (GenBank accession number AF248959). BLAST analysis in the GenBank, NCBI database showed 99.07% similarity to a '*Ca*. P. solani' strain from Japan (GenBank accession number LC460259). The partial *stamp* gene of KRI-M-4 strain was cloned, sequenced, and analyzed in the GenBank, NCBI database showing 100% identity to a '*Ca*. P. solani' strain from Thailand (GenBank accession number MW464308). Phylogenetic tree analysis and a heatmap of pairwise distance analysis of the 16S rDNA sequences and partial *stamp* gene sequences revealed that the KRI-M-4 strain clustered with phytoplasmas in the 16SrXII group. This is first report of presence of '*Ca*. P. solani' strain associated with CWB disease, exhibiting witches' broom, yellow leaf, and short internode symptoms in Thailand.

Keywords: phytoplasmas, disease, *stamp* gene, identification, phylogenetic tree

Introduction

Cassava is one of economic crop of Thailand. The planting area in Kanchanaburi Province was about 573,000 rai, producing approximately 1,862,000 tons in 2022, making it the largest planting area and producer in the central region (Office of Agricultural Economics, 2022). The Kanchanaburi Provincial Agricultural Office has issued warning about the outbreak of cassava witches' broom disease associated with the presence of phytoplasmas, which is characterized by proliferation, short internodes, phloem necrosis, yield losses of up to 80%, and reduction in yield and starch content of up to 30% (Alvarez *et al.,* 2013; Kanchanaburi Provincial Agricultural Extension Office, 2023). Phytoplasmas are

classified in the 'Candidatus Phytoplasma' genus as diverse species following specific rules reported in IRPCM (2004). The proposal for defining new 'Ca. Phytoplasma' species as recently updated recommends the use of the whole 16S rRNA gene and of two out of five housekeeping genes (groEL, tuf, rp, secA, and secY), with the criteria that a strain must share >98.65% identity in the 16S rRNA gene sequence and >95% average nucleotide identity (ANI) with a previously reported species (Bertaccini et al., 2022). Classification of phytoplasmas is further supported by restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene F2nR2 fragment using a set of 17 endonucleases (Lee et al., 1998). A computer-simulated method was developed for phytoplasma differentiation based on 16S rRNA RFLP patterns, providing accurate and reliable differentiation across a wide range of phytoplasmas (Wei et al., 2007).

Several reports have documented the identification of CWB associated phytoplasmas in Thailand, belonging to the 16SrI, -II, -III, -VI, and -XV groups (Mejia, 2014; Klinkong et al., 2016; Moonjuntha et al., 2022). Meanwhile, the 16SrI, -III, -V, -VI, -X, -XII, and -XV groups have been reported in Vietnam (Alvarez et al., 2013; Mejia, 2014), the 16SrVIII group in the Philippines (Dolores et al., 2023), the 16SrI group in Cambodia (Alvarez et al., 2013), the 16SrIII group in Brazil and Argentina (Flores et al., 2013; Fernandez et al., 2018) and the 16SrII group in Uganda (Arocha et al., 2009). The objective of this study was to identify the phytoplasma present in cassava witches' broom diseased plantations in Kanchanaburi Province, Thailand.

Materials and Methods

Survey and collection

The CWB disease was surveyed in a total of 9 cassava cultivation plots in Kanchanaburi Province, Thailand, in November 2023, and CWB-symptomatic cassava samples were collected for phytoplasma detection.

Detection of phytoplasmas by amplification of partial 16S rRNA and stamp genes

Total genomic DNA was extracted from cassava midribs by using a modified cetyltrimethylammonium

bromide (CTAB) method (Sambrook et al., 1989). Detection of the 16S rRNA gene region for phytoplasmas was performed using the nested PCR technique with the KOD FX NeO reaction kit (Toyobo, Japan). The PCR was performed, in a total volume of 20 µl, containing 10 µl of 2x PCR buffer KOD FX NeO, 4 µl of 25 mM dNTPs (Toyobo, Japan), 1 µl of genomic DNA template (100 ng/µl), 0.5 µl each of 20 µM P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995), 0.4 µl KOD FX NeO DNA polymerase, and 3.6 µl of ddH$_2$O. The ddH$_2$O was also used as a negative control, while rice orange leaf phytoplasma DNA (GenBank accession number PX474365) was used as a positive control. PCR was performed in a Labcycler Basic thermal cycler (SensoQuest, Germany) with the following condition: initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 10 minutes. The PCR products were diluted 1:10 with ddH$_2$O and used as the template for the nested PCR that was carried out in a 20 µl reaction mixture prepared as described above, except with primers R16F2n and R16R2 (Gundersen and Lee, 1996). The nested PCR was performed with the following conditions: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. The nested PCR products were visualized by gel electrophoresis using 1.0% agarose gel in 0.5x Tris-Borate-EDTA buffer (TBE) buffer (5x stock solution: 0.445 mol/l tris(hydroxymethyl)aminomethane, 0.445 mol/l boric acid, 0.5 mol/l ethylenediaminetetraacetic acid, pH 8.0), For each reaction, 3 µl of PCR product were mixed with 1 µl of loading dye (SMOBio, Taiwan), loaded into the agarose gel, and electrophoresed at 100 V for 40 minutes. DNA bands were visualized and photographed under UV light using the UVITEC FireReader V10 system (Uvitec, United Kingdom).

PCR detection of the partial stamp gene of phytoplasmas was performed with the KOD FX NeO reaction kit (Toyobo, Japan) following the procedure described above. A total of 0.5 µl each of 20 µM Stamp-

XII-F (5'- ACTTCAGCTTTTGCTGCTTTCG-3') forward primer and 20 µM Stamp-XII-R (5'-ACCAGCTACAACGTAAAGAACG-3') reverse primer (this study), plus 0.4 µl KOD FX NeO, and 3.6 µl ddH$_2$O were used. The ddH$_2$O was used as the negative control, and papaya yellows phytoplasma (Donnua *et al.,* 2021) was used as the positive control. The PCR reaction was performed in the same thermal cycler described above with the following conditions: initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 68°C for 1 minute; followed by a final extension at 68°C for 7 minutes. The PCR products were visualized by gel electrophoresis using 1.5% agarose gel in 0.5x TBE buffer. For each reaction, 3 µl of PCR product was mixed with 1 µl of loading dye (SMOBio, Taiwan), loaded into the agarose gel, and electrophoresed at 100 V for 45 minutes. DNA bands were visualized and photographed as described above.

Gene cloning and sequence analysis

The representative sample KRI-M-4, which tested positive by nested PCR on 16S rDNA using the R16F2n/R16R2 primers, was directly sequenced (Solgent, South Korea). The nucleotide sequence was aligned and edited in MEGA 11 and analyzed by BLAST in the GenBank database of the National Center for Biotechnology Information (NCBI). The KRI-M-4 sample that tested positive by PCR also in the partial *stamp* gene using Stamp-XII-F/ Stamp-XII-R primers, was cloned and sequenced. The DNA product was separated by gel electrophoresis, and a single band corresponding to the target DNA was excised and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified DNA was ligated into the pGEM®-T Easy Vector (Promega, USA), and the recombinant plasmid DNA was heat-shock transformed into *Escherichia coli* DH5α cells. The bacterium was cultured on solid LB medium (Sunitha *et al.,* 1999) containing 100 µg/ml ampicillin for 16 h at 37°C. Colonies were selected by PCR using the Stamp-XII-F /Stamp-XII-R primers. Positive colonies were then cultured in LB broth for 16 h at 37°C with shaking at 100 rpm. The bacterial cell suspension was centrifuged to pellet the cells, which were used for plasmid extraction using a modified CTAB method (Sambrook *et al.,* 1989). Extracted plasmids were tested by PCR and purified as described above. The PCR product from a selected clone was sent for nucleotide sequence analysis (SolGent, South Korea). The nucleotide sequences were aligned and edited in the MEGA 11, and the edited sequence was analyzed by BLAST in the GenBank database of the National Center for Biotechnology Information (NCBI).

Molecular identification of phytoplasmas

The partial 16S rRNA and *stamp* gene sequences of the KRI-M-4 strain were aligned in MEGA 11. Phytoplasma strains from 10 ribosomal groups, including 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVIII, 16SrXI, 16SrXII, 16SrXIV, 16SrXV, and 16SrXXII, were retrieved from GenBank, NCBI, and aligned in MEGA 11 for comparison with the KRI-M-4 strain. *Acholeplasma laidlawii* was used as outgroup. The length of each nucleotide sequence used for analysis was 852 base pairs. The phylogenetic tree was constructed by Neighbor-Joining method with a distance matrix based on the p-distance model in the MEGA 11. Bootstrapping with 1,000 replicates was performed to assess the reliability of the inferred trees. Pairwise distance analysis was conducted in R Studio, and a heatmap was generated using the phylogenetic tree combined with pairwise distance data.

The nucleotide sequences of the partial *stamp* gene of CWB phytoplasma were retrieved from GenBank, NCBI, and then aligned and edited in MEGA 11 for comparison with the KRI-M-4 strain. The length of each nucleotide sequence used for analysis was 282 bp. A phylogenetic tree was constructed using the Neighbor-Joining method with a distance matrix based on the p-distance model in MEGA 11. Bootstrapping with 1,000 replicates was performed to assess the reliability of the inferred trees. Pairwise distance analysis was conducted in R Studio, and a heatmap was generated using the phylogenetic tree combined with pairwise distance data.

Results

The survey of cassava from 9 plots in Kanchanaburi Province comprised 25 samples, showing symptoms

leaf distortion, short internodes, bud proliferation and shoot proliferation (Figure 1). Out of these samples, 15 tested positive, showing an approximately 1,800 bp target band when amplified with P1/P7 primers while 24 samples were positive, showing an approximately 1,200 bp band when amplified with R16F2n/R16R2 primers in nested PCR (Figure 2). Detection of the *stamp* gene by PCR showed that only one sample (KRI-M-4) was positive, exhibiting an approximately 342 bp target band when using Stamp-XII-F /Stamp-XII-R primers. Nineteen samples (KRI-M-1, KRI-M-2, KRI-M-3, KRI-M-5, KRI-M-6, KRI-M-7, KRI-M-8, KRI-M-9, KRI-M-10, KRI-M-11, KRI-M-12, KRI-M-15, KRI-M-16, KRI-M-17, KRI-M-18, KRI-M-21, KRI-M-22, KRI-M-24, and KRI-M-25) showed DNA bands, but none corresponded to the target fragment. Five samples (KRI-M-13, KRI-M-14, KRI-M-19, KRI-M-20, and KRI-M-23) showed no DNA bands (Figure 2).

The KRI-M-4 strain (GenBank accession number PQ333137) showed 98.65% identity with the reference strain of '*Ca.* P. solani' (GenBank accession number AF248959) and 99.07% identity with the strain Kamibun from pepper from Japan (GenBank accession number LC460259) and 100% identity in the *stamp* gene of a '*Ca.* P. solani' strain from papaya from Thailand (GenBank accession number MW464308)

when analyzed using BLAST in GenBank, NCBI. A phylogenetic tree and a heatmap of pairwise distance analysis of the 16S rDNA of the KRI-M-4 strain was compared with phytoplasmas from the 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVIII, 16SrXI, 16SrXII, 16SrXIV, 16SrXV, and 16SrXXII groups. The KRI-M-4 resulted closely related to the 16SrXII group, showing 90–98.6% similarity in the heatmap (Figure 3).

A phylogenetic tree and a heatmap of pairwise distance analysis of the *stamp* gene sequences of KRI-M-4 (PV261035) were compared with phytoplasma sequences in the 16SrXII group retrieved from GenBank, NCBI. KRI-M-4 (GenBank accession number PV261035) was closely related to the 16SXII-A group, a phytoplasma infecting papaya in Thailand, showing >98.6% similarity in the heatmap confirming the similarity data obtained in the 16D rRNA gene (Figure 4).

Discussion

In Thailand, cassava samples were surveyed and collected from Chachoengsao, Kamphaeng Phet, Rayong, Nong Khai, Nakhon Phanom, Mukdahan, Buriram, Sisaket and Surin Provinces. Symptoms such as witches' broom, dwarf, exaggerated bud proliferation, short internodes and small leaves were observed. The phytoplasma detected was classified in the 16SrI group

Figure 1. Comparison of cassava witches' broom symptom to healthy cassava plant in Kanchanaburi Province, Thailand: (a) leaf distortion, short internodes, bud proliferation and shoot proliferation (KRI-M-4 strain) and (b) healthy cassava plant.
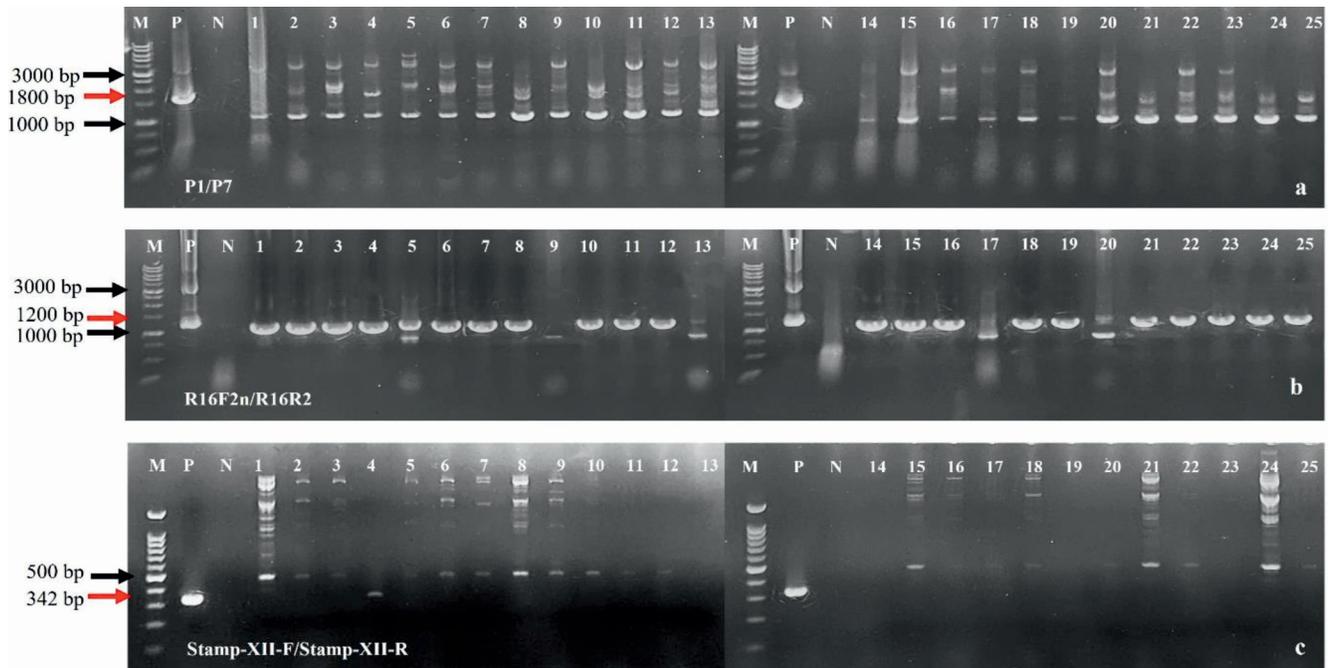
Figure 2. PCR amplification of 16S rRNA gene performed using P1/P7 followed by R16F2n/R16R2 primers in nested PCR (a and b, respectively). PCR amplification of the partial *stamp* gene of phytoplasmas carried out using the Stamp-XII-F /Stamp-XII-R primers (c). M = 1 kb DNA ladder, SMOBio, Taiwan); N = negative control (ddH$_2$O). P = positive control (rice orange leaf phytoplasma; GenBank accession number PX474365) in a and b; P = positive control (16SrXII group papaya yellows phytoplasma; Donnua *et al.,* 2021) in c. Lanes 1-25 represent CWB cassava samples: 1 = KRI-M-1, 2 = KRI-M-2, 3 = KRI-M-3, 4 = KRI-M-4, 5 = KRI-M-5, 6 = KRI-M-6, 7 = KRI-M-7, 8 = KRI-M-8, 9 = KRI-M-9, 10 = KRI-M-10, 11 = KRI-M-11, 12 = KRI-M-12, 13 = KRI-M-13, 14 = KRI-M-14, 15 = KRI-M-15, 16 = KRI-M-16, 17 = KRI-M-17, 18 = KRI-M-18, 19 = KRI-M-19, 20 = KRI-M-20, 21 =KRI-M-21, 22 = KRI-M-22, 23 = KRI-M-23, 24 =KRI-M-24, 25 = KRI-M-25.
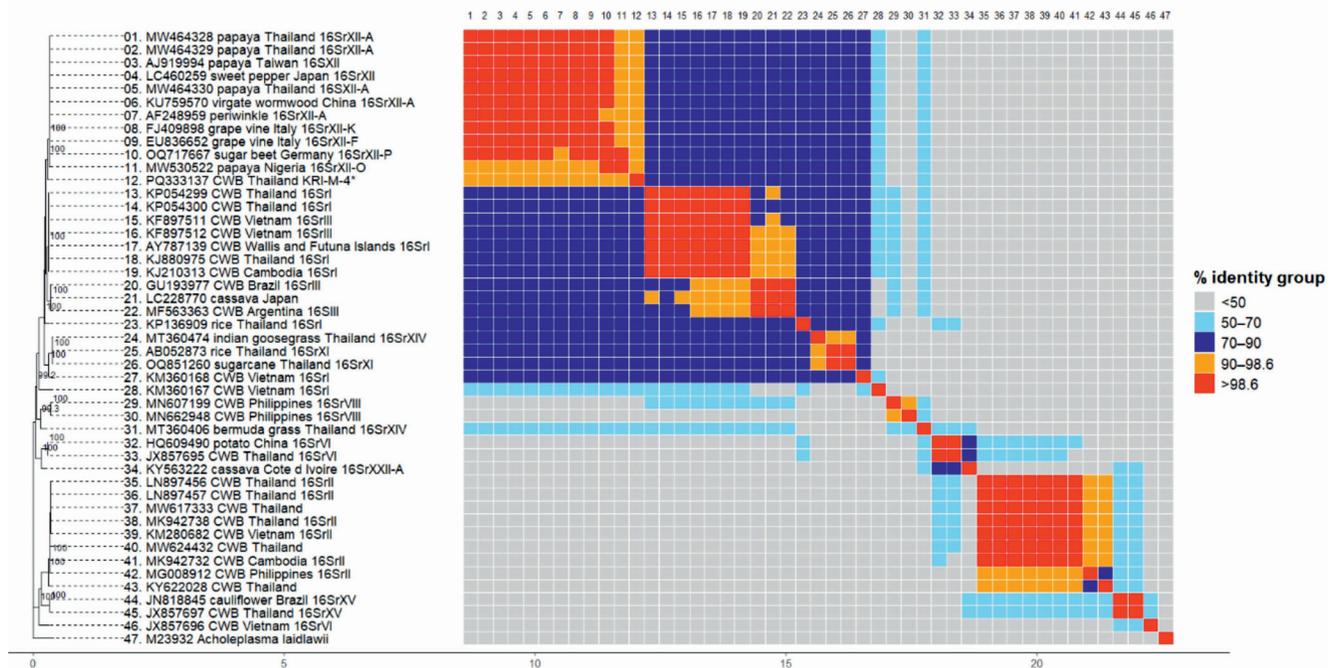
Figure 3. Phylogenetic tree and pairwise nucleotide identity matrix based on the 16S rRNA gene. The heatmap shows the pairwise percentage of nucleotide identity, while the dendrograms represent the phylogenetic relationships inferred from the multiple sequence alignments using the Neighbor-Joining method. The color gradient indicates identity values ranging from <50% (light grey), 50–70% (sky blue), 70–90% (dark blue), 90–98.6% (orange), to >98.6% (red). Bootstrap values (>98.6%) are indicated at the nodes. The sequence obtained in this study (GenBank accession number PQ333137) is marked with an asterisk (*) in the tree and compared to phytoplasma sequences in 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVIII, 16SrXI, 16SrXII, 16SrXIV, 16SrXV, and 16SrXXII groups retrieved from GenBank, NCBI.
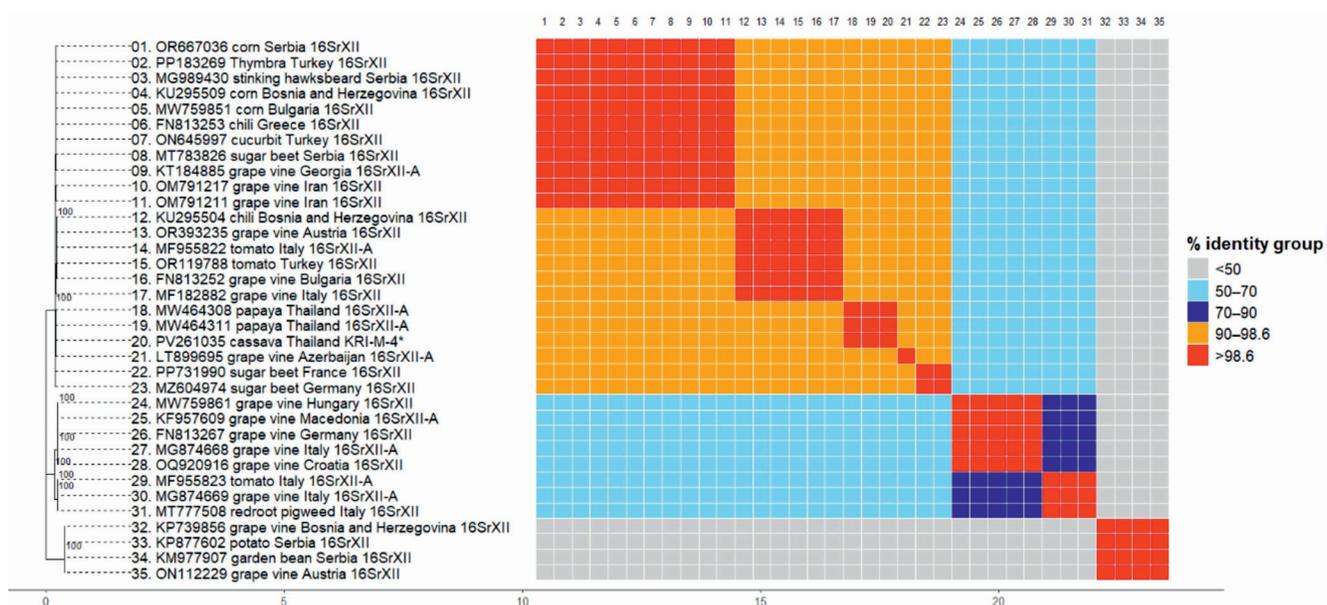
Figure 4. Phylogenetic tree and pairwise nucleotide identity matrix based on the partial *stamp* gene. The heatmap shows the pairwise percentage of nucleotide identity, while the dendrograms represent the phylogenetic relationships inferred from the multiple sequence alignments using the Neighbor-Joining method. The color gradient indicates identity values ranging from <50% (light grey), 50–70% (sky blue), 70–90% (dark blue), 90–98.6% (orange), to >98.6% (red). Bootstrap values (100%) are indicated at the nodes. The sequence obtained in this study (GenBank accession number PV261035) is marked with an asterisk (*) in the tree and compared to phytoplasma sequences in 16SrXII group retrieved from GenBank, NCBI.

(Klinkong *et al.*, 2016). Saengsai *et al.* (2019) surveyed cassava in Nakhon Ratchasima province, and observed witches' broom, leaf distortion and yellowing leaves. The phytoplasma was classified in the 16SrII group. Mejia (2014) surveyed cassava in Thailand showed symptoms of short internodes, small yellow leaves and witches' broom in the middle and/or lower parts of plant, mealybug were not present, and the phytoplasma was classified in the 16SrVI group. Plant exhibiting severe mealybug infestation on shoots and apices were associated with phytoplasma of the 16SrXV group. Moreover, in plants apparently healthy, phytoplasmas in the 16SrI, -VI, -XV groups were detected. Previous reports of CWB phytoplasmas in Thailand have classified them into several groups including 16SrI, -II, -III, -VI, and -XV (Mejia, 2014; Klinkong *et al.*, 2016; Moonjuntha *et al.*, 2022). In Thailand, phytoplasma in the 16SrXII group have been reported in infected papaya, causing symptoms such as yellow necrosis, bunchy top-like growth, shortening of internodes, excessive proliferation of axillary shoots, green vien, yellow shoots, crinkle mosaic (Donnua *et al.*, 2021).

In this study, cassava fields were surveyed, and samples were collected from Kanchanaburi Province.

The observed symptoms were leaf distortion, short internodes, bud proliferation and shoot proliferation and were consistent with those reported by Klinkong *et al.* (2016) in Thailand. The results of molecular identification of the phytoplasma detected in cassava provide new evidence of a 'Ca. P. solani' strain associated with CWB in Thailand. This phytoplasma is in the same ribosomal group reported by Mejia (2014) in Vietnam where cassava showed short internodes, small-yellow leaves and witches' broom in the middle and/or lower parts of plant.

Conclusion

The CWB phytoplasma found in Kanchanaburi Province was associated with the presence of leaf distortion, bud proliferation and shoot proliferation. The phytoplasma is a strain of 'Ca. P. solani' with 98.65% similarity to the reference strain. In addition, BLAST analysis of the *stamp* gene of this KRI-M-4 strain showed 100% identity to the 'Ca. P. solani' strain from papaya from Thailand (GenBank accession number MW464308). A heatmap generated using the phylogenetic tree combined with pairwise distance data indicated that both 16S rRNA and the *stamp* genes of

KRI-M-4 are closely related to phytoplasmas in the 16SrXII group. Further research should be carried out to verify the epidemiological implications of this finding.

Acknowledgments

References

Alvarez E, Pardo JM, Mejia JF, Bertaccini A, Thanh ND and Hoat TX 2013. Detection and identification of '*Candidatus* Phytoplasma asteris'-related phytoplasmas associated with a witches' broom disease of cassava in Vietnam. *Phytopathogenic Mollicutes*, 3(2): 77-81.

Arocha Y, Echodu R, Talengera D, Muhangi J, Rockefeller E, Asher O, Nakacwa R, Serugga R, Gumisiriza G, Tripathi J, Kabuye D, Otipa M, Vutseme LK and Boa E 2008. Occurrence of '*Candidatus* Phytoplasma aurantifolia' (16SrII group) in cassava and four other species in Uganda. *New Disease Reports*, 17: 28.

Bertaccini A, Arocha-Rosete Y, Contaldo N, Duduk B, Fiore N, Guglielmi Montano H, Kube M, Kuo C-H, Martini M, Oshima K, Quaglino F, Schneider B, Wei W and Zamorano A 2022. Revision of the '*Candidatus* Phytoplasma' species description guidelines. *International Journal of Systematic and Evolutionary Microbiology*, 72: 005353.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and non-culturable Mollicutes. *Journal of Microbiological Methods*, 14: 53–61.

Dolores LM, Langres JA, Pinili MS, Caasi-Lit MT, Cortaga CQ, Retuta YM and Cueva FMD 2023. Incidence, distribution, and genetic diversity of '*Candidatus* Phytoplasma luffae'-related strain (16SrVIII) associated with the cassava witches' broom (CWB) disease in the Philippines. *Crop Protection*, 169: 106244.

Donnua S, Parkpoom B and Thaipong K 2021. '*Candidatus* Phytoplasma solani' associated with papaya phytoplasma disease in Thailand. *Khon Kaen Agriculture Journal*, 49(5): 1249-1258.

Fernandez F, Uset A, Baumgratz G and Conci L 2018. Detection and identification of a 16SrIII-J phytoplasma affecting cassava (*Manihot esculenta* Crantz) in Argentina. *Australasian Plant Disease Notes*, 13: 24.

Flores D, Haas IC, Canale MC and Bedendo IP 2013. Molecular identification of a 16SrIII-B phytoplasma associated with cassava witches' broom disease. *European Journal of Plant Pathology*, 137: 237–242.

Gundersen DE and Lee I-M 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, 35: 144–151.

IRPCM 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology*, 54: 1243–1255.

Kanchanaburi Provincial Agricultural Extension Office 2023. Pest outbreak warning. https://kanchanaburi.doae.go.th/province/?cat=5.

Klinkong S, Rungsawang W, Khwantongyim P and Reanwarakorn K 2016. Molecular characterization of phytoplasma associated with witches' broom disease - emerging disease of cassava in Thailand. *Agricultural Science Journal*, 47(2): 175-188.

Lee I-M, Gundersen-Rindal DE, Davis RE and Bartoszyk IM 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology*, 48: 1153–1169.

Mejia JF 2014. Identification and genetic diversity in phytoplasmas associated with diseases of cassava and other agronomic relevant crops in south-east Asia and Latin America. PhD thesis, *Alma Mater Studiorum* - Università di Bologna, 139 pp.

Moonjuntha P, Lankaew S, Maneechoat P, Doungsaard C, Hoat TX, Pao S, Pinili MS and Natsuki KT 2022. Cassava witches' broom phytoplasma in Southeast Asia. *The 15th National Plant Protection Conference "Modern farming, modern life and environmental care". Rama Gardens hotel, Bangkok, Thailand*, 99-100.

Office of Agricultural Economics 2022. Cassava for factories: cultivated area, harvested area, yield and yield per rai at national, regional and provincial levels in 2022. Office of Agricultural Economics, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Saengsai W, Sakuanrungsirikul S, Thongmee W and Ratthawat B 2019. Screening cassava cutting infected by cassava witches' broom phytoplasma using LAMP and nested PCR. *Proceedings of the 16th National Seed Conference 2019. Thepsatri Rajabhat University, Lopburi*, 272-278.

Sambrook J, Fritsch ER and Maniatis T 1989. Molecular Cloning: A Laboratory Manual, (2nd ed). Cold Spring Harbor Laboratory Press, New York, 1-1626.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC 1995. Phylogenetic classification of plant pathogenic mycoplasma like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology*. Eds S Razin and JG Tully San Diego, CA: Academic Press, 369-380.

Sunitha K, Lee JK and Oh TK 1999. Optimization of medium components for phytase production by *E. coli* using response surface methodology. *Bioprocess Engineering*, 21(6): 477-481.

Wei W, Davis RE, Lee I-M and Zhao Y 2007. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *International Journal of Systematic and Evolutionary Microbiology*, 57: 1855–1867.

Research Article

Identification of a 'Candidatus Phytoplasma asteris' 16SrI-F strain infecting periwinkle in Iran

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Abstract

Since 2021, symptoms of little leaf disease have been observed in periwinkle plants grown in Eram garden, Fars province, Iran. The agent of the Eram garden periwinkle little leaf (EGPLL) disease was transmitted to periwinkle and eggplant plants through dodder and induced phytoplasma type symptoms. Phylogenetic and *i*PhyClassifier analyses using a 1250 bp fragment of the 16S rRNA gene, obtained via nested PCR, cloning and sequencing identified the EGPLL phytoplasma as a 'Candidatus Phytoplasma asteris' strain belonging to the 16Sr group I, subgroup F. This is the first report of a 16SrI-F phytoplasma strain naturally occurring in periwinkle in Iran.

Introduction

Phytoplasmas are microscopic, wall-less bacteria that colonize plant phloem tissues and insect emolimph. Responsible for numerous plant diseases, collectively known as phytoplasma-associated diseases, they can cause significant economic losses in agriculture and horticulture worldwide. Symptoms of these diseases often include virescence, phyllody, witches' broom, abnormal proliferation of shoots and roots, leaf yellowing or reddening, reduced leaf and fruit size, phloem necrosis, overall decline, and stunting (Bertaccini and Duduk, 2009). Phytoplasmas are mainly associated with numerous destructive plant diseases (Bertaccini *et al.*, 2014; Bertaccini, 2022) and are transmitted by phloem-feeding insects such as leafhoppers, psyllids and planthoppers, which play a crucial role in their epidemic dissemination (Weintraub and Beanland, 2006). They are currently classified within the provisional genus 'Candidatus Phytoplasma' based primarily on 16S rRNA gene sequence analysis (IRPCM, 2004; Bertaccini *et al.*, 2022). Multilocus sequence analysis of less-conserved genes has also proven useful for differentiating genetically close but epidemiologically distinct strains within a given phytoplasma taxon (Martini *et al.*, 2019).

Periwinkle [*Catharanthus roseus* (L.) G. Don.] belongs to the Apocynaceae family, is native to the Madagascar islands in the Indian Ocean. This plant is currently cultivated in many tropical and subtropical regions worldwide. In addition to its ornamental value

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and rapid growth, it produces medicinal compounds such as vincristine and vinblastine used in therapy to human cancer diseases (Duke, 1985; Quadri *et al.*, 2024). Periwinkle hosts many phytoplasma strains globally and plays a crucial role in the verification of the presence of phytoplasma diseases. This plant is highly susceptible to phytoplasma infections, making it an ideal host for studying these pathogens.

To date, more than 50 '*Ca.* Phytoplasma' species have been identified based on 16S rRNA gene sequencing (IRPCM 2004; Bertaccini *et al.*, 2022; Rodrigues *et al.*, 2023, 2024). Important phytoplasma diseases of periwinkle are associated with the presence of '*Ca.* P. asteris' strains (16SrI) (Lee *et al.*, 2004; Marcone *et al.*, 2000; Torres *et al.*, 2004; Nejat *et al.*, 2010), '*Ca.* P. trifolii' (16SrVI-A) (Hiruki and Wang, 2004; Lee *et al.*, 1998), and '*Ca.* P. hispanicum' (16SrXIII-A) (Davis *et al.*, 2016; Gundersen *et al.*, 1994).

In Iran, periwinkle diseases due to phytoplasmas in the aster yellows ribosomal group have been reported from Fars (Salehi *et al.*, 2005) and Markazi (Babaie *et al.*, 2007) provinces. Periwinkle phyllody due to phytoplasmas enclosed in group 16SrII was reported in Kerman and Sistan-Baluchistan provinces (Salehi *et al.*, 2005; Siampour *et al.*, 2019). Additionally, periwinkle phyllody due to the '*Ca.* P. trifolii' (16SrVI-A subgroup) has been reported in Mazandaran province (Fattahi *et al.*, 2016), and periwinkle phyllody due to 16SrIX phytoplasmas in Fars province (G. Shaygan and M. Salehi, GenBank accession number KC332292).

Since a 2021 survey on phytoplasma disease, symptoms of little leaf disease were observed in a patch of periwinkle plants in Eram garden, a historic Persian garden in Shiraz, located in the central area of Shiraz city, Fars province, Iran. The objective of this work was to determine the presence and identity of the phytoplasmas associated with EGPLL disease and their ability to induce symptoms in experimental host plants after dodder inoculation.

Material and Methods

Source of the disease. Five periwinkle plants showing symptoms of little leaf disease were selected from a patch of symptomatic periwinkles in Eram garden in Shiraz (Fars province of Iran). These plants were then transferred to an insect-free greenhouse to serve as sources for biological and molecular studies. A visual evaluation of the symptoms was performed to verify the disease incidence. The percentage of disease incidence was calculated by counting the number of plants with symptoms out of total number of plants observed in a plot and this value was multiplied by 100.

Dodder and graft inoculation. To transmit the Eram garden periwinkle little leaf (EGPLL) agent to periwinkle and eggplant (*Solanum melongena* L.) *via* dodder (*Cuscuta campestris* Yunck) inoculation, dodder seeds were germinated on moist filter paper and seedlings were transferred to a healthy seed grown and PCR negative beet plants. Three weeks later, the new strands of dodder grown on beet plants were transferred to a periwinkle plant suffering from little leaf disease, which had been moved from the field and maintained in a pot. One month later, connections between beet dodder and periwinkle were cut, and dodder infested periwinkle pot was placed adjacent to periwinkle and eggplant pots (each containing six healthy plants) grown from seed for connection via dodder. Dodder strands from diseased periwinkle were gradually established on periwinkle and eggplant plants and after six weeks, periwinkle and eggplant plants were freed of dodder and kept in the insect-free greenhouse to verify symptom expression. For long-term maintenance, the EGPLL agent was transmitted from symptomatic dodder-inoculated periwinkle to healthy periwinkle plants by side grafting. For side grafting small axillary shoots from a symptomatic dodder-inoculated plant served as scions. Each of the six-recipient plant received two scions. The graft unions were wrapped with parafilm, and the grafted plants were covered with plastic bags for two weeks to maintain humidity.

DNA extraction and PCR detection. Total DNA was extracted from 0.2 g of midrib tissue of fresh leaves of naturally diseased and inoculated plants using the Healey *et al.* (2014) procedure. Total DNA extracted from a periwinkle plant infected with an alfalfa witches' broom phytoplasma strain (16SrII-C) (Salehi *et al.*, 2011) was used as a positive control.

Asymptomatic periwinkles collected in the Eram garden and samples devoid of DNA templated were used as negative control. The detection of phytoplasmas in extracted DNA samples was verified by nested PCR using primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by R16F2n/R16R2 (Gundersen and Lee, 1996). The R16F2n/R16R2 primer pair amplifies approximately 1250 bp of the phytoplasma 16S rRNA gene. The PCR reagents and conditions for phytoplasma detection were as described by Salehi *et al.* (2020). PCR products were separated in 1% agarose gels in 1X TBE buffer (108 g Tris-HCl, 55 g boric acid, 40 ml EDTA 0.5 M, pH 8.0). DNA bands were stained with 0.5 mg/ml ethidium bromide and visualized with a UV transilluminator. The molecular weight of the PCR products was estimated by comparison with 100 bp DNA ladder (Fermentas, Vilnius, Lithuania).

Cloning and sequencing of PCR products. Five amplicons of the 16S rRNA gene from naturally infected periwinkle plants, along with six from each of the dodder-inoculated eggplant and periwinkle plants, were obtained using R16F2n/R16R2 primers in nested PCR. These amplicons were ligated into the pTZ57R/T vector and cloned into *Escherichia coli* DH5α using the Ins TD A clone PCR Product Cloning Kit (Sinaclone, Tehran, Iran) according to the manufacturer's instructions. The presence of the correct insert was confirmed with *EcoR*I and *Pst*I restriction endonucleases. Plasmid DNA from recombinant colonies was purified using the GF-1 PCR Clean-Up Kit (Vivantis, Malaysia, HQ). Sequencing was performed by Macrogen (South Korea) in both directions using forward and reverse M13 (Messing, 1983) primers and one representative sequence, was then submitted to NCBI GenBank.

Phylogenetic analyses. The obtained partial 16S rDNA sequence from EGPLL phytoplasma was compared with 16S rDNA sequences of phytoplasmas in GenBank using BLASTn from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The R16F2n/R2 16S rDNA sequences of 30 phytoplasmas, including EGPLL, were aligned using the Clustal W program and evolutionary analyses were conducted in MEGA12 (Kumar *et al.*, 2024) utilizing up to 2 parallel computing threads. Phylogenetic trees based on partial 16S rDNA sequences were constructed by the neighbour joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). *Acholeplasma laidlawii* was used as an outgroup to root the tree. Bootstrapping was performed 100 times to estimate stability and support for the branches.

Virtual RFLP analysis. The virtual RFLP patterns of 16S rDNA (1250 bp) fragment from EGPLL phytoplasma were analyzed and compared to those of other phytoplasmas using *i*PhyClassifier and each 16S rDNA fragment was digested *in silico* with 17 distinct restriction enzymes (Zhao *et al.*, 2009).

Results and Discussion

Characteristic symptoms of EGPLL were virescence, phyllody, little leaf, internode shortening, witches' broom, and yellowing (Figure 1). In the surveyed patch of periwinkle plants grown in the Eram garden, the disease incidence was 3.4%.

All dodder and graft-inoculated periwinkle plants developed phyllody, virescence, little leaf, and witches' broom symptoms seven to ten weeks after inoculation. Additionally, four of five dodder-inoculated eggplants

Figure 1. Little leaf, phyllody, virescence, and witches' broom symptoms observed in periwinkle plants grown in Eram garden, Fars province, Iran.

Figure 2. Eggplant (left) and periwinkle (right) plants dodder inoculated with periwinkle little leaf agent showing virescence and phyllody disease symptoms.

displayed virescence and phyllody symptoms eight to twelve weeks after inoculation (Figure 2). DNA samples from all inoculated plants positively reacted in nested-PCR tests. A DNA fragment of approximately 1.25 kbp (F2n/R2 fragment) was amplified in nested PCR from five naturally symptomatic periwinkle potted plants and from all the symptomatic dodder-inoculated plants, as well as from the positive control (data not shown). No PCR products were obtained from symptomless periwinkle plants and from the PCR mixture devoid of DNA (negative control). After sequencing and alignment all R16F2/R16R2 primed PCR products were identical to each other (sequence identity 100%) and one 1.250 kbp DNA fragment from one of the naturally symptomatic periwinkle plants was submitted to GenBank data base under accession number PP316099. The BLAST search showed that this strain, had 99.20% identity to 'Ca. P. asteris' (GenBank accession number M30790).

The phylogenetic analysis placed the EGPLL phytoplasma strain in the 16SrI phytoplasma group, specifically clustering with strains of the 16SrI-F subgroup (Figure 3). The *i*PhyClassifier analysis (Figure 4) showed that the phytoplasma associated with EGPLL disease in periwinkle from Fars had no differences, and its virtual RFLP pattern was identical (similarity coefficient 1.00) to the pattern of phytoplasmas attributed to 16Sr group I, subgroup F (GenBank accession number AY265211).

This is the first report of the association of a member of 16SrI-F phytoplasma with the periwinkle little leaf

Figure 3. Phylogenetic tree based on partial 16S rDNA sequences constructed by the neighbour joining method showing the relationship among periwinkle little leaf phytoplasma and selected reference phytoplasmas from GenBank. *Acholeplasma laidlawii* was used as an outgroup to root the tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1.000 replicates) are shown below the branches, only values above 60 are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA12. The scale bar indicates 0.02 substitution per nucleotide position.
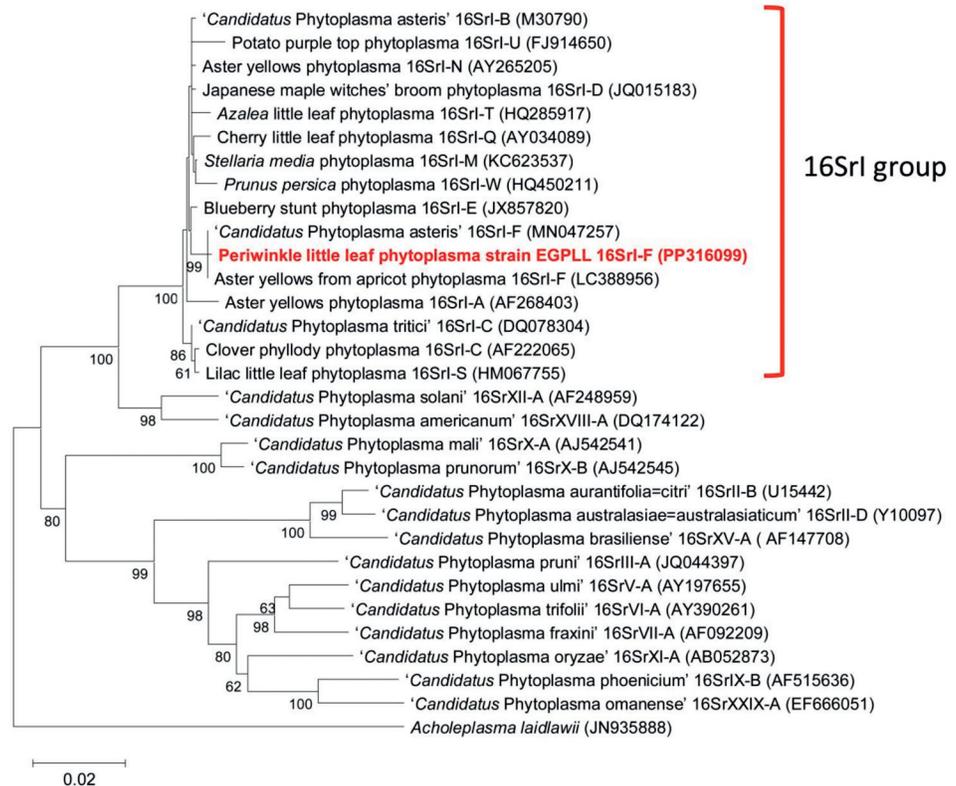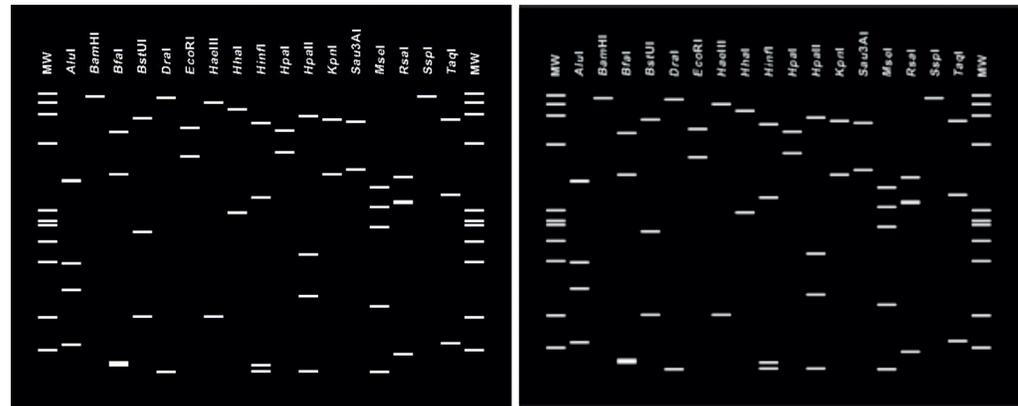
Figure 4. Virtual RFLP profiles using *i*PhyClassifier. Left, periwinkle little leaf phytoplasma (GenBank accession number PP316099) and right '*Candidatus* Phytoplasma asteris' reference strain of 16Sr group I, subgroup F (GenBank accession number AY265211), respectively.

disease in Iran. The aster yellows (16SrI) phytoplasma group is the third most widespread phytoplasma identified in Iran, with four subgroups reported: 16SrI-B, 16SrI-F, 16SrI-R, and 16SrI-S (Esmailzadeh Hosseini *et al.*, 2023a, 2023b). Phytoplasmas belonging to subgroup 16SrI-F have been reported in apricot and periwinkle in Spain and Germany, respectively (Lee *et al.*, 1998; Bertaccini, 2023), and more recently in potato with purple top symptoms in Ecuador (Castillo-Carrillo *et al.*, 2018).

Acknowledgments

References

Babaie G, Khatabi B, Bayat H, Rastgou M, Hosseini A and Salekdeh GH 2007. Detection and characterization of phytoplasma infecting ornamental and weed plants in Iran. *Journal of Phytopathology*, 155(6): 368–372.

Bertaccini A 2022. Plants and phytoplasmas: when bacteria modify plants. *Plants*, 11: 1425.

Bertaccini A 2023. Phytoplasma collection. https://www.ipwgnet.org/collection

Bertaccini A, Duduk B, Paltrinieri S, Contaldo N 2014. Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. *American Journal of Plant Sciences*, 5: 1763-1788.

Bertaccini A, Arocha-Rosete Y, Contaldo N, Duduk B, Fiore N, Guglielmi Montano H, Kube M, Kuo C-H, Martini M, Oshima K, Quaglino F, Schneider B, Wei W and Zamorano A 2022. Revision of the '*Candidatus* Phytoplasma' species description guidelines. *International Journal of Systematic and Evolutionary Microbiology*, 72: 005353.

Castillo-Carrillo C, Paltrinieri S, Buitrón Bustamante J and Bertaccini A 2018. Detection and molecular characterization of a 16SrI-F phytoplasma in potato showing purple top disease in Ecuador. *Australasian Plant Pathology*, 47: 311-315.

Davis RE, Harrison NA, Zhao Y, Wei W and Dally EL 2016. '*Candidatus* Phytoplasma hispanicum', a novel taxon associated with Mexican periwinkle virescence disease of *Catharanthus roseus*. *International Journal of Systematic and Evolutionary Microbiology*, 66: 3463-3467.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *Journal of Microbiological Methods*, 14: 53–61.

Duke JA 1985. Handbook of Medicinal Herbs. CRC Press, pp 168.

Esmailzadeh Hosseini SA, Azadvar M, Babaei G, Salehi M and Bertaccini A 2023a. Diversity, distribution and status of phytoplasma diseases in Iran. In: *Phytoplasma Diseases in Asian countries. Diversity, Distribution and Current Status*. Eds Tiwari AK, Caglayan K, Al-Sadi A, Azadvar M and Abeysinghe S, Academic Press, 39-83.

Esmailzadeh Hosseini SA, Azadvar M, Babaei G, Salehi M and Bertaccini A 2023b. Important phytoplasma ribosomal subgroups distributed in Iran. *Phytopathogenic Mollicutes*, 13(1): 125-126.

Fattahi M, Salehi M, Sharzehi A and Hosseini SE 2016. Partial biological and molecular characteristics of a phytoplasma associated with Behshahr (Mazandaran) periwinkle phyllody. *Iranian Journal of Plant Pathology*, 52: 135-141.

Gundersen DE and Lee I-M 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer sets. *Phytopathologia Mediterranea*, 35: 144-151.

Gundersen DE, Lee I-M, Rehner SA, Davis RE and Kingsbury DT 1994. Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. *Journal of Bacteriology*, 176: 5244-5254.

Healey A, Furtado A, Cooper T and Henry RJ 2014. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant Methods*, 10: 21.

Hiruki C and Wang K 2004. Clover proliferation phytoplasma: '*Candidatus* Phytoplasma trifolii'. *International Journal of Systematic and Evolutionary Microbiology,* 54: 1349-1353.

IRPCM 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology*, 54: 1243-1255.

Kumar S, Stecher G, Suleski M, Sanderford M, Sharma S and Tamura K 2024. Molecular evolutionary genetics analysis version 12 for adaptive and green computing. *Molecular Biology and Evolution*, 41: 1-9.

Lee I-M, Gundersen-Rindal DE, Davis RE and Bartoszyk IM 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, 48: 1153-1169.

Lee I-M, Gundersen-Rindal DE, Davis RE, Bottner KD, Marcone C and Seemüller E 2004. '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *International Journal of Systematic and Evolutionary Microbiology*, 54: 1037-1048.

Marcone C, Lee I-M, Davis RE, Ragozzino A and Seemüller E 2000. Classification of aster yellows-group phytoplasmas based on combined analysis of rRNA and *tuf* gene sequence. *International Journal of Systematic and Evolutionary Microbiology*, 50: 1703-1713.

Martini M, Quaglino F and Bertaccini A 2019. Multilocus genetic characterization of phytoplasmas. In: *Phytoplasmas: Plant Pathogenic Bacteria - III Genomics, host pathogen interactions, and diagnosis.* Eds Bertaccini A, Oshima K, Kube M and Rao GP, Springer, Singapore, 161-200.

Messing J 1983. New M13 vectors for cloning. *Methods in Enzymology*, 101: 20-78.

Nejat N, Sijam K, Abdullah SNA, Vadamalai G and Dickinson M 2010. Molecular characterization of an aster yellows phytoplasma associated with proliferation of periwinkle in Malaysia. *African Journal of Biotechnology*, 9: 2305-2315.

Quadri A, Barbaresi A, Tassinari P, Bertaccini A, Contaldo N, Mercolini L, Protti M, Montalbetti R, Laurita R and Torreggiani D 2024. Enhancement of vindoline and catharanthine production in *Catharanthus roseus* by LED light and plasma activated water. *Plos One*, 19(12): e0315542.

Rodrigues JB, Tran-Nguyen LTT, Gambley C, Al-Sadi AM, Al-Subhi AM, Foissac X, Salar P, Cai H, Yang J-Y, Davis R, Jones L, Rodoni B and Constable FE 2023. The observation of taxonomic boundaries for the 16SrII and 16SrXXV phytoplasmas using genome-based delimitation. *International Journal of Systematic and Evolutionary Microbiology*, 73: 7.

Rodrigues JB, Tran-Nguyen, Gambley C, Webster C, Kehoe M, Bond S, Rodoni B and Constable FE 2024. '*Candidatus* Phytoplasma vignae', assigning a species description to a long-known phytoplasma occurring in northern Australia. *International Journal of Systematic and Evolutionary Microbiology*, 74: 8.

Saitou N and Nei M 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4: 406-425.

Salehi M, Esmailzadeh Hosseini SA, Salehi E, Quaglino F and Bianco PA 2020. Peach witches' broom, an emerging disease associated with '*Candidatus* Phytoplasma phoenicium' and '*Candidatus* Phytoplasma aurantifolia' in Iran. *Crop Protection*, 127: 104946.

Salehi M, Heydarnejad J and Izadpanah K 2005. Molecular characterization and grouping of 35 phytoplasmas from central and southern provinces of Iran. *Iranian Journal of Plant Pathology*, 41: 62-64.

Salehi M, Izadpanah K, Siampour M and Esmailzadeh Hosseini SA 2011. Polyclonal antibodies for the detection and identification of Fars alfalfa witches' broom phytoplasma. *Bulletin of Insectology*, 64(Supplement): 59-60.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: *Molecular and diagnostic procedures in Mycoplasmology.* S Razin and JG Tully (eds), Academic Press. San Diego, California (USA), 369–380.

Siampour M, Izadpanah K, Martini M and Salehi M 2019. Multilocus sequence analysis of phytoplasma strains of 16SrII group in Iran and their comparison with related strains. *Annals of Applied Biology*, 175(1): 83-97.

Tamura K, Nei M and Kumar S 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)*, 101: 11030-11035.

Torres L, Galdeano E, Docampo D and Conci L 2004. Characterization of aster yellows phytoplasma associated with *Catharanthus* little leaf in Argentina. *Journal of Plant Pathology*, 86: 209-214.

Weintraub PG and Beanland L 2006. Insect vectors of phytoplasmas. *Annual Revue of Entomology*, 51: 91-111.

Zhao Y, Wei W, Lee I-M, Shao J, Suo X and Davis RE 2009. Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *International Journal of Systematic and Evolutionary Microbiology*, 59: 2582-2593.

Prevalence and molecular identification of '*Candidatus* Phytoplasma asteris' strains in okra crops across three Indian states

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Abstract

Besides viral diseases, phytoplasma-associated diseases pose a significant threat to okra cultivation in India. During 2022-2025 seasons, okra plants exhibiting characteristic phytoplasma symptoms such as little leaf, shoot proliferation, bud proliferation and bunchy crown were observed in Kerala, New Delhi and Tripura states of India. Molecular detection using phytoplasma-specific primers P1/Tint and R16F2n/R16R2 amplified DNA fragments of about 1.25 kb of the 16S rRNA gene from all the symptomatic okra leaf samples, while asymptomatic samples showed no amplifications. Pairwise sequence analysis of the 16S rRNA gene revealed almost 100% identity among the okra phytoplasma strains in the study and '*Candidatus* Phytoplasma asteris' strains. Phylogenetic analysis further clustered these okra strains with '*Ca.* P. asteris' strains. *In silico* RFLP analysis employing 17 restriction endonucleases confirmed the affiliation of the okra phytoplasma strains within the 16SrI-B subgroup, with a similarity coefficient of 1.00. Although the '*Ca.* P. asteris' has been reported earlier in India, the present study highlights the prevalence and importance of '*Ca.* P. asteris' strains occurrence in okra crops across three Indian states, underscoring their epidemiological importance.

Keywords: *Abelmoschus esculentus,* 16SrI-B subgroup, nested PCR assay, 16S rRNA gene, *in silico* RFLP analysis

Introduction

Okra (*Abelmoschus esculentus* L.), a member of the Malvaceae family, is a widely cultivated vegetable crop in tropical and subtropical regions globally (Massrie, 2025). It is also known as lady's finger and bhindi and its pods are rich in essential nutrients, including fibre, protein, vitamins, and minerals (Jain *et al.,* 2012; Patra *et al.,* 2023). India leads in the world okra production, accounting for 7.158 million tons from 0.54022 million hectares, making it the seventh most important vegetable crop in the country (Elkhalifa *et al.,* 2021; Ali *et al.,* 2022). Okra's economic importance extends beyond its consumption as food, with its mature fruits and stems being used in the paper industry and mucilage as food additives (Dhakne and Pansare,

2024). However, there are several diseases including powdery mildew, fusarium wilt and begomovirus infections which significantly reduce the pod yields (Singh, 2023). Absence of improved disease resistant cultivars exacerbates these biotic stress challenges (Benti *et al.*, 2017; Massrie, 2025). Phytoplasmas are pleomorphic, wall-less, intracellular plant-pathogenic, phloem- inhabiting bacteria associated with diseases in hundreds of crop plants, weeds, forest plants across the world which led to huge economic losses (Wang *et al.*, 2024). Various phytoplasma strains have been reported to infect okra in different regions of India and globally, highlighting the widespread impact of these pathogens (Kumar *et al.*, 2012; Gungoosingh-Bunwaree *et al.*, 2011; Kumari *et al.*, 2019, 2023; Reddy *et al.*, 2025).

During a survey on okra fields in Delhi, Tripura, and Kerala in 2022-2025, phytoplasma like symptoms were observed, prompting this study to identify the associated phytoplasma strains using molecular detection and sequence comparison analysis of the 16S rRNA gene.

Materials and Methods

Surveys were conducted during 2022-2025 in okra fields of three states of India located at Indian Agricultural Research Institute, Pusa experimental fields, New Delhi; Kottarakkara, Kollam district, Kerala and Shipahijala district, Tripura. Two asymptomatic and three symptomatic okra leaf samples were collected from each different farmers/experimental fields of the three states. The midribs from the leaves of symptomatic and asymptomatic plants were processed for DNA extraction by using Plant DNA Kit (Qiagen) following the manufacturer's instructions. The DNA was suspended in 100 µl of elution buffer and kept at -20°C. Before PCR assays DNA samples were quantified and diluted with nuclease free water to a final concentration of 20 ng/µl. The genomic DNA samples were assayed for amplification of a 16S rRNA gene region using phytoplasma specific primer pairs, P1/Tint (Deng and Hiruki, 1991; Smart *et al.*, 1996) followed by nested primer pair R16F2n/R2 (Gundersen and Lee, 1996). The amplified products were diluted at 1: 30 ratio with nuclease free water (Sisco Research

Laboratory, Mumbai, India) and 2 µl were used as template in nested PCR assays. DNA extracted from a sesame phyllody phytoplasma strain maintained in *Catharanthus roseus* in greenhouse (16SrI group; GenBank accession number KC920747) was used as positive control. PCR reactions were carried out in a thermal cycler (Mastercycler, Eppendorf, Germany).

The amplified 16S rRNA gene fragments from the symptomatic okra leaf samples, two per each different location, were purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, USA). The purified PCR products were then ligated into the pGEM®T vector (Promega, Madison, USA) and cloned into competent *Escherichia coli* (DH5-α) cells. The cloned products were outsourced for sequencing using the M13Fwd/M13Rev universal primer pair in both directions at Barcode Bioscience, Bengaluru, India. The resulting sequences of the 16S rRNA gene products from six okra strains (two each from the three states) were submitted to GenBank. The sequences obtained were compared with those of phytoplasmas strains in GenBank using the Basic Local Alignment Search Tool (BLASTn) system of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). Sequence assembly and construction of phylogeny were carried out using the software MEGA 12 (Kumar *et al.*, 2024) with 1,000 bootstrap replications. The sequence of *Acholeplasma laidlawii* was used as the outgroup to root the tree. The phylogeny was inferred using the Maximum Likelihood method and Tamura-Nei (1993) model of nucleotide substitutions. The initial tree for the heuristic search was selected by choosing the tree with the superior log-likelihood between a Neighbor-Joining tree (Saitou and Nei, 1987) and a Maximum Parsimony tree. The MP tree had the shortest length among 10 MP tree searches; each performed with a randomly generated starting tree.

Partial sequences from the 16S rRNA gene corresponding to the R16F2n/R2 fragments were subjected to *in silico* RFLP analyses with the *i*PhyClassifier tool and coefficient values were calculated (Zhao *et al.*, 2009).

Results and Discussion

Distinctive phytoplasma symptoms in okra, including little leaf, phyllody and apical shoot proliferation were observed at Farmer's field, Kottarakkara, Kollam district, Kerala (Figures 1a, b and c). Similar symptoms, such as vertical side shoot proliferation along with little leaf, were observed at IARI fields, New Delhi (Figures 1d and e), while bunchy crown with little leaf was the common symptoms observed at Shipahijala district of Tripura (Figures 1f and g). The symptoms were more severe at the harvesting stage of the crop in all the surveyed fields, resulting in a significant reduction in the yield of marketable fruits. Based on visual observations of symptomatic *versus* non-symptomatic okra plants in 10 x 10 meter plots (average of 5 plots/ field) the disease incidence rate ranged from 8 to 15% across the different surveyed locations.

The nested PCR amplification of the 16S rRNA gene yielded products of about 1.25 kb in all symptomatic okra samples and in the positive control. In contrast, no

Figure 1. Okra symptoms associated with phytoplasma presence a, b and c: little leaf, phyllody, and apical shoot proliferation at Kollam district, Kerala; d and e: vertical side shoot proliferation along with little leaf at IARI fields, New Delhi; f and g: bunchy crown with little leaves at Shipahijala, Tripura.
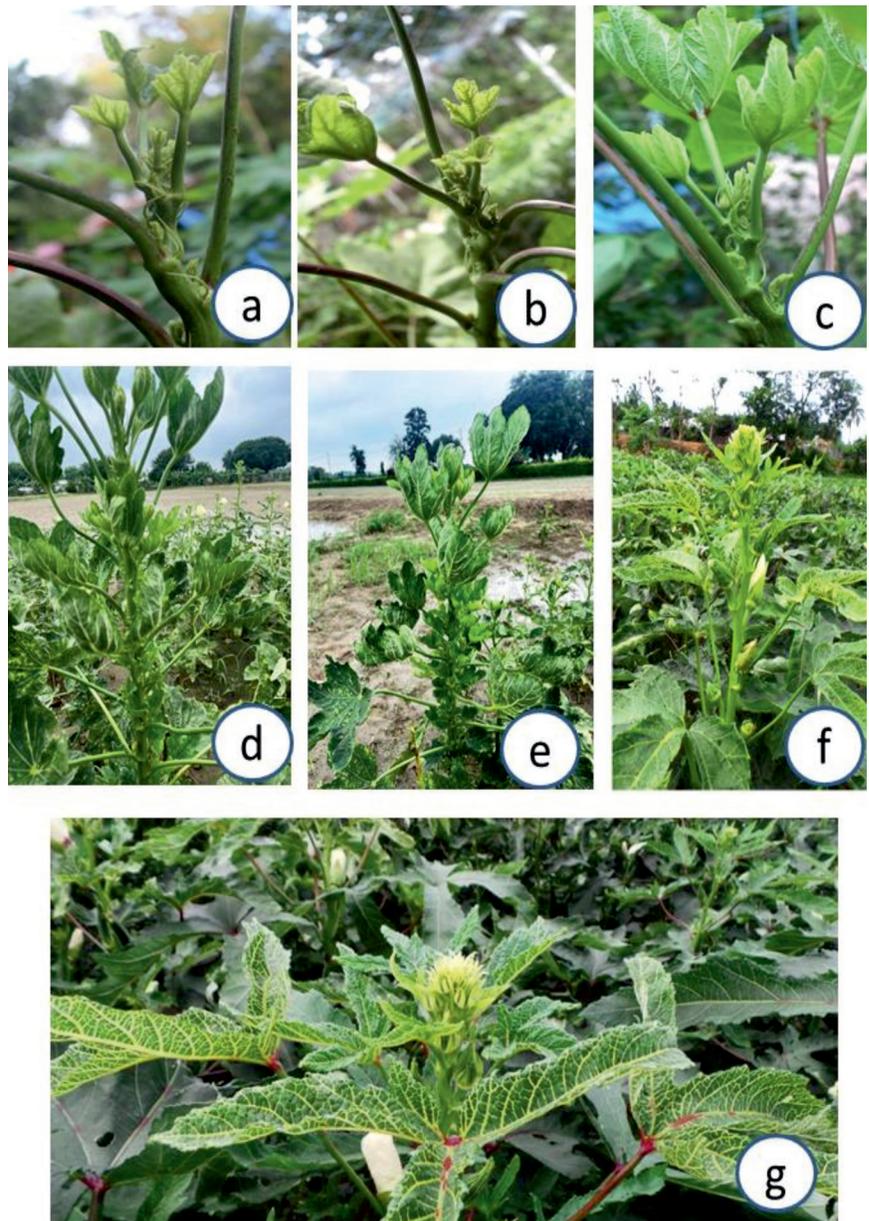
amplification was observed in nested PCR assays of asymptomatic okra samples collected from fields distant from the three locations where the disease was present (data not shown). Since there was 100% sequence identity among the 16S rRNA gene sequences of okra phytoplasma strains within each state, two okra sequences from each location were submitted to GenBank under the accession numbers reported in Figure 2. The BLAST analysis unveiled that the partial 16S rRNA gene sequences of the okra phytoplasma strains from plants showing the symptoms of shoot proliferation, little leaf and crown bunching, respectively, exhibited nucleotide identities ranging from 99.52% to 100% with 'Candidatus Phytoplasma asteris' strains. Pairwise sequence comparison of the 16S rRNA gene sequences from okra phytoplasma strains revealed 99.59%-99.83% identity among the okra strains from Kerala 1 and 2 (GenBank accession numbers PX249779 and PX271126), New Delhi-IARI 1 and 2 (GenBank accession numbers PX249811 and PX271135), and Tripura 1 and 2 (GenBank accession numbers PX271156 and PX271136). Each of the two okra phytoplasma sequences from the same locality showed 100% identity to each other, but only the two strains from Kerala showed 100% identity with 'Ca. P. asteris' from *Oenothera* from USA (GenBank accession number M30790) that is the reference strains for this 'Ca. Phytoplasma'. The phytoplasma strains from Tripura showed the presence of two GAPs at positions 307 and 330 when aligned with the 'Ca. P. asteris' reference strain (GenBank accession number M30790) and were 100%

identical to the sequence of a *Celosia* phytoplasma strain from Tripura (GenBank accession number MH523450), while the two strains from New Delhi – IARI showed the same two GAPs of the Tripura strains plus one G/A SNP at position 37 producing 100% identity with a oilseed rape phyllody phytoplasma from Poland (GenBank accession number HM561990).

Phylogenetic analysis results confirmed that the okra phytoplasma strains were clustering with 'Ca. P. asteris'-related strains from India and abroad (Figure 2). *In silico* RFLP analysis of the 16S rRNA gene sequences of okra strains confirmed identical restriction endonuclease profiles with 16SrI-B subgroup representative strain (GenBank accession number AP006628), yielding a similarity coefficient of 1.00 (Figure 3) for all the detected strains.

Phytoplasma diseases pose significant constraints to the production of economically important crops in India, with over 22 vegetable species reported as hosts for phytoplasmas affiliated to seven ribosomal groups (Rao, 2021; Kumari *et al.*, 2019, 2023). Various phytoplasmas have been identified associated with okra diseases worldwide. Specifically, phytoplasmas in the 16SrXII and 16SrV groups were identified in okra from Mauritius (Gungoosingh-Bunwaree *et al.*, 2011). Moreover, Kumar *et al.* (2012) and Reddy *et al.* (2025) reported the presence of 16SrI and 16SrII phytoplasma groups in okra with bunchy top, big bud and virescence.

Although various phytoplasma strains have been reported earlier as associated with okra diseases

Figure 2. Phylogenetic tree constructed by maximum likelihood method and Tamura-Nei (1993) model based on 16S rRNA gene sequences of okra phytoplasma strains with selected phytoplasma strains from GenBank. The okra strains are highlighted in red, green and blue according with their 100% identity to each other. Accession numbers are specified in the tree, *Acholeplasma laidlawii* was used as an outgroup. Numbers on branches are bootstrap values obtained for 1000 replicates.
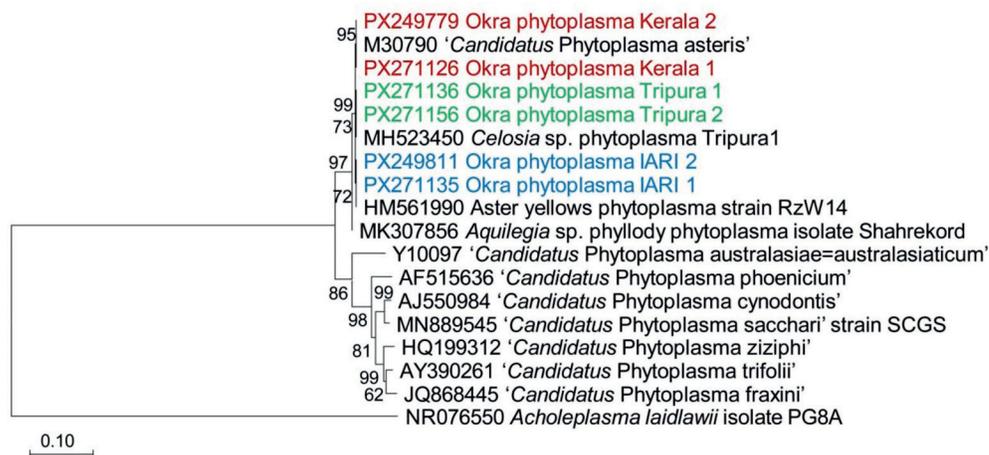
Figure 3. Comparison of virtual RFLP patterns generated from *in silico* digestion of 1.25 kb 16S rDNA sequences of (a) '*Candidatus* Phytoplasma asteris' (GenBank accession number AP006628), (b) phytoplasma from okra Kerala (GenBank accession number PX249779), (c) phytoplasma from okra IARI (GenBank accession number PX249811), (d) phytoplasma from okra Tripura (GenBank accession number PX271156), digested using 17 different endonucleases (*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I) confirmed that the okra phytoplasma strains belong to 16SrI-B subgroup.
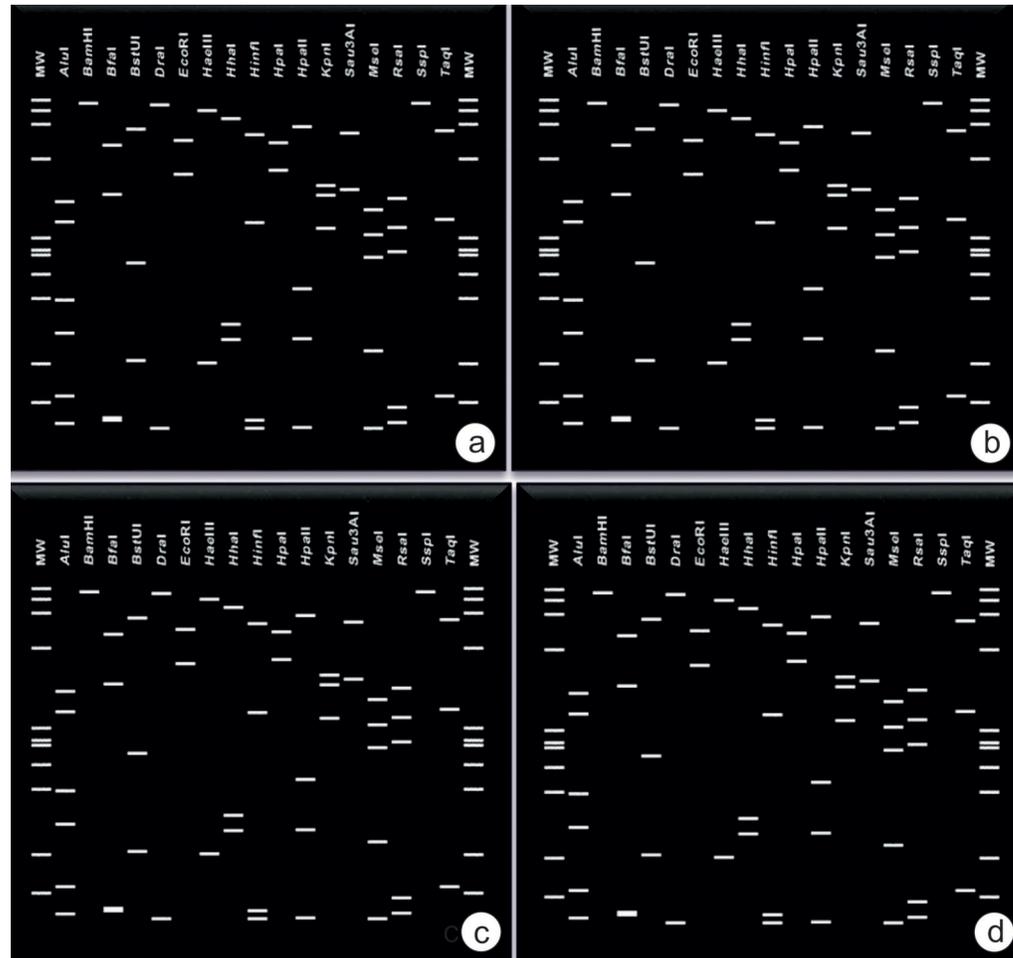
globally, this study revealed the occurrence of '*Ca*. P. asteris' strains (16SrI-B) infecting okra crop in three Indian states (Kerala, Tripura, and New Delhi), highlighting the epidemiological significance of the phytoplasma infections in this crop.

The 16SrI-B phytoplasma subgroup is known for its widespread distribution and ability to infect diverse crops, including vegetables, ornamentals, fruits, legumes, and oil crops also in India (Rao, 2021). Phytoplasmas in this subgroup were reported in several weed species and transmitted by identified leafhopper vectors in India (Rao, 2021, 2025). Considering the geographic overlap between this study and previous reports, it is possible to speculate that okra serves as a natural host facilitating the transmission of this phytoplasma strain especially considering that three possible lineages, distinguished by specific GAPs and SNPs were detected according with

the diverse localities surveyed. The detection of '*Ca*. P. asteris' in okra across three states warrants immediate attention from the scientific community and farmers to facilitate timely diagnosis and develop effective management strategies.

Moreover, considering okra's susceptibility to various begomovirus strains in India and worldwide (Davis *et al.*, 2024), the possibility of mixed infections with phytoplasmas cannot be ruled out. Further studies are necessary to investigate this potential co-infection and its impact on yield losses. To develop a comprehensive management strategy, additional research is required to survey, detect, and characterize novel phytoplasma strains in okra from diverse geographic locations in India, ultimately providing a clearer understanding of the genetic diversity of phytoplasmas associated with diseases of this crop.

References

Ali AM, Teshome A and Abebe D 2022. Nutritional composition and health benefits of okra: a review. *Ethiopian Journal of Health Science*, 32: 19–30.

Benti T, Getahun M and Admasu S 2017. Management of okra pests and diseases: a review. *Ethiopian Journal of Crop Science*, 25: 145–156.

Davis TW and Thompson AN 2024. Begomoviruses associated with okra yellow vein mosaic disease (OYVMD): diversity, transmission mechanism, and management strategies. *Molecular Horticulture*, 4(1): 36.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods*, 14: 53-61.

Dhakne V and Pansare U 2024. Production technology of okra. *Agrobios Newsletter*, 6(23): 12.

Elkhalifa AEO, Alshammari E, Adnan M, Alcantara JC, Awadelkareem AM, Eltoum NE, Mehmood K, Panda BP and Ashraf SA 2021. Okra (*Abelmoschus Esculentus*) as a potential dietary medicine with nutraceutical importance for sustainable health applications. *Molecules*, 26(3): 696.

Gundersen DE and Lee I-M 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, 35: 144–151.

Gungoosingh-Bunwaree A, Contaldo N, Paltrinieri S, Benimadhu SP and Bertaccini A 2011. Detection of phytoplasma infection in okra in Mauritius. *Bulletin of Insectology*, 64(Supplement): 103–104.

Jain N, Jain R, Jain V and Jain S 2012. A review on *Abelmoschus esculentus. Pharmacia*, 1: 84–89.

Kumar S, Singh V and Lakhanpaul S 2012. Molecular characterization and phylogeny of phytoplasma associated with bunchy top disease in its new host okra (*Abelmoschus esculentus*) in India reveal a novel lineage within the 16SrI group. *European Journal of Plant Pathology*, 133: 371-378.

Kumar S, Stecher G, Suleski M, Sanderford M, Sharma S and Tamura K 2024. MEGA12: molecular evolutionary genetic analysis version 12 for adaptive and green computing. *Molecular Biology Evolution*, 41(12): 263.

Kumari S, Nagendran K, Rai AB, Singh B, Rao GP and Bertaccini A 2019. Global status of phytoplasma diseases in vegetable crops. *Frontiers of Microbiology*, 10: 1349.

Kumari S, Sertkaya G, Krishnan N, Pandey KK, Singh J, Caglayan K, Rao GP, Bertaccini A and Tiwari, AK 2023. Update on phytoplasma diseases associated with vegetable crops in Asian countries. In: *Phytoplasma Diseases in Asian Countries. 2. Phytoplasma Diseases of Major Crops, Trees, and Weeds in Asia*; eds Tiwari AK, Caglayan K, Hoat TH, Al Subhi A, Nejat N and Reddy G, Academic Press, Elsevier, UK, 20-44.

Massrie KD 2025. Constraints and opportunities on okra (*Abelmoschus esculentus*) production in Ethiopia: a review. *Frontiers in Sustainable Food Systematics*, 9: 1546995.

Patra SK, Poddar R, Pramanik S, Bandopadhyay P, Gaber A and Hossain A 2023. Growth, yield water productivity and economics of okra (*Abelmoschus esculentus* L.) in response to gravity drip irrigation under mulch and without-mulch conditions. *Scientia Horticulturae*, 321(1): 112327.

Rao GP 2021. Our understanding about phytoplasma research scenario in India. *Indian Phytopathology*, 74: 371-401.

Rao GP 2025. Insect vector transmission of phytoplasmas and citrus greening bacteria in diverse agricultural crops of India. *Phytopathogenic Mollicutes*, 15(1): 37-38.

Reddy CNL, Muttappagol M, Madhu GS, Devaraju A and Venkataravanappa V 2025. Characterization of phytoplasma associated with big bud and virescence disease of okra in India. *Indian Phytopathology*, 78: 171–175.

Saitou N and Nei M 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4: 406-425.

Singh RP 2023. Managing okra diseases: current practices and future directions. *International Journal Plant Pathology*, 19: 234-246.

Smart CD, Schneider B, Blomquist CL, Guerra LJ, Harrison NA, Ahrens U and Kirkpatrick BC 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Applied Environment Microbiology*, 62(8): 2988–2993.

Tamura K and Nei M 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, 10: 512-526.

Wang R, Bai B, Li D, Wang J, Huang W, Wu Y and Zhao L 2024. Phytoplasma: a plant pathogen that cannot be ignored in agricultural production. Research progress and outlook. *Molecular Plant Pathology*, 25(2): e13437.

Zhao Y, Wei W, Lee I-M, Shao J, Suo X and Davis RE 2009. Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *International Journal of Systematics and Evolutionary Microbiology*, 59: 2582-2593.

Research Article

From field to lab: best practices for phytoplasma-infected sample storage and processing

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Abstract

Phytoplasmas represent a group of plant pathogens implicated in numerous diseases affecting a wide range of wild and cultivated plant species across the globe. They are prokaryotes that lack cell wall, and are localized in plant tissues with active phloem, such as immature shoots, midribs, and petioles of young leaves, making these organs ideal for sampling. Rapid and accurate phytoplasma detection, together with understanding their relationship with host plants, is crucial for managing phytoplasma-associated diseases. Most scientific articles emphasize the importance of rapidly processing phytoplasma-infected plant samples to avoid damaging effects of oxidation. However, dealing with a large number of samples and biological replicates, processing samples within a reasonable period is almost impossible. This motivated us to conduct experiments on long-term sample storage and the faster processing of large sample numbers. Additional difficulties were encountered during the sample processing, particularly when homogenizing the phloem of woody plants. Producing high-quality DNA is crucial for molecular studies, as is facilitating the processing of numerous samples. By combining a specific storage method for samples and using a dedicated grinding machine, it was possible to obtain phytoplasma DNA with a higher quality efficiently and extend the seasonal work throughout the year.

Keywords: apricot, woody plant, long-term storage, DNA extraction

Introduction

Phytoplasmas represent a group of plant pathogens implicated in numerous diseases affecting a wide range of wild and cultivated plant species across the globe. They are prokaryotes that lack cell wall, and are localized in plant tissues with active phloem, such as immature shoots, midribs, and petioles of young leaves, making these organs ideal for sampling.

In the case of woody plants, it is important to select the appropriate sampling time due to the seasonal changes in phytoplasma concentration (Jarausch *et al.*, 1999; Necas *et al.*, 2008). Phytoplasmas in the phloem tubes of the above-ground parts of woody plants perish during the annual decay of vascular tissues. During this period, phytoplasmas overwinter in the living phloem sap of the roots. Therefore, the pathogen can theoretically be detected in the roots. In spring, the newly formed phloem tissues are recolonized, and by late August or early September in the northern hemisphere, the tree becomes systemically infected. The most suitable phytoplasma detection periods are the summer and early autumn months (Maskova *et al.*, 2009).

Differences can be observed in the detection of phytoplasma presence in the above-ground woody parts of plants during the winter and early spring months. In stone fruits, the so-called secondary phloem tissues enable phytoplasmas to be detected throughout the winter. In spring, recolonization of the aerial parts of the tree occurs, followed by systemic colonization from July through late autumn (Marcone *et al.*, 2010). However, the lowest phytoplasma concentration is observed during the formation of new phloem tissues from March to May (Seemüller *et al.*, 1998). The results of Kiss *et al.* (2024) indicated that phytoplasma was detectable at high rates (above 90%) in annual shoots from August to December, and in one-year-old shoots from January to March and in May. In contrast, lower detection rates in April, June, and July suggest that these months are less reliable for phytoplasma detection. Conversely, secondary phloem elements are absent in apple trees, rendering phytoplasma infections rare during the winter or early spring (Schaper and Seemüller, 1982; Seemüller *et al.*, 1984).

The uneven distribution of phytoplasmas within plants is an important consideration when collecting samples. This is particularly true of woody plants, where the uneven distribution and low concentration of phytoplasmas are common phenomena (Errea *et al.*, 2002). Phytoplasma concentration can vary even within one shoot at the same sampling time (Kiss and Necas, 2022).

Since extracting DNA from phloem tissue is challenging, many researchers choose to use leaf petioles or even whole leaf blades for DNA extraction. However, this method only yields reliable results during specific periods of the year (May–July) (Green *et al.*, 1999; Mergenthaler, 2004). A key advantage of this approach is its enhanced practicality, as it simplifies handling and reduces the presence of inhibitors (Jones *et al.*, 2023), resulting in cleaner DNA extracts.

Although DNA-based detection techniques have significantly improved the reliable identification of phytoplasmas, purifying phytoplasma DNA from plant tissues - especially woody, fiber-rich tissues- poses numerous challenges. In problematic plant species, where phytoplasmas occur in extremely low concentrations. One solution is to transfer them to a host plant (*e.g.*, *Catharanthus roseus*) that is manageable and suitable for DNA extraction, using phytoplasma-free dodder. However, this method is not suitable for routine diagnosis and often proves to be unreliable, yields misleading results.

An alternative solution for phytoplasma detection is to prepare phloem tissues, but this is time-consuming, labor-intensive and is not feasible with simple collection systems (*e.g.*, single-use syringes). Phytoplasma DNA enrichment (and thus the DNA content enrichment) can be achieved through differential centrifugation using sucrose gradients (Lee *et al.*, 1993; Neimark and Kirkpatrick, 1993), or by separating the thymine/adenine-rich phytoplasma DNA through cesium chloride-bisbenzimidazole gradients (Sears *et al.*, 1989; Kollar and Seemüller, 1989). However, these methods are also time-consuming, and require significant resources, making them impractical for large sample numbers.

DNA extracts from woody plants often contain various inhibitors (such as polyphenols and polysaccharides) that can inhibit PCR amplification. The absence of phytoplasma-specific amplification products does not necessarily indicate that the sample analyzed was free from phytoplasmas. Several DNA extraction methods have been developed for problematic plant species or parts (*e.g.*, roots or bark), to improve the reliability of phytoplasma detection (Gibb and Padovan, 1994; Zhang *et al.*, 1998; Green *et al.*, 1999).

In Hungary, '*Candidatus* Phytoplasma prunorum' is associated with severe diseases and significant economic losses in apricot orchards. In light of this, significant effort was made to control the disease and to develop methods that facilitate laboratory detection work.

Materials and Methods

Samples were collected from five important apricot-growing areas of Hungary: Somogytúr, Balatonvilágos, Érd, Domoszló and Boldogkőváralja. Table 1 presents the examined locations, number of trees, varieties, years, and examination frequency.

Table 1. Number of apricot trees annually monitored and tested for '*Candidatus* Phytoplasma prunorum' presence from different growing areas of Hungary.

Location	Number of apricot trees	Apricot variety	Years
Érd	250	Ladycot	2020–2023
Domoszló	200	Pinkcot	2020–2023
Boldogkőváralja	100	Magyar kajszi	2020–2023
Somogytúr	200	Bergarouge	2020–2021
Balatonvilágos	200	Perlecot, Sunnycot, Tomcot	2020–2021

To compare the effects of a developed long-term freeze storage and homogenization method on the quantity and quality of extracted DNA, it was processed a total of 60 samples using three different protocols. For each method, twenty samples were processed, consisting of randomly chosen four samples from each of the five regions. Samples 1-4 were originated from Érd, samples 5-8 from Domoszló, samples 9-12 from Boldogkőváralja, samples 13-16 from Somogytúr and samples 17-20 from Balatonvilágos. The studies were conducted in 2020. In all cases, it was applied the method described by Ahrens and Seemüller (1992) to extract the DNA using the below listed methods.

Method 1: samples were processed involving manual homogenization of fresh material in a mortar and pestle; method 2: samples were flash-frozen in liquid nitrogen and then manually homogenized in a pre-chilled mortar and pestle with additional liquid nitrogen; method 3: the frozen samples were homogenized using a TissueLyser device.

Long-term storage of petiole and phloem samples

The leaf petioles were placed in 2 ml sample storage tubes (Eppendorf) and kept on ice until the end of the fieldwork. By the evening of the collection day at the latest, the samples were transferred to a -70°C freezer, where they remained until processing (in some cases for up to a year).

To process phloem samples, branch or root segments that were approximately 0.5-1 cm in diameter and 20 cm in length were collected. These were placed in bags and stored on ice until the end of the collection period. No later than the following day, these samples were transferred to a refrigerator at 4°C, where they were kept until phloem preparation. This storage period could extend to 10 days in some instances.

The phloem prepared was immersed in liquid nitrogen and placed in Falcon tubes, which were also immersed in liquid nitrogen. The samples were then transferred to a -70°C freezer, where they remained until processing (for up to a year in some cases).

Sample preparation and homogenization using Qiagen TissueLyser

To homogenize plant tissues and disrupt cell structures, the Qiagen TissueLyser II (Model: SN) tissue grinding apparatus was used. The homogenization process involves the following steps:

1. Equipment preparation: The 50 ml stainless steel grinding jars supplied with the device along with their corresponding 20 mm diameter stainless steel balls were immersed in liquid nitrogen. The tools were kept in the cryogenic fluid until complete temperature equilibration was achieved.

2. Sample preparation: The plant samples to be analyzed were placed in liquid nitrogen immediately prior to processing. This step ensures the tissues freeze rapidly, preventing cells and, specifically, DNA molecule degradation.

3. Homogenization: The pre-cooled grinding jars were removed from the liquid nitrogen, and the frozen plant sample was placed inside immediately. The jar was then placed in the TissueLyser II apparatus, and the homogenization process was initiated.

Homogenization was performed on frozen samples using a TissueLyser device using the homogenization parameters listed in Table 2. This protocol effectively disrupts plant tissues while minimizing heat-induced molecular degradation. The process results in a fine powder homogenate that is suitable for further molecular analysis.

Phytoplasma detection was then performed by PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996) universal primers, as well as specific fECA1/rECA2 primers (Jarausch *et al.*, 1998). Thermocycler conditions consisted of 98°C for 2 minutes, 30 cycles at 98°C for

Table 2. Specific settings for the Qiagen TissueLyser II. Model: SN machine and the usability of samples obtained with these settings

Settings	Time (seconds)	Frequency (Hz)	Sample consistency	Usability for DNA extraction
1	15	20	Homogeneous fine powder	Phytoplasma cells likely ruptured, floating during extraction
2	15	15	Homogeneous fine powder	DNA extraction worked perfectly
3	15	10	Coarse	Unsuitable
4	10	15	Rough powder	Unsuitable

30 seconds, 55°C, 60°C or 50.2°C (P1/P7, R16F2n/R16R2 and fECA1/rECA2 respectively) for 30 seconds and 72°C for 1 minute, followed by a final extension of 72°C for 10 minutes with KAPA2G Robust HotStart PCR Kit (Merck KGaA, Darmstadt, Germany).

Results and Discussion

Long-term sample storage and DNA extraction optimization

Over the past decade, over 3000 samples have been processed, providing substantial empirical evidence for the applied methodologies. Storage at -70°C is suitable for the long-term preservation of petiole and phloem samples, with DNA quality remaining unaffected for up to one year.

Storage and extraction methods

Following storage at -70°C, traditional DNA extraction methods were compared (using mortar and pestle) with the TissueLyser-based extraction method. Both approaches yielded high quality DNA, demonstrating the efficacy of the storage protocol.

TissueLyser protocol optimization

As mentioned above, it was evaluated four distinct settings on the TissueLyser apparatus for grinding frozen samples: 1. 15 seconds at 20 Hz, 2. 15 seconds at 15 Hz, 3. 15 seconds at 10 Hz and 4. 10 seconds at 15 Hz (Table 2).

Settings 3 and 4 resulted in coarse, inadequately homogenized samples that were unsuitable for DNA extraction. In contrast, settings 1 and 2 produced fine powder that was suitable for further processing.

A comprehensive DNA extraction was conducted using both extraction methods on samples ground with all settings. However, when setting 1 was used (15 seconds at 20 Hz), a significant issue arose during centrifugation: the pellet failed to adhere to the bottom of the centrifuge tube and instead floated, resulting in substantial sample loss. This problem was particularly critical given the low concentration of phytoplasmas in samples.

The most effective protocol identified involved grinding the samples for 15 seconds at a frequency of 15 Hz (setting 2). This approach had no drawbacks and significantly enhanced the efficiency of tissue disruption. Importantly, this method yielded a higher quantity of phytoplasma-enriched DNA of sufficient purity for molecular analyses than the other tested protocols (Table 2.). This optimized protocol ensures the efficient disruption of plant tissue while minimizing heat-induced molecular degradation. The result is high-quality DNA that is suitable for subsequent molecular analyses, particularly for the detection and identification of phytoplasmas.

Following DNA extraction, it was used a Maestrogen MN-913 spectrophotometer device (Maestrogen, Hsinchu city, Taiwan) to measure the concentration, degradation and purity of the obtained DNA. Starting from 1 g of plant material, it was possible to extract 120-200 ng/µl of DNA from as little as 100-150 mg of plant powder. The pellet was then resuspended in 100 µl of nuclease-free water. Unlike traditional extraction methods, which required 1 g of plant material for DNA extraction, the above method proved sufficient for extracting the same amount of DNA using only 100–150 mg of sample (Figure 1). This improvement is attributed to the efficient pulverization process and the reduction in DNA degradation.

Conclusions

Based on processing approximately 3000 samples it is possible to confidently assert that storage at -70°C is suitable for preserving apricot petiole and phloem samples long-term for up to one year without

Figure 1. DNA concentration results from different DNA extraction methods. Method 1: conventional method, the DNA pellet obtained from 1 g of plant material was resuspended in 100 μl of nuclease-free water. Method 2: combined extraction method involving a frozen sample and manual homogenization. The DNA pellet from 1 g of plant material was resuspended in 100 μl of nuclease-free water. Method 3: frozen sample and homogenization using a TissueLyser device, the DNA pellet from 1 g of plant material was resuspended in 100 μl of nuclease-free water. Samples 1-4 were originated from Érd, samples 5-8 from Domoszló, samples 9-12 from Boldogkőváralja, samples 13-16 from Somogytúr and samples 17-20 from Balatonvilágos.
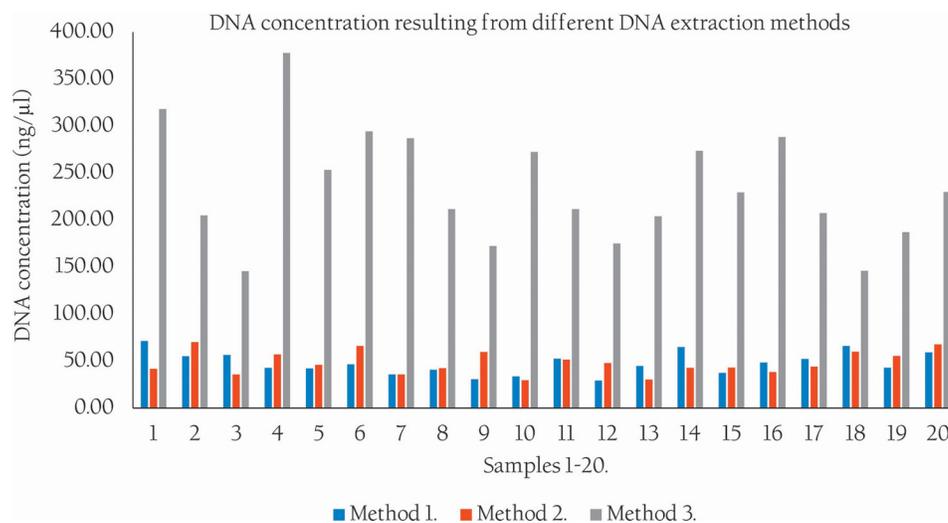
compromising the quality or quantity of extracted DNA. The simultaneous collection of samples, which inevitably leads to accumulation was required from the testing performed. Processing massive quantities of samples within a short period has always presented a significant challenge. Storing samples at -70°C has provided a solution to this problem.

The sample cryogenic storage i) preserves DNA quality and quantity for up to one year; ii) allows batch processing of samples collected simultaneously and iii) provides flexibility in sample processing timelines.

The TissueLyser apparatus used for grinding frozen samples offers substantial advantages when processing numerous samples within a short period and extracting larger quantities of high-quality DNA. The fine powder obtained through this method offers several benefits such as: i) rapid processing of multiple samples; ii) higher quantity and quality of extracted DNA and iii) space-efficient storage, the small volume of the resulting powder enables samples, including backup aliquots, to be stored in 2 ml tubes, thus conserving freezer space at -70°C.

This approach ensures that potentially valuable samples remain available for future analysis and allows the storage of larger quantities when necessary. Combining cryogenic storage with TissueLyser-based sample processing significantly enhances the capacity for high numbers sample management and DNA extraction in phytoplasma research.

References

Ahrens U and Seemüller E 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies sequence of the 16S rRNA gene. *Phytopathology*, 82: 828-832.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods*, 14: 53-61.

Errea P, Aguelo V and Hormaza JI 2002. Seasonal variations in detection and transmission of pear decline phytoplasma. *Journal of Phytopathology*, 150: 439-443.

Gibb KS and Padovan AC 1994. A DNA method that allows reliable PCR amplification of MLO DNA from difficult plant host species. *PCR Methods and Applications*, 4(1): 56–58.

Green MJ, Thompson DA and MacKenzie DJ 1999. Easy and efficient DNA extraction from woody plants for the detection of phytoplasmas by polymerase chain reaction. *Plant Disease*, 83(5): 482–485.

Gundersen DE and Lee I-M 1996. Ultrasensitive detection of phytoplasmas by nested PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, 35: 144-151.

Jarausch W, Lansac M, Saillard C, Broquaire J-M and Dosba F 1998. PCR assay for specific detection of European stone fruit yellows phytoplasmas and its use for epidemiological studies in France. *European Journal of Plant Pathology*, 104: 12-27.

Jarausch W, Lansac M and Dosba F 1999. Seasonal colonization pattern of European stone fruit yellows phytoplasmas in different Prunus species detected by specific PCR. *Journal of Phytopathology*, 147(1): 47–54.

Jones MM, Nagalingum NS and Handley VM 2023. Testing protocols to optimize DNA extraction from tough leaf tissue: a case study in *Encephalartos*. *Applied Plant Science*, 11: 11525.

Kiss T and Necas T 2022. Quantification of 'Candidatus Phytoplasma prunorum' in apricot trees exhibiting uneven European stone fruit yellows symptoms. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 70(1): 31–36.

Kiss T, Trempetic G, Morvay P and Necas T 2024. Analysis of ÈCandidatus Phytoplasma prunorumÈ titer in the tissues of apricot trees (*Prunus armeniaca* L.) during the year. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 72(5-6): 177-182.

Kollar A and Seemüller E 1989. Base composition of the DNA of mycoplasmalike organisms associated with various plant diseases. *Journal of Phytopathology*, 127(2): 177-186.

Lee I-M, Hammond RW, Davis RE and Gundersen DE 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology*, 83(8): 834-842.

Marcone C, Jarausch B and Jarausch W 2010. 'Candidatus Phytoplasma prunorum', the causal agent of European stone fruit yellows: an overview. *Journal of Plant Pathology*, 92(1): 19-34.

Masková V, Krska B and Necas T 2009. Comparison of methods of isolating DNA for ESFY phytoplasma detection. *Acta Horticulturae*, 825: 213-220.

Mergenthaler E 2004. Phytoplasma diseases in Hungary: development of advanced diagnostic methods. Ph. D. Thesis.

Necas T, Krska B, Maskova V, Michal A 2008. Influence of the time of sampling on detection of ESFY phytoplasma. *Acta Horticulturae*, 781: 435–441.

Neimark H and Kirkpatrick BC 1993. Isolation and characterization of full length chromosomes from non culturable plant pathogenic mycoplasma like organisms. *Molecular Microbiology*, 7: 21-28.

Sears BB, Li PO, Holland N, Kirkpatrick BC and Klomparens KL 1989. Isolation and characterization of DNA from a mycoplasmalike organism. *Molecular Plant-Microbe Interactions*, 2(4): 175-180.

Schaper U and Seemüller E 1982. Condition of the phloem and the persistence of mycoplasma-like organisms associated with apple proliferation and pear decline. *Phytopathology*, 72: 736-742.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC 1995. Phylogenetic classification of plant pathogenic mycoplasma like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology*. Eds S Razin and JG Tully, Academic Press, San Diego, California, USA, 369-380.

Seemüller E, Schaper U and Zimbelmann F 1984. Seasonal variation in the colonization patterns of mycoplasmalike organisms associated with apple proliferation and pear decline. *Pflanzenschutz*, 91: 371.

Seemüller E, Stolz H and Kison H 1998. Persistence of the European stone fruit yellows phytoplasma and areal parts of *Prunus* taxa during dormant season. *Journal of Phytopathology*, 146: 407-410.

Zhang YP, Uyemoto JK and Kirkpatrick BC 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. *Journal of Virological Methods*, 71(1): 45–50.

Research Article

First report of '*Candidatus* Phytoplasma asteris' strains associated with phyllody of rudbeckia in Türkiye

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Abstract

During field surveys conducted in 2024, *Rudbeckia* species plants (Asteraceae) exhibiting symptoms of phyllody, virescence, axillary shoot proliferation, and stunting were observed in public gardens in Istanbul, Türkiye. To investigate possible phytoplasma presence, symptomatic plants were analyzed by polymerase chain reaction (PCR) using universal primers targeting ribosomal RNA gene. All symptomatic samples tested positive. PCR with primer pair P1/P7, followed by nested PCR with R16F2n/R16R2, consistently yielded amplicons of the expected size (1.25 kb). Sanger sequencing of the 16S rRNA gene and BLASTn analysis against the NCBI database revealed 99.30–99.44% sequence identity with '*Candidatus* Phytoplasma asteris' and other members of the aster yellows group (16SrI). Phylogenetic analysis further confirmed that the sequences clustered within phytoplasma strains enclosed in subgroup 16SrI-B. This study provides the first evidence of phytoplasma infection in *Rudbeckia* species in Türkiye and expands the documented host range of phytoplasmas in this region of Türkiye.

Keywords: phytoplasma, aster yellows, 16SrI group, ornamental plants

Introduction

Phytoplasmas are wall-less, phloem-restricted *Mollicutes* that are transmitted primarily by phloem-feeding insects, particularly leafhoppers and planthoppers. They are responsible for numerous plant diseases worldwide, characterized by symptoms such as witches' broom, virescence, phyllody, flower malformations, and stunting (Bertaccini and Duduk, 2009). The 16SrI (aster yellows) group represents one of the most widespread phytoplasma groups, infecting hundreds of plant species across diverse families (Lee *et al.*, 2004). Ornamental plants often serve as reservoirs of phytoplasmas, facilitating pathogen persistence and transmission in urban and peri-urban environments. *Rudbeckia* species (commonly known as black-eyed Susan) are perennial ornamentals of the family Asteraceae, widely cultivated in Türkiye for landscaping. Despite global reports of phytoplasma diseases in ornamentals such as chrysanthemum, petunia, and periwinkle (Marcone, 2014) no evidence has been reported in *Rudbeckia* from Türkiye (Caglayan, 2023). The aim of this study was to document and molecularly identify the phytoplasmas infecting symptomatic rudbeckia plants in Istanbul, Türkiye.

Materials and Methods

During spring and summer 2024, rudbeckia plants showing phytoplasma-like symptoms were observed in two public garden sites (Fenerbahce and Bostanc Public Gardens) in Istanbul, Türkiye. Symptoms included phyllody (conversion of flower organs to leaf-like structures), virescence (greening of flower tissues), stunting, and proliferation of axillary shoots. Seven symptomatic plants were sampled along with three asymptomatic plants from the same locations, stored in cold box and transported to the laboratory. Total nucleic acids were extracted from midribs and young stems by a CTAB-based protocol using a CTAB extraction buffer with slightly modified composition [1% cetyltrimethylammonium bromide, 1 M Tris–HCl pH 8.0, 10 mM EDTA, 1.4 M NaCl, 1.2% polyvinylpyrrolidone, and 0.1% (v/v) 2-mercaptoethanol]. Approximately 50 mg of tissue was ground in 1 ml of 1% CTAB buffer supplemented with 30 µl of proteinase K (20 mg/ml). The mixture was briefly vortexed and incubated for 2 h at 65°C. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was then added, vortexed, and centrifuged at 13,000 g for 15 minutes. The aqueous phase was recovered, and DNA was precipitated with an equal volume of cold isopropanol. The sample was mixed by gentle inversion and incubated at –80°C for 15 min, followed by centrifugation at 13,000 g for 20 min at 4°C to recover the precipitate. The resulting pellet was washed with 70% ethanol, air-dried, and dissolved in 40 µl of nuclease-free water.

Initial amplification of phytoplasma DNA employed the universal phytoplasma primers P1/P7 targeting a region enclosing the 16S rRNA operon (Deng and Hiruki, 1991; Schneider et al., 1995). Nested PCR was performed using R16F2n/R16R2 primers to increase sensitivity (Gundersen and Lee, 1996). Reaction conditions followed reported protocols for phytoplasma detection (Boztas et al., 2024). Nested PCR products from selected samples were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel, Duren, Germany) and sequenced bidirectionally using R16F2n/R16R2 primers by Sanger method (Macrogen Europe, Amsterdam, Netherlands).

Consensus sequences were assembled using BioEdit v7.7. BLASTn searches were performed against NCBI GenBank to identify closest phytoplasma relatives. Multiple alignments were prepared with MUSCLE, and phylogenetic trees were constructed using the neighbour-joining method in MEGA 11 with default values and 1,000 replicates for bootstrap analysis (Tamura et al., 2021). Acholeplasma laidlawii PG-8A strain was used as the out-group to root the tree. Virtual restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA sequences was conducted using iPhyClassifier to assign ribosomal subgroup (Zhao et al., 2009).

Results

Symptomatic rudbeckia plants displayed stunting, virescence, phyllody, and excessive branching (Figure 1). Healthy plants appeared normal with bright yellow flowers. PCR yielded faint bands in some samples, while nested PCR consistently amplified ˜1.2 kb fragments from positive samples. All the 7 symptomatic plants tested positive for phytoplasma after nested PCR, while the 3 asymptomatic plants were negative. BLASTn analysis of the two sequenced amplicons (˜1,250 bp) revealed 99.30% and 99.44% identity for the Rudbekia 3 and 4 phytoplasma strains, respectively with members of the 16SrI (aster yellows)

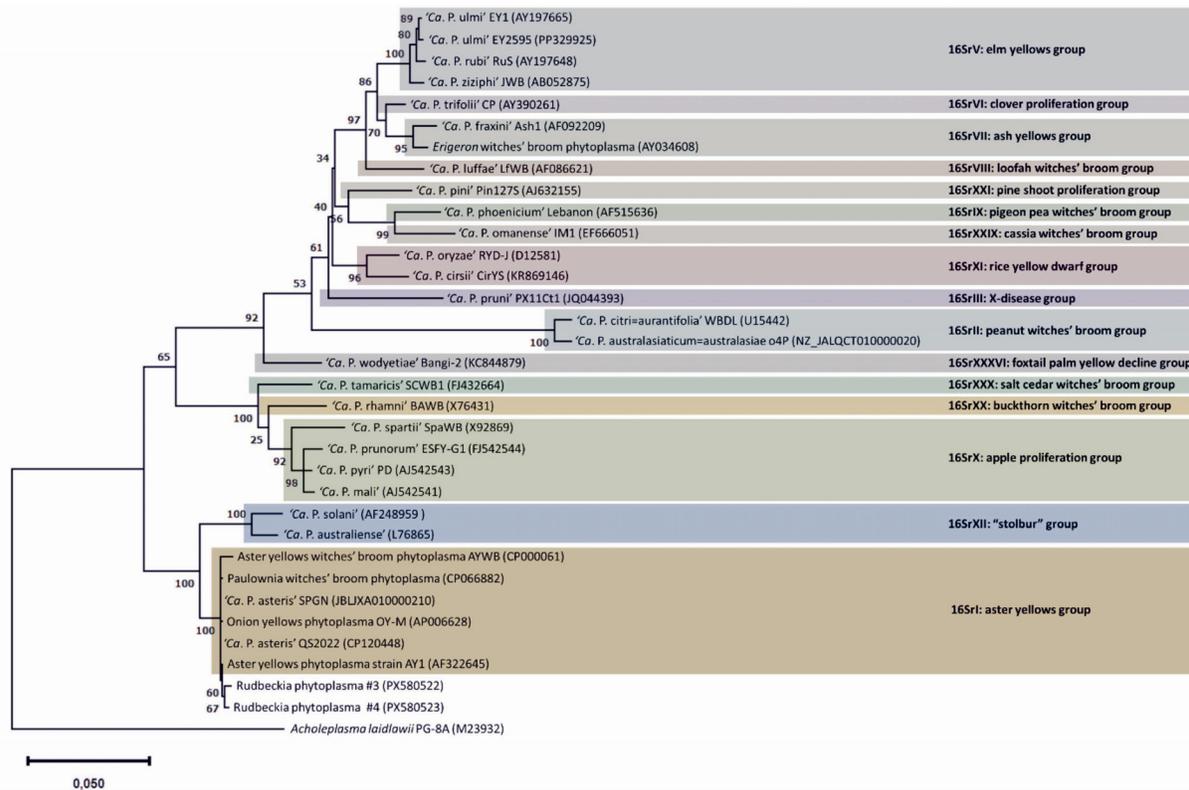**Figure 1.** Symptoms of phytoplasma presence in rudbeckia plants.

Figure 2. Phylogenetic tree constructed by maximum likelihood method of two representative 16S rRNA gene sequences from the phytoplasmas infecting *Rudbeckia* #3, and #4 samples and selected reference phytoplasmas from the GenBank database. *Acholeplasma laidlawii* PG-8A was used as the outgroup.

group. Phylogenetic analysis grouped the *Rudbeckia* sequences with 'Candidatus Phytoplasma asteris' (16SrI-B) with a strong bootstrap support (Figure 2). Virtual RFLP analysis confirmed that the sequences corresponded to those of phytoplasmas enclosed in the 16SrI-B subgroup since they produced identical profiles with a coefficient of identity of 1 with the 'Ca. P. asteris' reference strain.

Discussion and Conclusion

This study provides the first molecular evidence of phytoplasma infection in *Rudbeckia* species in Türkiye. The observed symptoms are consistent with those observed as associated with phytoplasma-associated diseases reported in other ornamentals. Detection of 16SrI-B subgroup phytoplasma is epidemiologically significant, as this group is widespread, infecting diverse plant families, and is transmitted by polyphagous leafhoppers. Urban and peri-urban landscapes provide continuous vegetation cover and

a stable environment that favors insect vector populations such as leafhoppers (Cicadellidae) and planthoppers (Fulgoromorpha), which are known to transmit 16SrI group phytoplasmas (Weintraub and Beanland, 2006). Once infected, these polyphagous vectors can move between ornamental hosts and spontaneous weeds. Thus, phytoplasma-infected rudbeckia plants located in public gardens could contribute to the spread of aster yellows phytoplasmas into the nearby fields and environments. Similar epidemiological roles have been proposed for other ornamentals such as *Petunia hybrida*, *Chrysanthemum morifolium*, and *Catharanthus roseus* that were reported to harbor aster yellows or related phytoplasmas in both Europe and North America (Galetto *et al.*, 2011; Himeno *et al.*, 2011; Marcone and Ragozzino, 1995). These species were shown to support both phytoplasma multiplication and acquisition by insect vectors, serving as bridge hosts between natural vegetation and cultivated plants. The presence of

phytoplasma in *Rudbeckia* species in Türkiye likely represents a comparable scenario, emphasizing the need for insect vector surveillance and host–vector–pathogen interaction studies also in the garden areas. While this study relied on 16S rRNA gene analysis, further genomic characterization (*e.g.*, multilocus sequence typing, whole genome sequencing) would be valuable for more precise epidemiological tracing. These findings highlight the importance of monitoring ornamental plants in epidemiological surveys and encourage further studies on insect vector populations and plant species host range also in Türkiye.

Acknowledgements

References

Bertaccini A and Duduk B 2009. Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopathologia mediterranea*, 48(3): 355-378.

Boztas K, Derecik K, Caglayan K and Tulum I 2024. Molecular detection and characterization of 'Candidatus Phytoplasma mali' associated with phyllody on *Narcissus tazetta* in Türkiye. *Journal of Crop Health*, 76(3): 683-691.

Caglayan K 2023. Diversity, distribution, and status of phytoplasmas diseases in Turkey. In: *Phytoplasma Diseases in Asian Countries. 1. Diversity, Distribution, and Current Status*. Eds AK Tiwari, K Caglayan, AM Al-Sadi, M Azadvar and S Abeysinghe, Academic Press, Elsevier, UK, 249-267.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods*, 14(1): 53-61.

Galetto L, Bosco D, Balestrini R, Genre A, Fletcher J and Marzachì C 2011. The major antigenic membrane protein of 'Candidatus Phytoplasma asteris' selectively interacts with ATP synthase and actin of leafhopper vectors. *PLoS One*, 6(7): e22571.

Gundersen DE and Lee I-M 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, 35: 144–151.

Himeno M, Neriya Y, Minato N, Miura C, Sugawara K, Ishii Y, Yamaji Y, Kakizawa S, Oshima K and Namba S 2011. Unique morphological changes in plant pathogenic phytoplasma infected petunia flowers are related to transcriptional regulation of floral homeotic genes in an organ specific manner. *The Plant Journal*, 67(6): 971-979.

Lee I-M, Gundersen-Rindal D, Davis RE, Bottner K, Marcone C and Seemüller E 2004. 'Candidatus Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *International Journal of Systematic and Evolutionary Microbiology*, 54(4): 1037-1048.

Marcone C 2014. Molecular biology and pathogenicity of phytoplasmas. *Annals of Applied Biology*, 165(2): 199-221.

Marcone C and Ragozzino A 1995. Natural occurence of aster yellows phytoplasmas on periwinkle in Southern Italy and their molecular characterization. *Rivista di Patologia Vegetale*, 5(2): 49-59.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC 1995. Phylogenetic classification of plant pathogenic mycoplasma like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology*. Eds S Razin and JG Tully San Diego, CA: Academic Press, 369-380.

Tamura K, Stecher G and Kumar S 2021. MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, 38(7): 3022-3027.

Weintraub PG and Beanland L 2006. Insect vectors of phytoplasmas. *Annual Revue of Entomology*, 51, 91-111.

Zhao Y, Wei W, Lee I-M, Shao J, Suo X and Davis RE 2009. Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *International Journal of Systematic and Evolutionary Microbiology*, 59(10): 2582-2593.

Research Article

Effect of phytoplasma presence on the biochemical profile of *Cicer arietinum*

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Abstract

Phytoplasmas are wall-less and pleomorphic bacterial pathogens that cause considerable yield losses in more than 1,000 plant species worldwide. They are associated with the presence of different symptoms including witches' broom, phyllody, virescence, yellowing. The aim of the present work was to analyze the changes in protein contents, phenylalanine ammonia-lyase (PAL), peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO) in field-grown chickpea plants (*Cicer arietinum*) in the presence of phytoplasma infection. The activities of PAL, PPO and POX were observed to increase in the infected plants compared with healthy plants. The amount of total soluble proteins, chlorophyll content and catalase activity was significantly higher in healthy leaves. In conclusion the observed alterations could be considered as the response and the adaptation of chickpea plants in the presence of phytoplasmas.

Keywords: chickpea, phytoplasma, phyllody, biochemical changes

Introduction

Chickpea (*Cicer arietinum* L.) is an important, cool-season legume in central India and is commonly referred to as the "king of pulses". It is a rich source of good quality protein with the ability to sustain soil fertility when included in different cropping systems. It is mostly grown under rain fed conditions in arid and semi-arid areas around the world (Millan *et al.*, 2006). The major producers of chickpea are India, Myanmar, Pakistan and Turkey. In India it covers an area of 9.94 million hectares with an average annual production of 11.5 million tons. More than 50 diseases and 54 insect pests have been reported to date affecting chickpea in different parts of the world (Chen *et al.*, 2011). The situation is further aggravated due to the recent presence of prevalent infections of 16SrII-D ('*Candidatus* Phytoplasma australasiaticum') phytoplasmas associated with chickpea phyllody

disease in various parts of India (Akram *et al.*, 2016; Reddy *et al.*, 2021).

Phytoplasmas are wall-less and pleomorphic bacterial pathogens that cause considerable yield losses in more than 1,000 plant species worldwide and transmitted by phloem feeding insects, mostly leafhoppers (Weintraub and Beanland, 2006). A wide range of symptoms are associated with the presence of phytoplasmas in the diseased plants and may vary depending on the strain, host, time of infection, age of the plant and environmental conditions (Bertaccini and Lee, 2018). The major symptoms observed were flower virescence, phyllody and extensive proliferation of branches. At the time of crop maturity when the healthy plants are drying, the diseased plants in the field remain green. All these morphological changes affecting phenotypical behaviour are closely related to biochemical alterations in infected plants. One of the earliest responses of plants to pathogen infections is the intensification of the synthesis of reactive oxygen species (ROS). One of the main reasons for the formation of ROS, including hydrogen peroxide (H_2O_2) was found to be the increasing intensity of photorespiration during pathogenesis. The H_2O_2 is the most stable compound among ROS, and it plays a signaling role in plant responses to stress (van Breusegem *et al.*, 2008). Phytoplasmas harm infected plants by affecting various physio-biochemical and metabolic processes and altering gene expression, with the biosynthesis and accumulation of carbohydrates being the most highly affected processes. Phytoplasma-induced symptoms also include stomatal closure, photosynthetic impairment due to declining leaf area, and photosynthetic pigments, leading to limited transportation of photo-assimilates to sink organs, causing yield and quality weakness. One of the main effects of phytoplasma infection is the decrease in plant productivity (Bertaccini and Duduk, 2009).

The occurrence of phyllody disease of chickpea is a new entrance to the plant disease scenario in India where it was firstly reported from Coimbatore in Tamil Nadu, during 1959 (Siddique *et al.*, 2014). This syndrome has been observed in Ethiopia, Myanmar, Australia, Oman and Pakistan (Akhtar *et al.*, 2009).

The phytoplasma presence was confirmed by molecular techniques using PCR and sequence information's. The aim of the present work was to investigate the alterations in various biochemical activities in field-grown chickpea plants infected with '*Ca.* P. australasiaticum'.

Materials and Methods

Plant materials

The chickpea leaves used in this study were collected from field-grown plants with symptoms of phytoplasma infection. Symptomless plant leaves were also used as control. A total of 30 samples were utilized, comprising 15 from asymptomatic and 15 from symptomatic plants. No symptoms induced by other pathogens were observed on the plants from which these samples were taken. Biochemical analyses were performed in three replications for each treatment group (diseased and symptomless plants).

Detection and identification of phytoplasmas

DNA was extracted from 1 g of fresh leaf midribs collected from both symptomatic and asymptomatic (negative control) plants using a CTAB method (Maixner *et al.*, 1995). The extracted DNA concentration was assessed using a nanoDrop device (Thermo Fisher Scientific, USA). DNA samples were analyzed using nested PCR amplifying the 16S rRNA gene with universal phytoplasma primers P1/P7 and R16F2n/R16R2 (Deng and Hiruki 1991, Schneider *et al.*, 1995, Gundersen and Lee, 1996). The reaction was performed in solutions (25 µl total volume) containing 100 ng of nucleic acid, 2.5 µl of 1X PCR Buffer (HiMedia, Einhausen, Germany), 0.5 µl of 2.5 mM dNTP mix (Thermo Fisher Scientific, USA), 1 µl of each primer (Eurofins Genomics, Bangalore, India), 0.2 µl of Taq DNA polymerase (Thermo Fisher Scientific, USA). The following conditions were used for the cycling: denaturation at 94°C for 4 minutes (94°C for 4 minutes for the first cycle), annealing at 56°C for 45 seconds (55°C for nested reaction), and primer extension at 72°C for 1 minute and 10 minutes in the final cycle. A total of 5 µl of nested PCR products was analyzed by agarose

gel electrophoresis and visualized by ethidium bromide under UV transillumination.

Protein activity

To estimate total protein content, 0.5 g of fully expanded fresh leaves from both phytoplasma-infected and healthy chickpea plants were ground in cold potassium phosphate buffer (pH 7.0). The homogenized samples were then centrifuged, and the supernatant was collected. Total soluble protein concentration was determined using the dye-binding method described (Bradford *et al.*, 1976).

Phenylalanine ammonia-lyase (PAL)

PAL activity was assayed following the method reported by Ross and Sederoff (1992). Fresh leaf tissue samples (2.5 g) from phytoplasma infected and healthy plants of chickpea was ground with 5 ml of ice-cold 0.1 M borate buffer (pH 8.8) and filtered through four layers of muslin cloth. The filtrated and then it was centrifuged at 13,000 rpm for 5 minutes at 4°C. One milliliter of supernatant was mixed with 2 ml of 0.05 M borate buffer (pH 8.8) and 1 ml of 0.02 M L-phenylalanine. The samples were incubated at 30°C for 1 hour. The reaction was stopped by adding 0.2 ml of 6 M trichloroacetic acid (TCA) and PAL activity was measured. One activity unit was defined as a change in absorbance of 0.01 at 290 nm h^{-1} g^{-1} protein. PAL activity was expressed as mg/g fresh weight.

Peroxidase activity (POX)

For the estimation of POD activity, fresh leaves from phytoplasma infected and asymptomatic plants of chickpea were homogenized in a solution composed of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA and 1 mM dithiothreitol (DTT). The activity of POD was measured using the method of Chance and Maehly (1955) with modifications in the extraction buffer compositions to include EDTA and DTT for enzyme stabilization. One unit POD activity was defined as an absorbance change of 0.01unit min^{-1}.

Catalase activity (CAT)

Fresh leaves from phytoplasma infected and asymptomatic plants were separately mixed in a medium composed of 50 mM potassium phosphate buffer, pH 7.0 and 1 mM dithiothreitol (DTT) for the measurement of CAT activity. Assay solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O and 0.1 ml enzyme extract (min^{-1} g^{-1}). Reduction in absorbance of the reaction solution at 240 nm was recorded after every 20 seconds. An absorbance change of 0.01 unit's min^{-1} was defined as one unit CAT activity. Enzyme activity was expressed as on fresh weight basis (Hameed *et al.*, 2011).

Polyphenol oxidase activity (PPO)

To measure PPO activity fresh leaf tissue samples were cut into small pieces of about 5 mm long from each of the treatment and were ground in liquid nitrogen using a mortar and pestle. PPO activity was measured as described by Ngadze *et al.* (2012). The absorbance at 546 nm was measured for 4 minutes at 20 second intervals, the values per minute were calculated and the results were presented as U μL^{-1} min^{-1}.

Chlorophyll content

The chlorophyll a, chlorophyll b and total chlorophyll contents were estimated from fresh leaves of both phytoplasma infected and healthy plants using the standard procedure of Lichtenthaler (1987). For these purpose leaf samples of 0.25 g were added in 10 ml 80% acetone, grounded in the presence of sand with pestle in mortar and then filtered through muslin cloths. The absorbance of extract was measured at 663, 645, 505, 453 and 470 nm wavelengths, and the concentration of the above mentioned pigments were calculated (Davis *et al.*, 1976).

Results and Discussion

Phytoplasma detection and identification

The infected chickpea plants have shown typical symptoms, including sterility of the flowers and reduced internodal length. The presence of the phytoplasmas was detected by 16SrRNA gene nested PCR with the universal primers P1/ P7 and R16F2n / R16R2. While asymptomatic control gave no amplification, an expected 1250 bp fragments were
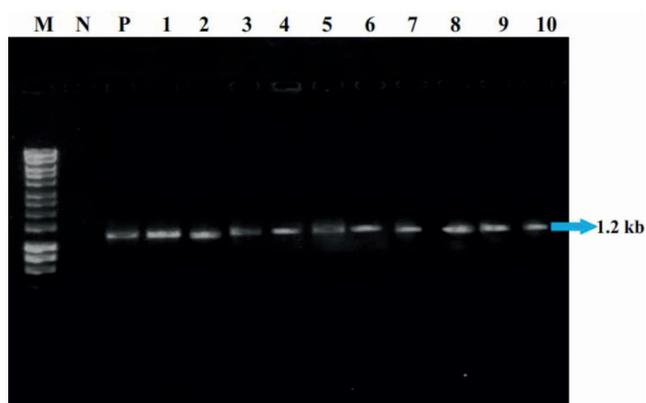
Figure 1. Nested PCR amplification of phytoplasma 16S rDNA with the universal primer pairs P1/P7 followed by R16F2n/R16R2. Lane M, 1 kb DNA ladder, lane N, negative control, lane P, positive control, lanes 1 to 10 are infected chickpea samples.

obtained from all symptomatic chickpea plant DNA samples (Figure 1).

Protein content

A significant reduction in total soluble protein content was observed in infected leaves (149.8 mg g^{-1}) compared to asymptomatic leaves (272 mg g^{-1}) (Figure 2). The reduced protein content in diseased leaves may be attributed to the degradation of proteins or inhibition of protein synthesis, particularly that of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the most abundant and functionally vital soluble protein involved in the Calvin cycle (Eichelmann *et al.*, 1999). Involvement of proteins in plant disease resistance has been documented in many plant pathogenic interactions (Hameed *et al.*, 2017). The reduced protein
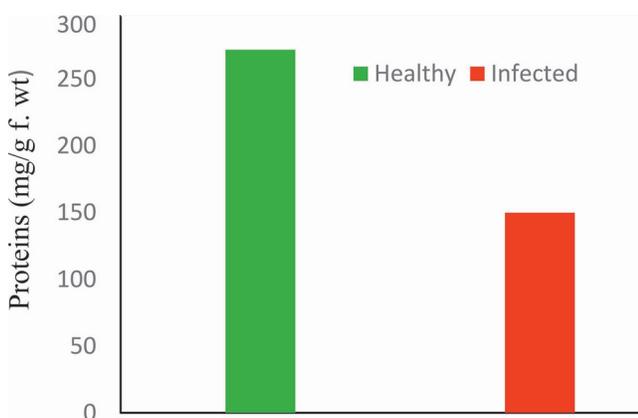
levels may also result from increased proteolytic activity and oxidative stress induced by phytoplasma infection, leading to cellular damage and impairment of physiological functions. Similar reductions in protein levels have been reported in various phytoplasma-infected plant species such as maize, tomato, grapevine and apple (Favali *et al.*, 2001; Bertamini *et al.*, 2001). This reduction in protein synthesis ultimately weakens the plant's overall metabolic capacity and contributes to the manifestation of disease symptoms.

Phenylalanine ammonia-lyase activity (PAL)

PAL is a key enzyme in the synthesis of secondary compounds, endogenous signaling molecule salicylic acid, which in turn activates the expression of a variety of pathogenesis-related proteins. A notable increase in PAL activity was observed in chickpea leaves infected with phytoplasma, compared to asymptomatic plants. Infected leaves showed 0.18 mg g^{-1} compared to healthy plants 0.15 mg g^{-1} (Figure 3). The enhanced PAL activity in infected leaves suggests an active defense response, as this enzyme contributes to strengthening the cell wall and producing antimicrobial phenolics that inhibit pathogen multiplication. These results agree with the findings of Kiprovski *et al.* (2018) and Hameed *et al.* (2017) who reported the increased activity of PAL in *Oenothera biennis* L. infected with 'Candidatus Phytoplasma solani' and mungbean infected by unidentified

Figure 2. Protein content in asymptomatic and phytoplasma-infected chickpea leaves. Values are expressed as mg protein per g of fresh weight (mg/g f.wt).
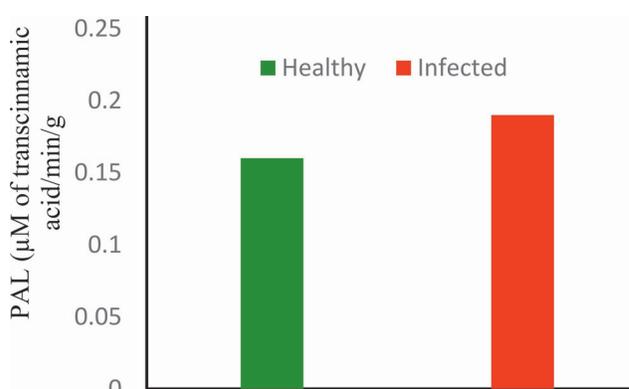

Figure 3. Phenylalanine ammonia-lyase (PAL) activity in asymptomatic and phytoplasma-infected chickpea plants, expressed as μM trans-cinnamic acid/min/mg of protein.

phytoplasmas, respectively. The high PAL activity observed in phytoplasma-infected chickpea leaves underscores its importance as a biochemical marker of defense activation and resistance mechanism in response to pathogen infection.

Peroxidase activity (POX)

Peroxidase is one of the first antioxidant enzymes responding and providing fast defense against plant pathogens by participating in a variety of defense mechanisms (Salari *et al.*, 2013). The phytoplasma-infected chickpea leaves exhibited significantly higher peroxidase activity compared to asymptomatic leaves. The infected chickpea leaves had the highest peroxidase activity (3.79) as compared to leaves from asymptomatic plants (Figure 4). The increased peroxidase activity in infected tissues is often associated with the reinforcement of plant cell walls through lignin and suberin deposition, as well as the cross-linking of cell wall proteins, which collectively contribute to pathogen containment (Hiraga *et al.*, 2001). Similar trends have been reported in sesame and potato infected with phytoplasmas or other pathogens, where elevated peroxidase activity correlated with the plant's attempt to restrict pathogen spread (Youssef *et al.*, 2018; Christopher *et al.*, 2012). The high peroxidase activity in phytoplasma-infected chickpea leaves likely represents an induced defense response aimed at enhancing structural barriers.
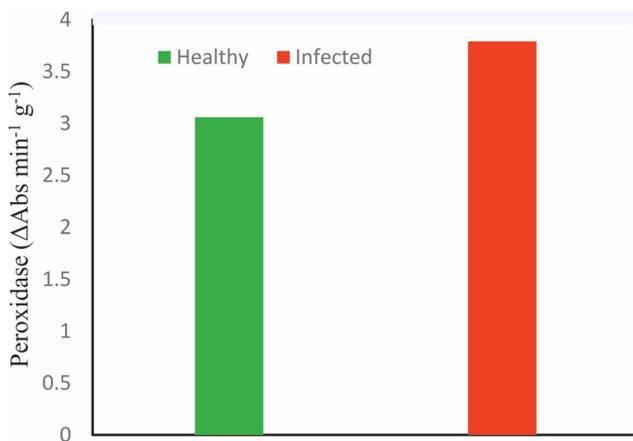
Catalase activity (CAT)

CAT is a crucial antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen, thereby protecting plant cells from oxidative damage during both normal metabolism and stress responses (Scandalios, 2005). CAT activity was found to be significantly decreased in phytoplasma-infected chickpea leaves when compared to the one in the leaves of asymptomatic plants. The catalase activity decreased in phytoplasma infected leaves from 199.8 to about 131.9 (Figure 5). The reduction in catalase activity could be a consequence of enhanced proteolysis caused by peroxisomal endopeptidases, which are induced by oxidative stress. Similar findings have been reported in phytoplasma-infected mungbean, sesame, and tomato plants, where reduced catalase activity was associated with enhanced resistance signaling (Hameed *et al.*, 2017; Youssef *et al.*, 2018). Moreover, the reduction of CAT activity usually increases plant resistance to pathogens by allowing the accumulation of hydrogen peroxide (H_2O_2), which functions as a signaling molecule in activating defense responses and also exert direct antimicrobial effects, thereby strengthening the plant's ability to combat pathogenic invasion (Magbanua *et al.*, 2007).

Polyphenol oxidase activity (PPO)

Polyphenol oxidase (PPO) activity was markedly

Figure 4. Peroxidase (POX) activity in asymptomatic and phytoplasma-infected chickpea plants. Values are expressed as change in absorbance (ΔAbs min^{-1} g^{-1} fresh weight).
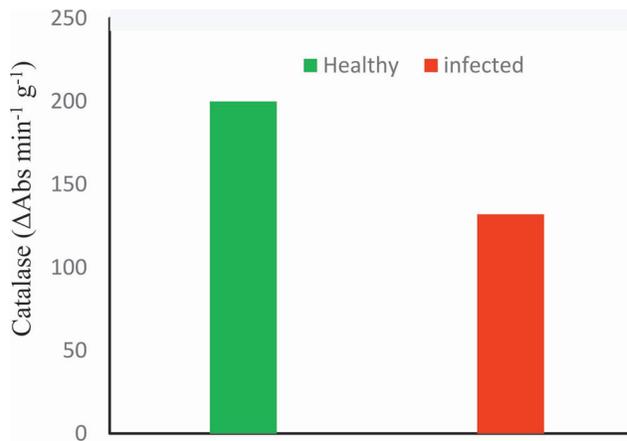
Figure 5. Catalase (CAT) activity in asymptomatic and phytoplasma-infected chickpea plants. Values are expressed as change in absorbance (ΔAbs min^{-1} g^{-1} fresh weight).
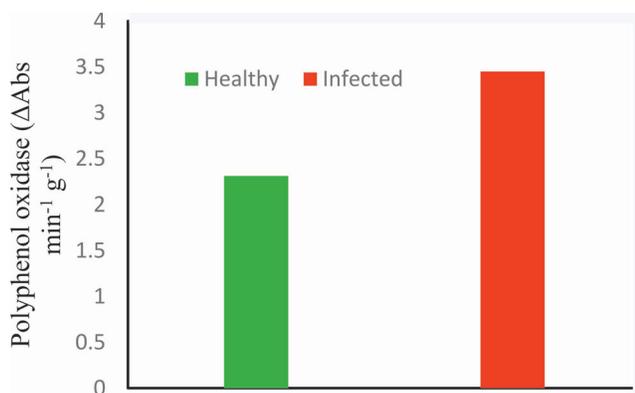
Figure 6. Polyphenol oxidase (PPO) activity in asymptomatic and phytoplasma-infected chickpea plants. Values are expressed as change in absorbance (ΔAbs min^{-1} g^{-1} fresh weight).

increased in phytoplasma-infected chickpea leaves compared to asymptomatic controls. The polyphenol oxidase activity from 2.31 was increased in infected leaves to about 3.45 (Figure 6). PPO plays a critical role in the oxidation of phenolic compounds to quinones, which are highly reactive and can form structural barriers by cross-linking with cell wall proteins, thereby limiting pathogen spread (Mayer, 2006). The upregulation of PPO in infected tissues is often triggered by signaling molecules such as salicylic and jasmonic acids, which accumulate in response to pathogen-induced oxidative stress (Thipyapong *et al.*, 2004). The polyphenol oxidase activity in infected plants was higher than in asymptomatic plants and this indicates the attempt to resist the plant pathogens. Present results agree with earlier findings of Youssef *et al.* (2018) who observed the activity of polyphenols oxidase in the phytoplasma infected sesame was higher than in the healthy sesame plant. Upon phytoplasma infection in *O. biennis*, the activity of polyphenoloxidase was significantly increased (Kiprovski *et al.*, 2018). The elevated PPO activity in infected chickpea leaves reflects an induced defense strategy, aimed at enhancing resistance by reinforcing structural defenses and limiting pathogen proliferation through oxidative toxicity.

Chlorophyll (Chl) pigments

Phytoplasma infected plants showed a significant decrease in the concentration of Chla, Chlb and total

Chl content as compared to uninfected plants. The marked reduction of total Chl in phytoplasma infected leaves in the present study may be due to the decrease of both Chla and Chlb contents as previously reported in many viruses and phytoplasma infected plants. The present findings confirm that phytoplasmas have a role in the inhibition of chlorophyll bio-synthesis in plant host leaves (Bertamini *et al.*, 2002). It can be assumed that the observed reduction in chlorophyll levels will probably interfere with the photosynthetic capacity in the chickpea leaves as previously observed for the papaya dieback and corn infected with maize bushy stunt phytoplasma (Junqueira *et al.*, 2004).
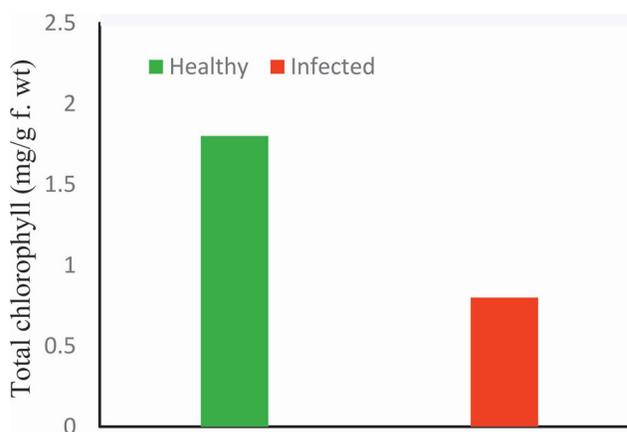
Figure 7. Total chlorophyll content in asymptomatic and phytoplasma-infected chickpea plants. Values are expressed as mg g^{-1} fresh weight.

Conclusions

The present finding indicates that phytoplasma infection triggers a broad, non-specific stress response in chickpea leaves. The observed alterations in phenolic content, total soluble proteins, and the activities of PAL, POX, CAT, PPO, along with reductions in chlorophyll a, chlorophyll b, and total chlorophyll in phytoplasma-infected chickpea leaves, suggest a substantial disruption of key physiological and biochemical pathways. These disturbances likely contribute to the manifestation of distinct symptoms, highlighting the complex nature of host–phytoplasma interactions. In conclusion, the present study offers valuable insights into the underlying mechanisms of plant response to pathogen infection, particularly phytoplasmas, in chickpea.

References

Akhtar KP, Shah TM, Atta BM, Dickinson M, Hodgetts J, Khan RA, Haq MA and Hameed S 2009. Symptomatology, etiology and transmission of chickpea phyllody disease in Pakistan. *Journal of Plant Pathology*, 91(3): 649-653.

Akram M, Sachan DK, Patil DK, Chavan RA, Sontakke PL, Saifulla M and Singh NP 2016. Characterization of phytoplasma associated with chickpea phyllody based on 16S rRNA gene sequence analysis. *Journal of Food Legumes*, 29(3-4): 232-236.

Bertaccini A and Duduk B 2009. Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopathologia Mediterranea*, 48: 355–378.

Bertaccini A and Lee I-M 2018. Phytoplasmas: an update. In: *Phytoplasmas: Plant pathogenic bacteria-I: Characterisation and epidemiology of phytoplasma-associated diseases*. Eds GP Rao, A Bertaccini, N Fiore and LW Liefting. Springer, Singapore, 1-29.

Bertamini MA and Nedunchezhian N 2001. Effects of phytoplasma ["stolbur"-subgroup ("bois noir"-BN)] on photosynthetic pigments, saccharides, ribulose 1, 5-bisphosphate carboxylase, nitrate and nitrite reductases, and photosynthetic activities in field-grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. *Photosynthetica*, 39: 119-122.

Bertamini M and Nedunchezhian N 2002. Leaf age effects on chlorophyll, rubisco, photosynthetic electron transport activities and thylakoid membrane protein in field grown grapevine leaves. *Journal of Plant Physiology*, 159(7): 799-803.

Bradford MM 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2): 248-254.

Chance M and Maehly AC 1955. Assay of catalases and peroxidases. *Methods in Enzymology*, 2: 764–775.

Chen W, Sharma HC and Muehlbauer FJ 2011. Compendium of chickpea and lentil diseases and pests. *American Phytopathological Society*, St. Paul, Minnesota, USA pp 164.

Christopher MW, Chen J and Civerolo EL 2012. Zebra chip-diseased potato tubers are characterized by increased levels of host phenolics, amino acids, and defense-related proteins. *Physiological and Molecular Plant Pathology*, 78: 66-72.

Davis BH 1976. Carotenoids. In: *Chemistry and Biochemistry of Plant Pigments*. Ed Goodwin TW, Academic Press, London, 38–165.

Deng S, Hiruki C 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods*, 14(1): 53-61.

Eichelmann H and Laisk A 1999. Ribulose-1, 5-bisphosphate carboxylase/oxygenase content, assimilatory charge, and mesophyll conductance in leaves. *Plant Physiology*, 119(1): 179-190.

Favali MA, Toppi LD, Vestena C, Fossati F and Musetti R 2001. Phytoplasmas associated with tomato "stolbur" disease. *Acta Horticulturae*, 551: 93–99.

Gundersen DE and Lee I-M 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, 35(3): 144-151.

Hameed A, Bibi N, Akhter J and Iqbal N 2011. Differential changes in antioxidants, proteases, and lipid peroxidation in flag leaves of wheat genotypes under different levels of water deficit conditions. *Plant Physiology and Biochemistry*, 49: 17–185.

Hameed S, Akhtar KP, Hameed A, Gulzar T, Kiran S, Yousaf S, Abbas G, Asghar MJ and Sarwar N 2017. Biochemical changes in the leaves of mungbean (*Vigna radiata*) plants infected by phytoplasma. *Turkish Journal of Biochemistry*, 42(6): 591-599.

Hiraga S, Sasaki K, Ito H, Ohashi Y and Matsui H 2001. A large family of class III plant peroxidases. *Plant and Cell Physiology*, 42(5): 462-468.

Junqueira A, Bedendo I and Pascholati S 2004. Biochemical changes in corn plants infected by the maize bushy stunt phytoplasma. *Physiological and Molecular Plant Pathology*, 65(4): 181-185

Kiprovski B, Dalovic I, Adamovic D, Mitrovic P, Marjanovic-Jeromela A, Malencic D and Popovic T 2018. Biochemical changes in *Oenothera biennis* plants infected by '*Candidatus* Phytoplasma solani'. *Journal of Plant Pathology*, 100: 209-214.

Lichtenthaler HK 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology*, 148: 350–382.

Magbanua ZV, De Moraes CM, Brooks TD, Williams WP and Luthe DS 2007. Is catalase activity one of the factors associated with maize resistance to *Aspergillus flavus*? *Molecular Plant-Microbe Interactions*, 20(6): 697-706.

Maixner M, Ahrens U and Seemüller E 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology*, 101: 241-250.

Mayer AM 2006. Polyphenol oxidases in plants and fungi: going places? A review. *Phytochemistry*, 67(21): 2318-2331.

Millan T, Clarke HJ, Siddique KH, Buhariwalla HK, Gaur PM, Kumar J, Gil J, Kahl G and Winter P 2006. Chickpea molecular breeding: new tools and concepts. *Euphytica*, 147: 81-103.

Ngadze E, Icishahayo D, Coutinho TA and van der Waals JE 2012. Role of polyphenol oxidase, peroxidase, phenylalanine ammonia lyase, chlorogenic acid, and total soluble phenols in resistance of potatoes to soft rot. *Plant Disease*, 96(2): 186-192.

Reddy MG, Baranwal VK, Sagar D and Rao GP 2021. Molecular characterization of chickpea chlorotic dwarf virus and peanut witches' broom phytoplasma associated with chickpea stunt disease and identification of new host crops and leafhopper vectors in India. *3 Biotech*, 11(3): 112.

Ross WW and Sederoff RR 1992. Phenylalanine ammonia lyase from loblolly pine: purification of the enzyme and isolation of complementary DNA clone. *Plant Physiology*, 98: 380-386.

Salari M, Panjehkeh N, Nasirpoor Z and Abkhoo J 2013. Reaction of melon (*Cucumis melo* L.) cultivars to *Monosporascus cannonballus* (Pollack & Uecker) and their effect on total phenol, total protein and peroxidase activities. *Journal of Phytopathology*, 161(5): 363-368.

Scandalios JG 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical and Biological Research*, 38: 995-1014.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology*. Eds S Razin and J Tully. Academic Press, San Diego, California, United States of America, 369-380.

Siddique AB, Rahman SM, Hossain MA and Rashid MA 2014. Phytochemical screening and comparative antimicrobial potential of different extracts of *Stevia rebaudiana* Bertoni leaves. *Asian Pacific Journal of Tropical Disease*, 4(4): 275-80.

Thipyapong P, Hunt MD and Steffens JC 2004. Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta*, 220: 105-117.

van Breusegem F, Bailey-Serres J, Mittler R 2008. Unraveling the tapestry of networks involving reactive oxygen species in plants. *Plant Physiology*, 147(3): 978-984.

Weintraub PG, Beanland L 2006. Insect vectors of phytoplasmas. *Annual Review of Entomology*, 51(1): 91-111.

Youssef SA, Safwat G, Shalaby AB and El-Beltagi HS 2018. Effect of phytoplasma infection on plant hormones, enzymes and their role in infected sesame. *Fresenius Environmental Bulletin*, 27(8): 5727-5735.

Short Communication

Identification of a new subgroup strain of 'Candidatus Phytoplasma cynodontis' associated with white leaf disease of Bermuda grass in Central India

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Abstract

Bermuda grass (*Cynodon dactylon*) is an invasive weed severely infected by Bermuda grass white leaf (BGWL) phytoplasma disease worldwide. During a survey in April 2025, Bermuda grass samples exhibiting white leaf symptoms were collected from Madhya Pradesh, Central India. The total DNA was extracted from symptomatic and asymptomatic leaf samples by a CTAB method and nested PCR assay was performed using universal primers for phytoplasma detection targeting 16S rRNA gene. Resulting amplicons (~ 1.2 kb) were purified, sequenced and submitted to GenBank under accession numbers PX442054-PX442057. The sequence analysis revealed 99-98% sequence identities and close phylogenetic relationships with 'Candidatus Phytoplasma cynodontis' (16SrXIV-A subgroup). The *i*PhyClassifier *in vitro* RFLP analysis of 1.2 kb 16S rRNA gene sequence indicated that these strains had a similarity coefficient of 0.85 to 0.89 to the reference strain of the 16SrRNA subgroup 16SrXIV-A suggesting that it may be a new subgroup strain or lineage of 'Ca. P. cynodontis' which needs further confirmation.

Bermuda grass (*Cynodon dactylon;* family *Poaceae*), which is native to Africa, is considered an excellent lawn grass (turf grass) in the tropics including India, where it can also be used as a ground cover to prevent soil erosion and also used as forage for livestock. It is a natural dominant flora in cultivated and uncultivated areas: roadsides, sea-coast sandy dunes, or along rivers and irrigated land, and a most common invasive weed. In India it is of great religious importance and commonly known as Bahama grass, "durva" grass, devil's grass. Bermuda grass also has medicinal importance as used in Ayurvedic, Unani, Nepalese, and Chinese systems of medicine. It contains active constituents of alkaloids, β-sitosterol, carotene, flavonoids, glycosides and triterpenoids, vitamin C, fats, and palmitic acid which also has excellent anti-inflammatory properties (Brosnan and Deputy, 2008). Phytoplasmas are insect-transmitted wall-less bacteria and live inside plant phloem tissues, which infect a wide variety of plants such as fruit crops, timber, vegetables, grasses, ornamental plants and many weed species (Asudi *et al.*, 2021). Highly conserved 16Sr RNA sequence is applied to identify and classify phytoplasmas (IRPCM, 2004; Bertaccini *et al.*, 2022).

Bermuda grass white leaf (BGWL) is a destructive, phytoplasma disease of Bermuda grass and it was first

described in Taiwan, where it is associated with 16SrXIV group of phytoplasma and characterized by whitening of the leaves and shortening of the stolons (Chen *et al.*, 1972). BGWL disease associated with phytoplasmas is reported worldwide (Snehi *et al.*, 2008; Kumar *et al.*, 2015; Duduk *et al.*, 2018; Mall *et al.*, 2023) reported witches' broom, leaf chlorosis, stunting and shortened rhizomes/stolons in *C. dactylon* and reported leafhopper *Exitianus indicus* as putative disease insect vector in India. This work was undertaken to verify the presence of phytoplasmas in symptomatic *C. dactylon* plants growing in areas of Central India.

The survey was conducted in the month of April 2025 at Mandideep (23°5'40" N, 77 31'5"E), District Raisen, Madhya Pradesh, Central India. Symptomatic Bermuda grass samples exhibiting whitening of the leaf symptoms were observed with an disease incidence varied from 7 to 10%. The symptomatic leaf samples were collected along with asymptomatic Bermuda grass leaf sample used as a negative control.

The genomic DNA was extracted using a CTAB method from symptomatic and asymptomatic leaves (100 mg) field collected during the survey (Ahrens and Seemuller, 1992). The phytoplasma 16S rRNA gene was amplified from DNA by PCR using P1/P7 primers (Deng and Hiruki, 1991; Schneider *et al.*, 1995), then the resulting PCR products were diluted 1:10 with sterile water and 1 µl was used for nested PCR using R16F2n/R16R2 primers (Gundersen and Lee, 1996). Each PCR

reaction consist of nuclease free water, 10x PCR buffer, dNTPs (10 mM), Taq polymerase of 5 U/µl (Takara Bio, Japan), forward and reverse primers (25 mM) and DNA template (40 ng). The nested PCR products were separated on 1% agarose gel, stained with ethidium bromide and image was documented using gel documentation system (Biorad, USA). The resulted amplicons obtained from nested PCR (about 1.2 kb) were purified (Hi-Media Purification Kit, Mumbai, India) and sequenced bidirectionally from Hi-media.

The sequence data obtained were analyzed on basic local alignment search tool (BLAST) for nucleotide identity within and with other reported strains of phytoplasmas and submitted to National Centre for Biotechnology Information GenBank database (NCBI). They were also analysed using the *i*PhyClassifier online tool (http://www.plantpathology.ba.ars.usda.gov/cgibin/resource/iphyclassifier.cgi) with 17 restriction endonucleases (Zhao *et al.*, 2013). Phylogenetic analyses were perused using Molecular Evolutionary Genetics Analysis (MEGA version 12) (Kumar *et al.*, 2024) program with 1000 replicates bootstrapping and phylogram were generated with Neighbour-joining method (Fernández *et al.*, 2023). Dendrograms were viewed by the NJ plot program.

During the survey in April 2025, the whitening of the leaf symptom was observed in Bermuda grass on agricultural roadside of Mandideep, District Raisen, Madhya Pradesh, India (Figure 1). Four symptomatic plants leaf samples were collected along with an

Figure 1. Asymptomatic Bermuda grass (A), Bermuda grass showing white leaves (B, C and D).

asymptomatic leaf sample used as negative control for the molecular identification of the phytoplasmas associated with the disease. The phytoplasma 16S rRNA gene was amplified by nested PCR with R16F2n/R16R2 primers from all the symptomatic (4/4) leaf samples but not from the asymptomatic one. Four nested PCR amplicons were sequenced and the consensus partial 16S rRNA gene nucleotide sequence data were submitted to NCBI GenBank under accession numbers PX442054-PX442057. BLASTn analysis showed 99-100% sequence identities with each other and also showed the highest nucleotide sequence identities with a *Triticum turgidum* phytoplasma from India (GenBank accession numbers MK829206, MK8229203, MK829201) and *Brachialis* grass white leaf phytoplasma from Thailand (GenBank accession number AB052872). 'Candidatus Phytoplasma cynodontis' reference strain (GenBank accession number KF234570) in *Cynodon dactylon* showed 99.20% identity and similar identities were calculated with other strains from India, Korea, Malaysia and Italy among many others present in GenBank database (Figure 2).

Phylogenetic analysis of 16S rRNA gene with MEGA 12 software using the neighbour-joining method revealed that all four BGWL phytoplasma isolates associated with Bermudagrass showed close phylogenetic relationships with each other forming a separate branch under 'Ca. P. cynodontis' clade (Figure 2). *In silico* restriction fragment length polymorphism (RFLP) patterns with 17 restriction enzymes of the F2nR2 16S rRNA encoding gene sequence of the under study BGWL phytoplasma strains were compared with the strain with GenBank accession number AJ550984 used as reference in this system for 16SrXIV-A subgroup (Figure 3). The similarity coefficient retrieved was noticed as of 0.97 with 16S rRNA genes of all the strains enclosed in this system and a similarity coefficient of 0.85 or less was calculated with all previously recognized phytoplasma groups and subgroups. Hence the present BGWL strains could be considered as variants of the reference BGWL 16SrXIV-A strain (Wei *et al.*, 2008). The phylogeny and *in silico* RFLP pattern comparison results confirmed that the phytoplasma associated with Bermuda grass in the present study is identified as 'Ca. P. cynodontis' related strain.

Figure 2. Phylogenetic analysis of the partial 16S rRNA gene (~1.2 kb) of under study phytoplasma strains (GenBank accession numbers PX442054; PX442055; PX442056 and PX442057) associated with Bermuda grass showed close phylogenetic relationships with 'Ca. P. cynodontis' phytoplasma strains and shared distinct relationships with other phytoplasma groups. The evolutionary relationships were constructed using the Neighbor-Joining method with bootstrap value of 1,000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004), only values above 40 are shown.
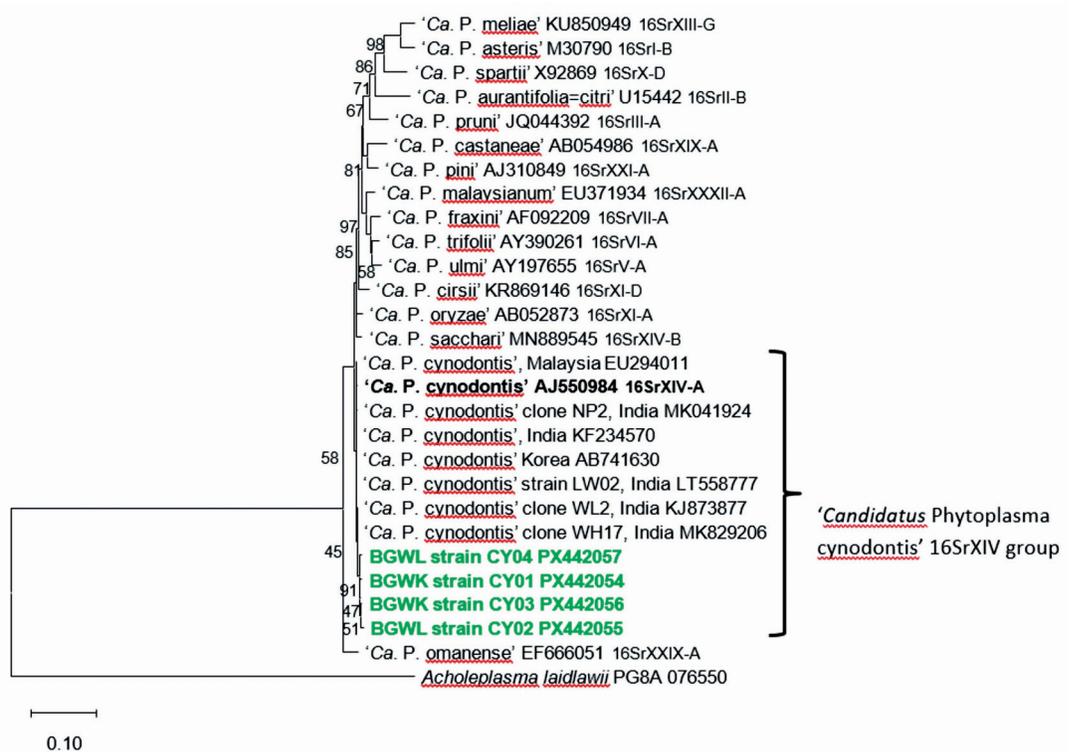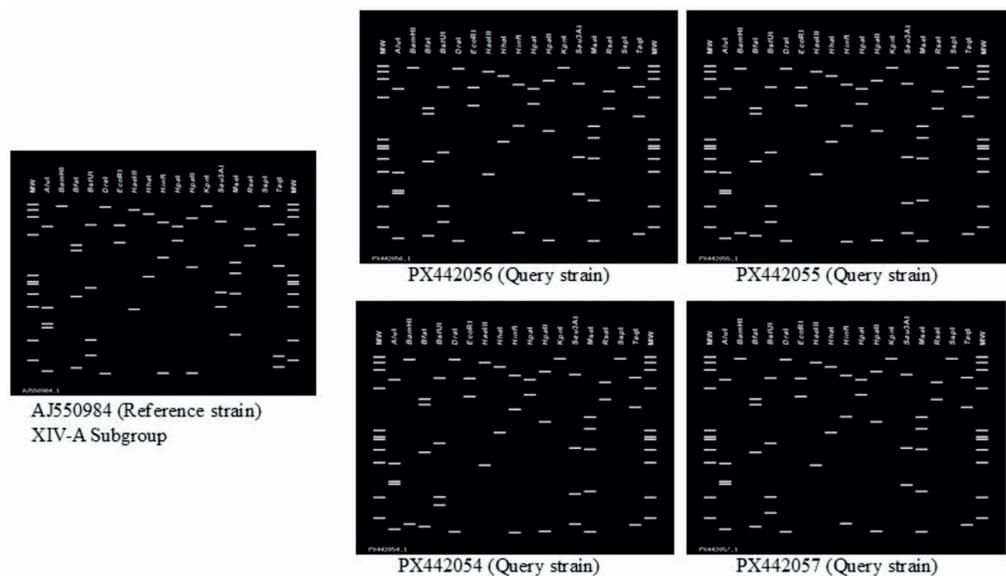
Figure 3. Virtual RFLP patterns from *in silico* digestion of 16S rRNA gene of F2n/R2 fragments of the BGWL strains under study with GenBank accession numbers PX442054; PX442055; PX442056; PX442057 compared with the reference strain for 16SrXIV-A subgroup (GenBank accession number AJ550984) generated using *i*PhyClassifier with 17 restriction enzymes.

Inside the 'Ca. P. cynodontis' three subgroups were reported with 16SrXIV-A being identified mainly in Europe and 16SrXIV-B and -C from Iran and Western Europe, respectively (Salehi *et al.*, 2009; Mitrovic *et al.*, 2015). 'Ca. P. cynodontis' (16SrXIV-A subgroup) associated with BGWL disease have been previously reported in India (Rao, 2021), however this is the first report of possibility new subgroup variant under 16SrXIV group and represent a different lineage from all those reported strain reported so far (Wei and Zhou, 2022). Further studies are required utilizing real RFLP and NGS to confirm the identity of this BGWL strain.

Acknowledgments

References

Ahrens U and Seemüller E 1992. Detection of DNA of plant pathogenic mycoplasma-like organism by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology*, 82: 828-832.

Asudi GO, Omenge KM, Paulmann MK, Reichelt M, Grabe V, Mithöfer A 2021 The physiological and biochemical effects on Napier grass plants following napier grass stunt phytoplasma infection. *Phytopathology* 111: 703-712.

Bertaccini A, Arocha-Rosete Y, Contaldo N, Duduk B, Fiore N, Montano HG, Kube M, Kuo C-H, Martini M, Oshima K, Quaglino F, Schneider B, Wei W and Zamorano A 2022. Revision of the 'Candidatus Phytoplasma' species description guidelines. *International Journal of Systematic and Evolutionary Microbiology*, 72(4): 5353.

Brosnan JT and Deputy J. 2008. Bermudagrass. *University of Hawai'i at Mānoa, and Human Resource, Cooperative Extension Service Publication*, TM-5: 1-6. http:// turfgrass.ctahr.hawaii.edu/downloads/Bermudagrass_NEW2.pdf

Chen TC, Lee CS and Chen MJ 1972. Mycoplasmalike organisms in *Cynodon dactylon* and *Brachiaria distachya* affected by white leaf disease. *Report of Taiwan Sugar Experimental Station*, 56: 49-55.

Deng S and Hiruki C 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiological Methods*, 14: 53-61.

Duduk B, Stepanovic J, Yadav A and Rao GP 2018. Phytoplasmas in weeds and wild plants. In: Phytoplasmas: Plant Pathogenic Bacteria-I-Characterization and Epidemiology of Phytoplasma Associated diseases, pp 313-345. Eds GP Rao, A Bertaccini, N Fiore and IW Liefting, Springer, Singapore.

Fernández A, Segura-Alabart N, Serratosa F 2023. The MultiFurcating Neighbor-Joining Algorithm for Reconstructing Polytomic Phylogenetic Trees. *Journal of Molecular Evolution*, 91: 773–779. doi.org/10.1007/s00239-023-10134-z.

Gundersen DE and Lee I-M 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer sets. *Phytopathologia Mediterranea*, 35: 144-151.

IRPCM 2004. 'Candidatus Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology*, 54: 1243–1255.

Kumar S, Jadon V, Tiwari AK and Rao GP 2015. *Exitianus indicus*

(Distant): a putative vector for '*Candidatus* Phytoplasma cynodontis' in India. *Phytopathogenic Mollicutes*, 5(Supplement): S51–S52.

Kumar S, Stecher G, Suleski M, Sanderford M, Sharma S and Tamura K 2024. Molecular evolutionary genetics analysis version 12 for adaptive and green computing. *Molecular Biology and Evolution*, 41: 1-9.

Mall S, Kirdat K, Singh A, Tiwarekar B, Sathe S, Marcone C, Yadav A 2023. Updates on phytoplasma diseases associated with weeds acting as alternate hosts in Asian countries. In: Phytoplasma Diseases in Asian Countries, Phytoplasma Diseases of Major Crops, Trees, and Weeds, pp 347-365, Vol 2. Eds AK Tiwari, K Caglayan, TX Hoat, AA Subhi, N Nejat, G Reddy, Academic Press.

Mitrovic J, Smiljkovic M, Seemüller E, Reinhardt R, Huttel B, Bertaccini A, Kube M and Duduk B 2015. Differentiation of '*Candidatus* Phytoplasma cynodontis' based on 16S rRNA and *groEl* genes and identification of a new subgroup, 16SrXIV-C. *Plant Disease*, 99: 1578–1583.

Rao GP, Sajeena A, Mathew D, John J, Dhanya MK, Ayisha R and Reddy MG 2021. First report of candidatus phytoplasma cynodontis (16SrXIV-A Subgroup) associated with cauliflower phyllody and flat stem in India. *Plant Disease*, 105(12): 4150.

Salehi M, Izadpanah K, Siampour M and Taghizadeh M 2009. Molecular characterization and transmission of Bermuda grass white leaf phytoplasma in Iran. *Journal of Plant Pathology*, 91: 655–661.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC 1995. Phylogenetic classification of plant pathogenic mycoplasma like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology*. Eds S Razin and JG Tully San Diego, CA: Academic Press, 369-380.

Snehi SK, Khan MS, Raj SK, Mall S, Singh M, Rao GP 2008. Molecular identification of '*Candidatus* Phytoplasma cynodontis' associated with Bermuda grass white leaf disease in India. Plant Pathology, 57: 770. doi: 10.1111/j.1365-3059.2008.01853.x

Tamura K, Nei M and Kumar S 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)*, 101: 11030-11035.

Wei W, Lee I-M, Davis RE, Suo X and Zhao Y 2008. Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. *International Journal of Systematic and Evolutionary Microbiology*, 58: 2368–2377.

Wei W and Zhao Y 2022. Phytoplasma taxonomy: Nomenclature, classification, and identification. *Biology*, 11(8): 1119. https://doi.org/10.3390/biology11081119.

Zhao Y, Wei W, Lee IM, Shao J, Suo X, Davis RE 2013. The *i*PhyClassifier, an Interactive Online Tool for Phytoplasma Classification and Taxonomic Assignment. In: Dickinson M, Hodgetts J Eds. Phytoplasma. Methods in Molecular Biology, vol 938. Humana Press, Totowa, NJ. doi.org/10.1007/978-1-62703-089-2_28

International Phytoplasmologist Working Group Workshop and Training Course (IPWG-2025)

Venue: ICAR-Indian Institute of Sugarcane Research, Lucknow, Uttar Pradesh, India (Feb 23-28, 2025)
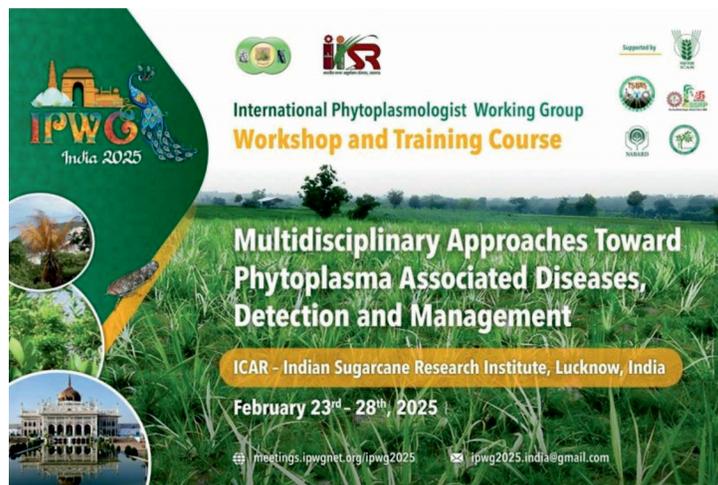
International training program on "Phytoplasma outbreaks: collect, preserve, identify phytoplasmas and their insect vectors" (February 23-24, 2025)
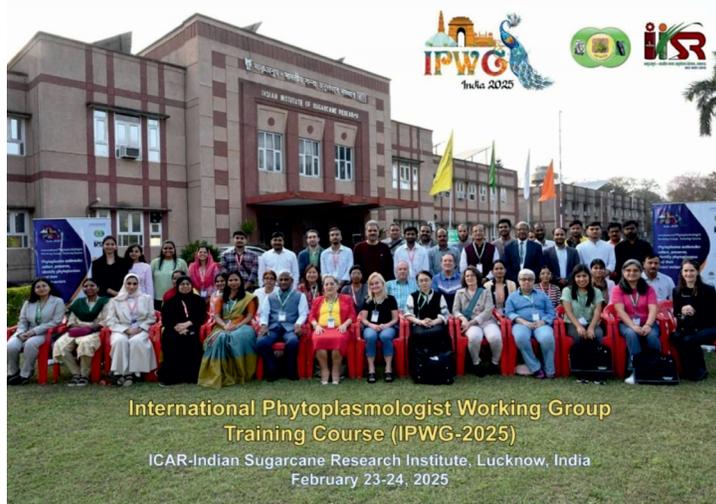
An international training program on "Phytoplasma outbreaks: collect, preserve, identify phytoplasmas and their insect vectors" was jointly organized by the International Phytoplasma Working Group and the Indian Sugarcane Research Institute, Lucknow, with the support of the Technology Society of Basic & Applied Sciences, New Delhi. Twenty-five training delegates registered from six countries participated. The training covered a field trip for the collection of phytoplasma-infected brinjal little leaf, sesame phyllody, and sugarcane grassy shoot samples and potential insects vectors; molecular detection of phytoplasmas in plants

Glimpses of the IPWG 2025 training program at ISRI, Lucknow, India (February 22-23, 2025)

and insects; and utilization of bioinformatics tools for phytoplasma molecular identification.

The primary objective of this training program was to provide participants with basic and practical knowledge, along with hands-on training on the symptomatology, detection, identification and taxonomy of phytoplasmas, as well as the identification of insect vectors responsible for phytoplasma transmission. In addition to lectures on various aspects of insect vector identification and transmission, updates on identification and characterization of phytoplasmas were delivered and discussed by senior plant pathologists from India, the USA, Italy and Germany. A technical manual was also

prepared and printed incorporating relevant protocols involved in the collection of field samples, recording symptoms, DNA extraction from plant and insect samples, PCR/nested PCR assays, gel electrophoresis, amplicon detection, and phytoplasma DNA sequence analysis through bioinformatics tools. A lecture series was also given by senior experts (Dr. Assunta Bertaccini, Italy; Dr. Barbara Jarausch, Germany; Dr. Wei Wei and Dr. Valeria Trivellone , USA), and hands-on training of related experiments was demonstrated for molecular identification of detected phytoplasm as.

This training program also introduced bioinformatics focusing on the analysis and interpretation of sequence data. All the trainees were requested to prepare and assess DNA sequences, performing sequence alignment with ClustalW, and using BLAST for sequence identification and annotation. The program also covered phylogenetic analysis with MEGA, as well as phytoplasma classification. Participants had gained experience and learnings in submitting annotated sequences to public repositories, equipping them with essential skills for genomic and molecular biology research.

The training manual will be very useful for the students and young career scientists involved in phytoplasma and phloem-limited microorganisms' research and also serve as good resource material. Each participant got the opportunity to discuss its individual queries with the training experts. Overall, the IPWG training program was a grand success.

International Workshop on "Multidisciplinary Approaches toward Phytoplasma-Associated Diseases Detection and Management" (February 25-28, 2025)

Besides the training program, an international workshop on "Multidisciplinary approaches toward phytoplasma-associated diseases detection and management" was also organized at the Indian Institute of Sugarcane Research, Lucknow, from 25-28 February 2025. Nearly 136 delegates from 16 countries participated in the workshop and discussed their research findings on the latest updates in the distribution, diagnosis, epidemiology, and management
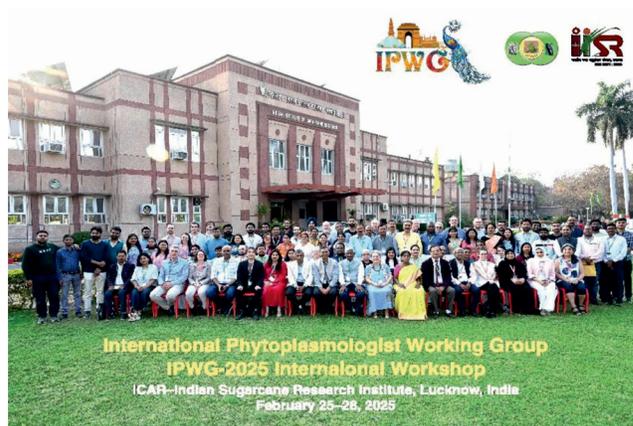
of phytoplasma associated diseases infecting important agricultural crops worldwide. There were nine technical sessions divided into 4 days: omics, new detection tools, interactions, country status, epidemiology and control, and special sessions on phytoplasma diseases in sugar and citrus crops, and palm trees. All the sessions were actively attended, and all the delegates participated in the answer and question session for each presentation. Six online presentations by delegates of the USA, India, Russia and Italy were also organized in different technical sessions of the workshop.

A cultural evening was organized on the 25th of February, and a social gala dinner was organized on the 27th of February 2025. A post-workshop tour to the Taj Mahal, Agra, India, was also organized for the foreign delegates.

All the papers accepted for the IPWG workshop have been published in the Phytopathogenic Mollicutes journal (SCOPUS indexed) June 2025 (Vol. 15, No. 1, 2025) issue and distributed to all workshop-registered delegates. An IPWG scientific committee meeting was held on 26th of February 2025 at the Indian Sugarcane Research Institute, to discuss the present and future activities and progress of the IPWG group.

This workshop was a grand success and provided updated knowledge and literature on the geographical distribution of phytoplasma diseases worldwide, which would help in focusing specific targets on the important diseases for developing disease management in respective countries and also alert about emerging phytoplasma diseases of quarantine concern. The

Glimpses of the IPWG 2025 Workshop at ISRI, Lucknow, India (February 25-28, 2025)

discussion on diagnosis, transmission, epidemiology, host-pathogen interactions and management would be helpful for rapid detection of phytoplasma diseases and accurate identification of both emerging and known phytoplasm as for their effective management strategies.

Chair IPWG: Dr. Assunta Bertaccini (Italy); e mail: assunta.bertaccini@unibo.it

Convenor: Dr. Rasappa Viswanathan (India); e mail: rasaviswanathan@gmail.com

Organizing Secretary: Dr. Govind P. Rao (India); e mail: gprao_gor@rediffmail.com

AIMS AND SCOPE

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- **Journal citation:** Smart CD, Schneider B, Blomquist CL, Guerra LJ, Harrison NA, Ahrens U, Loernz KH, Seemüller E and Kirkpatrick BC 1996. Phytoplasma-specific PCR primers based on sequences of the 16-23S rRNA spacer region. *Applied and Environmental Microbiology,* 62: 2988-2993.

- **Book citation:** Harrison NA, Rao GP and Marcone C (2008). Characterization, Diagnosis and Management of Phytoplasmas. (Harrison NA, Rao GP, Marcone C, Eds) Studium Press LLC, U.S.A. pp. 1-399.

- **Book chapter citation:** Schneider B, Seemüller E, Smart CD and Kirkpatrick BC 1995. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology*, pp. 369-380. Eds. S. Razin and J.G. Tully. San Diego, CA: Academic press.

- **DOI publication citation:** Malembic-Maher S, Salar P, Filippin L, Carle P, Angelini E and Foissac X 2010. Genetic diversity of European phytoplasmas of the 16SrV taxonomic group and proposal of 'Candidatus Phytoplasma rubi'. *International Journal of Systematic and Evolutionary Microbiology*, DOI 10.1099/ijs.0.025411-0, in press

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