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Welcome by the editors

We are delighted to edit the extended abstracts of the "Fifth International Phytoplasmologist Working Group Meeting" (IPWG) that will be held in Muscat, Oman. Phytoplasma associated diseases are a major limiting factor to quality and productivity of many economically important agriculture crops worldwide. Annual losses due to phytoplasma diseases in many crops vary, but under the pathogen favorable conditions they always lead to disastrous consequences to farming communities.

The IPWG group, born in Bologna, Italy, in 2007 is the forum for sharing information and strength and/or built new and more intense scientific interactions among participants. It is formed by worldwide scientists working on different aspects of these phloem-limited microorganisms and their interaction with plant and insect hosts to increase and disseminate the knowledge about phytoplasma-associated diseases worldwide. The group is organizing a meeting in different countries every four years, and it has successfully organized meetings in Italy (2007), Germany (2011), Mauritius (2015) and Spain (2019).

The 5th IPWG meeting will be held in Muscat, Oman at the Sultan Qaboos University from 21 to 25 May 2023 with expected attendance of about 100 scientists from about 30 countries worldwide. A round table discussion on recent updates on major emerging issues on phytoplasmas and closely related pathogens is also planned. An invited lecture by prof. Kenro Oshima from Japan on "Survival strategy of phytoplasmas suggested from genomic and virulence factor research" will open the meeting. The meeting session will have also focused presentations on phytoplasma recent achievements on detection and identification, epidemiology enclosing seed transmission and control methods aimed to environmentally friendly disease management solutions. Quarantine issues and activity related will also be discussed in a dedicated session.

The extended abstracts cover several aspects of phytoplasma research and are really imperative and valuable in redefining the research and development needs all over the world. Different aspects of phytoplasma-associated diseases are covered: phytoplasma-host interaction and omic applications, insect vectors, epidemiology and control, quarantine, mixed infection, disease reports worldwide and new detection methods. These topics will provide an authoritative scientific backdrop for informed discussions and debates on recent achievements in phytoplasma research and also share the global vision on growth of phytoplasma research.

The presentations of the meeting are included in this issue of Phytopathogenic Mollicutes, which is a half yearly official internationally known official publication of the Technology Society of Basic & Applied Sciences (TSBAS) and is published by Indianjournals.com, New Delhi, India. This issue contains 75 extended abstracts printed in the journal format. We are sure that this issue of Phytopathogenic Mollicutes will be helpful to update the phytoplasma research at a global level and for harnessing and understanding the full potential of this unique group of microorganisms. All the papers published in this issue have been reviewed and accepted by the IPWG Scientific Committee. Online information concerning these IPWG proceedings are available at the following web addresses:

- Indian Journals website: www.indianjournals.com
- IPWG website: www.ipwgnet.org

We want to thank the contributors for their diligence and timely submissions of extended abstracts. We apologize for errors that could have arisen during the editing process despite our careful attention. We want to thank all the meeting participants, the IPWG scientific committee members and Fabio Montanari for their consistent support in bringing out this issue of the Journal that is a very special one for the whole phytoplasma scientific community.

We welcome everyone attending the 5th IPWG meeting in Oman and wish them a comfortable stay and meaningful interactions with international colleagues.





Assunta Bertaccini and Govind Pratap Rao
Editors-in-Chief

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Interaction

Survival strategy of phytoplasmas suggested from genomic and virulence factor research

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Abstract

Phytoplasmas infect more than a thousand plant species worldwide and are transmitted by insect vectors. Phytoplasmas may alter the gene expression to colonize both plant and insect hosts. Despite their small genomes, they induce unique symptoms to their host plants such as phyllody and witches' broom. Although the molecular mechanisms behind the symptoms are not fully understood, several secreting effector proteins from phytoplasmas have been shown to induce the symptoms or manipulate the plant hosts.

Keywords: phytoplasma, genome, effector, phyllody

Introduction

Phytoplasmas are plant pathogenic bacteria that reside intracellularly within the plant phloem (Oshima, 2021). Phytoplasma-infected plants exhibit a wide range of unique symptoms, including phyllody, dwarfing, witches' broom, and purple tops. Phytoplasmas can infect more than a thousand plant species worldwide and are transmitted by insect vectors such as leafhoppers and psyllids (Oshima *et al*., 2011). Since the infected plants eventually decline and die in most cases, phytoplasmas cause great damage to agricultural production and forest trees. Although there is an agricultural need to develop effective methods to control phytoplasma diseases, phytoplasmas are still one of the most poorly characterized groups of plant pathogens.

Unique features of phytoplasma genomes

Genome analyses have been very useful for analyses of the feature of plant pathogens. Although phytoplasma genomes contain genes for basic cellular functions such as DNA replication, transcription, translation, and glycolysis, they lack genes for amino acid and fatty acid biosynthesis and the tricarboxylic acid cycle (Oshima *et al*., 2013). The phytoplasma genome encodes even fewer metabolic functional proteins than mycoplasma genomes, which were thought to have the minimum possible gene set. For example, phytoplasma genomes lack the genes encoding F_1Fo-type ATP synthase (Oshima *et al*., 2004). Since ATP synthase had been thought to be essential for life, the loss of its genes in the phytoplasma genome provided opportunities for reconsidering the question "what is life?". Interestingly, phytoplasmas harbour multiple copies of transporter-related genes not found in mycoplasmas, suggesting that they are highly dependent on metabolic compounds from their hosts.

Transcriptional changes during host switching

Since phytoplasmas are intracellular parasites of both plants and insects, their ability to adapt to two diverse environments is of considerable interest. Microarray analysis of '*Candidatus* Phytoplasma asteris' strain OY-M revealed that expression of approximately 33% of the genes changes during host switching between plant and insect, suggesting that the phytoplasma may dramatically alter the gene expression in response to their hosts (Oshima *et al*., 2011) and may use transporters, secreted proteins, and metabolic enzymes in a host-specific manner.

Two types of sigma factors, RpoD and FliA, are encoded in the phytoplasma genomes (Oshima *et al*., 2004). By an *in vitro* transcription assay, it has been demonstrated that RpoD regulates several housekeeping and virulence genes (Miura *et al*., 2015). The transcription starting sites have been identified from the genome of OY-M phytoplasma by using RNA-Seq technology. From these data, two promoter consensus sequences located upstream the transcription starting sites have been predicted. While one was almost identical to the RpoD-dependent consensus promoter sequence, the other was an unidentified novel motif, which might be recognized by another transcription factor such as FliA (Nijo *et al*., 2017).

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Virulence factors secreted from phytoplasmas

Despite their small genomes, phytoplasmas induce unique symptoms to host plants such as phyllody and witches' broom. Since phytoplasmas are cell wall-less and reside inside of host cells, their secreting proteins via Sec system function in the cytoplasm of the host plant cell and are predicted to have some important roles in host-parasite interactions and/or virulence as effector proteins. Although many candidate effectors have been found in the phytoplasma genomes so far, and the molecular mechanisms behind the symptoms are not fully understood, several effector proteins of phytoplasmas have been shown to induce the symptoms or manipulate plant hosts (Figure 1).

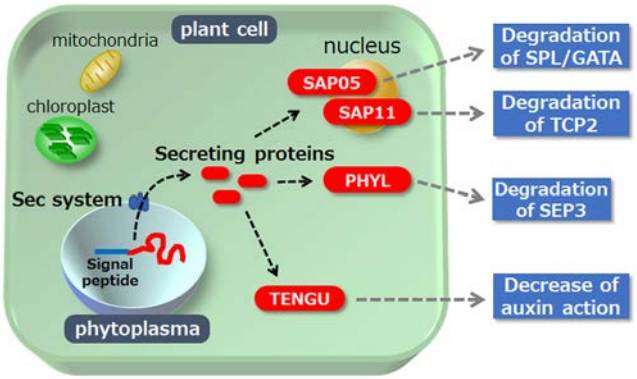


Figure 1. Schematic diagram of the functions of virulence proteins secreted from phytoplasmas. Secreting protein expressed in phytoplasma cell has a signal sequence that is removed during secretion via Sec system. Virulence proteins secreted from phytoplasmas locate to the cytoplasm or nucleus of a plant cell and manipulate plant proteins. For the functions of SAP05 and SAP11, see Oshima *et al.* (2023).

A small secreted protein, TENGU, encoded by the onion yellows phytoplasma, has been identified as a virulence factor that affects plant morphology (Hoshi *et al.*, 2009). Transient or transgenic expression of TENGU results in a short and bushy phenotype similar to the symptoms of phytoplasma-infected plants. Microarray analyses revealed that the expression of many auxin-related genes was significantly downregulated in TENGU-transgenic plants, suggesting that TENGU suppresses auxin signalling or biosynthesis pathways.

Flower malformations, such as phyllody and virescence, are a unique symptom of phytoplasma infection. It has been reported that phyllogen family proteins, such as SAP54 and PHYL, target and degrade MADS domain transcription factors (MTFs), the products of floral homeotic genes that constitute the floral quartet model (Maejima *et al.*, 2014; MacLean *et al.*, 2014). Phyllogens recognize A- and E-class MTFs of angiosperms and degrade them in a proteasome-dependent manner. Phyllogen-mediated phyllody induction requires the shuttle proteins Rad23s that translocate ubiquitinated proteins to the proteasome system. Phyllogens translocate MTFs to the proteasome system by mediating the interaction between MTF and Rad23 to form a ternary complex. This ternary complex is formed in the order MTF/phyllogen to MTF/phyllogen/Rad23 in a ubiquitin-independent manner (Kitazawa *et al.*, 2022). This indicates that phyllogens functionally mimic the ubiquitin to translocate their targets into the proteasome system. Further molecular insights into the secreting effector proteins will help elucidate the survival strategy of phytoplasmas.

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Interaction

Phytoplasma infection alters polar lipid composition and triggers chloroplast autophagy in host plants

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Abstract

Phytoplasmas are small, cell wall-less bacteria that infect a wide range of plant species. In this study, changes in the polar lipid composition of tomato plants infected with potato purple top (PPT) phytoplasma were investigated. The analysis revealed a decrease in the levels of monogalactosyldiacylglycerol (MGDG), phosphatidic acid, and phosphatidylglycerol in PPT phytoplasma-infected plants compared to controls. The MGDG/digalactosyldiacylglycerol ratio, an indicator of chloroplast thylakoid membrane structure and function, was reduced in PPT phytoplasma-infected plants. Additionally, the degradation of the chloroplast rubisco large and small subunits (RbcL and RbcS) was observed in infected plants, indicating the activation of chloroplast autophagy triggered by the accumulation of reactive oxygen species resulting from lipid composition changes. These findings suggest that PPT phytoplasma infection alters the lipid metabolism of host plants, which could contribute to development of yellowing symptoms and reduced photosynthesis efficiency. Understanding these mechanisms could lead to the development of new disease control strategies.

Keywords: polar lipids, thylakoid membranes, lipid composition, ROS, photosynthesis, chloroplast autophagy

Introduction

Phytoplasmas are small phloem-restricted and insect-transmissible bacteria that are responsible for a variety of diseases. Phytoplasma genomes have undergone reductive evolution, resulting in the loss of many genes and metabolic pathways that are essential for cellular life. Despite this reduction in genetic content, phytoplasmas have adapted to their unique niche as plant pathogens and are able to thrive within their host plants. This is believed to be due to the development of specialized strategies for obtaining nutrients from their plant hosts, allowing them to persist and evolve alongside plants over time (Tan *et al*., 2020).

Polar lipids are a vital component of cellular membranes in plants and play a crucial role in maintaining the structural integrity and proper function of these membranes. The unique composition and properties of polar lipids can promote cell signalling, stress responses, and plant hormone synthesis. However, in diseased or stressed plants, the composition and abundance of polar lipids can be altered, leading to changes in membrane structure and function that affect their permeability, make them more susceptible to damage, and disrupt essential cellular processes that depend on the proper function of the membrane (Mir *et al*., 2013). Several studies have shown that phytoplasma infection can lead to changes in the lipid metabolism of host plants, for example, sweet cherry virescence phytoplasma-induced alterations in the levels of polar and nonpolar lipids (Tan *et al*., 2020). These changes in lipid metabolism may reflect alterations in the host plant response to phytoplasma infection, may facilitate nutrient transport from host cells to phytoplasmas and promote their growth and replication. However, the specific roles of these lipids in the interaction between phytoplasmas and their host plants are not yet well understood. The present study aimed to address this knowledge gap by conducting an in-depth analysis of polar lipid composition in phytoplasma-infected plants. The study identified changes in the levels of specific polar lipids, which provided insights into the molecular mechanisms underlying the metabolic reprogramming of host plants in response to phytoplasma infection.

Materials and Methods

The Columbia Basin PPT phytoplasma was initially detected in diseased potatoes in Washington and Oregon. Tomato plants (cv. Moneymaker) were used as an alternative host to establish a PPT phytoplasma infection through grafting inoculation as previously described (Wei *et al*., 2013). To analyse polar lipids in plants infected with PPT phytoplasma,

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the upper leaves with witches' broom symptoms were harvested from both infected plants and not inoculated control plants at three months after grafting. A total of 0.1 gram of leaf tissues was incubated with 0.6 ml isopropanol containing 0.01% butylated hydroxytoluene at 75°C for 15 minutes. The lipids were then extracted by incubation with chloroform/methanol/water (30: 41.5: 3.5) at room temperature for 36 hours. The organic solvents were evaporated by nitrogen gas, and the dried extracts were analyzed by 4000 Q TRAP LC/MS/MS Mass Spectrometer at Kansas Lipidomics Research Center. Splash Lipidomix mass spec standard (Avanti Polar Lipids, Inc. 330707) was used as an internal control. Data were obtained from five biological repeated samples. The leaf samples were stained with 3,3'-diaminobenzidine (DAB) solution (1 mg/ml) overnight, and chlorophyll was removed by 96% ethanol at 40°C. The extracted total proteins from the leaves were either separated using SDS-PAGE and stained with Coomassie brilliant blue (CBB) or transferred onto a PVDF membrane to detect actin proteins.

Results and Discussion

The in-depth analysis of polar lipids in plants infected with PPT phytoplasmas showed a decrease in the overall quantity of polar lipids in the leaves of tomato plants PPT phytoplasma infected compared to non-inoculated controls. Specifically, the levels of three polar lipids including monogalactosyldiacylglycerol (MGDG) and phosphatidic acid (PA), and phosphatidylglycerol (PG) were significantly reduced. While digalactosyldiacylglycerol (DGDG) remained with no obvious changes. MGDG and DGDG are two major glycolipids that are found in the thylakoid membranes of plant chloroplasts. The MGDG/DGDG ratio is an important indicator of the structure and function of thylakoid membranes in chloroplasts. This ratio can vary depending on the growth conditions of the plant and the developmental stage of the chloroplasts (Moellering and Benning, 2011). In PPT phytoplasma-infected tomato plants, the MGDG/DGDG ratio was 4.3, while in not inoculated controls, it was 5.1. This result suggests that phytoplasma infection may have led to a decrease in the MGDG/DGDG ratio in the infected plants. Collectively, the shift in the lipid composition of the thylakoid membranes may be associated with stress conditions in the metabolic pathways involved in lipid synthesis, remodelling, and degradation. In addition, a decrease in MGDG/DGDG ratio could lead to the destabilization of the thylakoid structure, which may result in the production of reactive oxygen species (ROS). ROS accumulation in the infected leaves was confirmed by DAB staining (Figure 1a). When the thylakoid membranes of chloroplasts are damaged by the accumulation of ROS, chloroplast autophagy is triggered to remove and degrade the damaged membranes and proteins. The degradation of chloroplast rubisco large subunit (RbcL) and small subunit (RbcS) is a characteristic feature of chloroplast autophagy. In PPT phytoplasma-infected plants, a significant reduction in the levels of RbcL and RbcS was observed by SDS-PAGE analysis (Figure 1b).

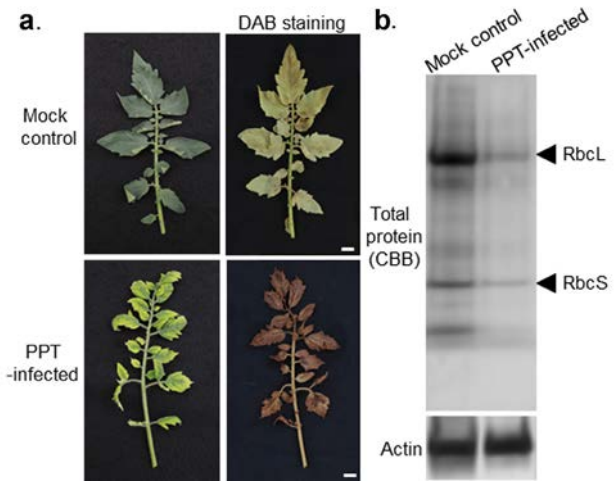


Figure 1. Potato purple top (PPT) phytoplasma induced the accumulation of reactive oxygen species (ROS) and reduced chloroplast rubisco protein levels in tomato plants. ROS accumulation was visualized by 3,3'-diaminobenzidine (DAB) staining (a). Brown coloration represents ROS generation. Rubisco protein levels were assessed by SDS-PAGE using total proteins isolated from leaves (b). CBB: Coomassie brilliant blue.

The disruption of polar lipid composition in phytoplasma-infected tomato leaves can have significant consequences for plant health. The reduction in MGDG levels and subsequent decrease in the MGDG/DGDG ratio could lead to destabilization of the thylakoid membrane structure, resulting in ROS production and damage to chloroplasts. This is supported by the reduction of chloroplast Rubisco proteins, which are known to be degraded during autophagy triggered by ROS accumulation. The alteration of polar lipids in phytoplasma-infected plants can severely hinder the process of photosynthesis, resulting in the emergence of yellowing symptoms and affecting growth and developmental processes. Further studies are needed to determine the underlying mechanisms of the effects of phytoplasma on polar lipid composition and their role in the development of yellowing symptoms, which could lead to the designing of effective strategies to mitigate the impact of phytoplasma infections on crop production.

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Interaction

Exploring changes in volatile organic compounds profiles of tomato plants infected with phytoplasmas

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Abstract

Volatile organic compounds (VOCs) emitted by plants play a crucial role in plant-pathogen interactions. This study investigated the changes in VOC profiles of tomato plants infected with potato purple top (PPT) phytoplasma to better understand the role of each VOC in disease symptom development. VOC profiles were systematically compared between PPT phytoplasma-infected and control tomatoes using gas chromatography mass spectrometry (GC-MS) with electron ionization (EI) mode. Among the VOCs identified in the study, at least eight were differentially emitted by infected and control plants. The work was then continued on a single VOC, α-copaene, which was emitted throughout the experiment, but its level differed in three different infection stages. Further studies will be performed to determine whether changes in VOC emission patterns could be used as a marker to detect plant diseases associated with phytoplasmas. This study provides new insights into the underlying mechanisms of phytoplasma pathogenesis and plant defence.

Keywords: α-copaene, phytoplasma, volatile organic compounds

Introduction

Phytoplasmas are minute bacteria that infect a wide range of plant species, including crops such as grapevines, wheat, and corn, as well as forest trees and ornamental plants. The symptoms of phytoplasma infection can vary greatly depending on the host plant and the strain of phytoplasma involved. Some common symptoms include virescence, phyllody, and witches' brooms (Wei *et al*., 2013). In nature, phytoplasmas are transmitted from one plant to another by insect vectors. Phytoplasma diseases can have significant economic impacts, leading to reduced yields, and reduced quality of products. So far, there are no effective management measures to control phytoplasma diseases.

Volatile organic compounds (VOCs) are a group of organic chemicals that are emitted by plants, as a consequence of their interactions with biotic and abiotic factors (Brilli *et al*., 2019). Some VOCs are used as a defence mechanism against herbivores, pathogens, and other environmental stressors. In addition, VOCs can also serve as signalling molecules, alerting neighbouring plants of a potential threat. These responses are part of a plant's overall defence system and play a role in protecting the plant from damage and ensuring its survival. By analysing the VOCs emitted from infected plants, it is possible to identify specific pathogen presence and possibly determine the level of severity of the infection. Furthermore, information can then be used to develop and implement studies on VOC emissions in plants infected with phytoplasmas (Tan *et al*., 2021). In the present study, the VOC emission profiling (volatilome) of potato purple top (PPT) phytoplasma-infected tomato plants at different symptom development stages, including big bud (BB), witches' broom (WB), and purple top (PT) was investigated. The differential volatile profiles among healthy (mock-inoculated) control and infected plants were determined. Most importantly, compared with other compounds identified in the study, a significantly higher amount of α-copaene was detected in PPT phytoplasma-infected tomato plants, especially at the WB infection stage.

Materials and Methods

PPT phytoplasma-infected tomato plants were established using single graft inoculation (Wei *et al*., 2013). The symptom development in plants was observed daily, and three plants at three infection stages [BB, around 5 weeks post-inoculation (WPI), WB at 10 WPI, and PT at 14 WPI] and three healthy (mock-inoculated) control plants at the same stages were used for VOC analysis. The plant VOCs were collected for 24

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h (16 hours in light and 8 hours in darkness) at room temperature with a dynamic headspace sampling system (Figure 1). The collected VOCs were eluted from the Super Q traps with about 300 µl methylene chloride. All eluents were stored in sealed glass vials in a freezer at -20°C until analyses. The VOCs were analysed by gas chromatographic mass spectrometry (GC-MS) with electron ionization (EI) mode according to the procedure previously described by Zhang *et al.* (1999). The chemical identification of the headspace volatiles was based on a comparison of their mass spectra with the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) and Wiley mass spectral libraries. Identities were confirmed by co-injection, mass spectra, and GC retention times of authentic standards on GC HP-5MS and DB-WAXETR capillary columns.



Figure 1. Plant volatile organic compounds (VOCs) collection by using a dynamic headspace sampling system. The left three are healthy (mock-inoculated) tomato plants, and the right three are potato purple top (PPT) phytoplasma infected plants.

Results

In this preliminary study, a total of 90 samples were collected at three infection stages (BB, WB, and PT) for VOC analysis. A total of 30 samples were collected for each stage, with samples collected from three infected and three healthy control plants every day for five consecutive days. Among the VOCs identified in healthy and PPT phytoplasma-infected tomato plants, at least eight compounds were differentially emitted.

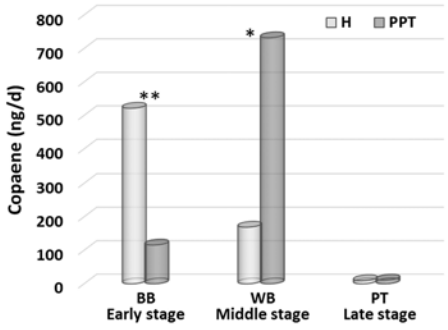


Figure 2. The emission patterns of α-copaene in PPT phytoplasma-infected tomato plants at different symptom development stages. H: healthy (mock-inoculated) plants; PPT: infected plants (**P<0.01 and *P<0.05).

Interestingly, one volatile, α-copaene, exhibited differential emission patterns in infected plants compared with healthy controls at three different infection stages. The α-copaene is a sesquiterpene compound found in a variety of plants and is a known attractant of certain insects. The amount of α-copaene decreased in the BB infection stage and increased in the WB infection stage. At the PT infection stage, α-copaene emission was significantly reduced and cannot be distinguished from that in healthy controls (Figure 2).

Discussion

Diseased plants often emit distinctive VOC profiles, which can provide valuable information about the nature of the disease and the severity of its effects on the plant. Detecting and analysing VOCs produced by diseased plants is an important tool to study plant health status.

These preliminary data showed that variations in VOC emissions in tomato plants infected with PPT phytoplasmas compared to healthy controls might be used as indicators to detect the presence of phytoplasmas and predict plant health status. In addition, among the differentially emitted VOCs, the emission amount and pattern of α-copaene varied significantly at different infection stages. α-copaene is a terpene, a class of natural compounds that is widely distributed in the plant kingdom. Phytoplasma infections decreased α-copaene emission at the BB infection stage (early infection stage manifested by flower deformation), which may be associated with the suppression of plant defence responses by the pathogen. At the middle (WB) infection stage (characterized by the production of more clustered small leaves in the proliferating branches), phytoplasma induced the increased α-copaene emission, which is probably related to plant defence responses to the pathogen or the increased number of leaves emitted more α-copaene. Changes in α-copaene emission can provide important information about the interactions between the pathogen and the plant host and the mechanisms by which phytoplasmas can manipulate plant defence responses. Further studies focusing on much earlier infection stages (before symptom development) will help to better understand the potential role of α-copaene in plant-phytoplasma interactions.

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Interaction

Lectin binding assay reveals phytoplasma infection-induced alteration of plant host protein glycosylation

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Abstract

Glycosylation is a common posttranslational modification that contributes to the activity and stability of diverse eukaryotic proteins. The present study performed a lectin binding assay using four biotinylated lectins and investigated changes in protein glycosylation patterns in tomato plants infected with potato purple top (PPT) phytoplasma. The results revealed that in PPT phytoplasma-infected tomato plants there was an increase in the levels of protein glycosylation involving mannose, galactose, and N-galactosamine glycans, while those of fucose glycans were decreased. Confocal microscopy analysis indicated that the altered glycosylation activities occurred mainly in the phloem tissue of infected plants. These findings suggest that PPT phytoplasma infection can significantly impact the glycosylation patterns of tomato plant proteins, which could provide valuable insights into the mechanisms of plant response to phytoplasma infection and potential strategies for crop protection.

Keywords: phytoplasma, lectin, ConA, glycosylation, tomato

Introduction

Glycosylation is one of the most important posttranslational protein modifications. Glycosylation occurs in diverse eukaryotic proteins and contributes to their folding, stability, mobility, and their abilities to interact with other molecules and mediate signal transduction (Roth *et al*., 2012). There are two types of glycosylation, namely N-glycosylation, and O-glycosylation. N-glycosylation starts in the endoplasmic reticulum and completes in the Golgi apparatus. O-glycosylation biogenesis occurs solely in the Golgi apparatus. Pathogen infection may alter host protein glycosylation, since many pathogenic bacteria secrete glycosidases into host cells. On the other hand, the expressions of glycosylation-related host genes may be up-or down-regulated in response to pathogen infection. Both could lead to changes in host glycosylation.

Phytoplasmas are a group of bacterial pathogens that infect a wide range of plant species and are associated with a variety of symptoms, including stunting, yellowing, witches' broom, and phyllody. Phytoplasmas are known to manipulate the host plant's physiology and metabolism, often by inducing changes in gene expression or signalling pathways. However, the specific mechanisms by which phytoplasmas manipulate host plants are not well understood. The present study investigated changes in glycosylation patterns in phytoplasma-infected plants by utilizing a lectin binding assay with four biotinylated lectins that specifically recognize and react with different sugar residues in glycosylated proteins. The results of the study shed light on how glycosylation patterns are regulated in response to phytoplasma infection and may have important implications for understanding plants defence mechanisms.

Materials and Methods

Tomato (*Solanum lycopersicum*) plants infected with potato purple top (PPT) phytoplasma and mock-inoculated plants were used. Frozen leaves were ground in 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl, 2 mM $MgCl_2$, and a complete protease inhibitor cocktail (CPIC). The resulting supernatants were collected as soluble fractions after centrifugation at 15,000 g for 10 minutes. The pellets were resuspended in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing CPIC. After brief centrifugation, the resulting supernatants were used as membrane fractions. SDS-PAGE was performed on the samples, followed by a reaction with four biotinylated lectins (Table 1).

Table 1. Lectins used and their sugar-binding properties.

Lectin (Abbreviation)	Sugar-binding specificity
Concanavalin A (ConA)	Mannose
Ricinus communis agglutinin 1 (RCA1)	Galactose; N-acetylgalactosamine
Ulex europaeus agglutinin 1 (UEA1)	Fucose
Wheat germ agglutinin (WGA)	N-acetylglucosamine

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The bound lectins were detected by western blot using alkaline phosphatase-conjugated streptavidin. Mouse actin antibody was used as a loading control. Paraffin sections were prepared using stems of PPT phytoplasma-infected and mock control plants. Deparaffined sections were treated with biotinylated ConA and UEA1, followed by visualization with Alexa Fluor 488 conjugated streptavidin. An anti-IDP antibody against the immunodominant protein of PPT phytoplasma was synthesized in another study (W. Wei, unpublished data) to determine the pathogen localization in plants. For visualization, Alexa Fluor 405 conjugated anti-rabbit secondary antibody was used. The Zeiss LSM710 confocal laser scanning microscopy (CLSM) system was utilized to acquire fluorescent images.

Results

Lectins are proteins that bind specifically to carbohydrates and are commonly used to identify glycan structures in complex mixtures. In this study, four lectins that specifically recognize and react to different sugar residues in the carbohydrate side chains (glycans) of glycosylated proteins were used to investigate changes in protein glycosylation patterns of phytoplasma-infected tomato plants (Table 1). The lectin ConA mainly reacts with α-mannose in N-glycosylation, while the lectin RCA1 specifically reacts with galactose and N-galactosamine residues in the glycosylated proteins.

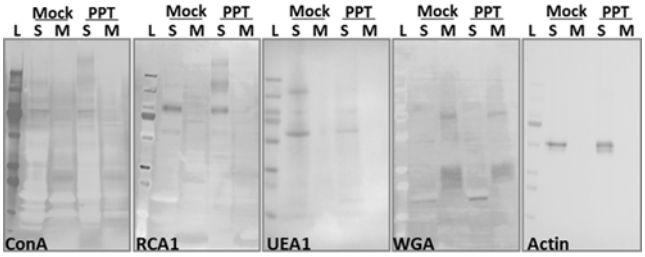


Figure 1. Lectin binding assay. Soluble (S) and membrane (M) protein fractions were isolated from leaf tissues of PPT phytoplasma-infected (PPT) and mock-inoculated (Mock) tomato plants. Biotinylated ConA, RCA1, UEA1, and WGA1 were used to probe targeted glycoproteins. Actin antibody was used as loading control. L: Protein size ladder.

The results of the lectin binding assay showed that higher levels of glycoproteins that react with ConA and RCA1 were observed in PPT phytoplasma-infected plants compared to mock-inoculated plants, as indicated by the relative intensities of the colorimetric detection signals (Figure 1). These results suggest that the host plants enhanced glycosylation activities involving α-mannose, galactose, and N-galactosamine glycans in response to the phytoplasma infection. This is particularly striking for high-molecular soluble glycoproteins. In addition to ConA and RCA1, lectin UEA1 which specifically reacts with fucose in N- and O-glycosylation was also used. The intensity of signals generated from UEA1-reactive glycosylated proteins in PPT phytoplasma-infected plants decreased compared to mock-inoculated plants (Figure 1), indicating a down-regulation of glycosylation involving fucose glycan in response to the infection. WGA is a lectin that specifically recognizes and binds to N-acetylglucosamine and sialic acid residues in glycosylated proteins. WGA did not show appreciable changes in response to PPT phytoplasma infection, except for a slight increase in a low molecular weight soluble protein (Figure 1). Further, confocal microscopy was conducted to pinpoint the sites of differential glycosylation. The results showed that the increased levels of mannose-type (ConA-reactive) glycosylation and decreased levels of fucose-type (UEA1-reactive) glycosylation occurred primarily in the phloem tissue of the infected tomato plants, which is also where the PPT phytoplasma is located.

Discussion

Phytoplasma pathogenesis is a multifaceted process that involves various epigenetic, biochemical, and physiological changes in the host. This study revealed that ConA-reactive, mannose N-glycosylation was upregulated in infected tomatoes. In general, N-glycosylation biogenesis is initiated with oligo-mannose and often ends up with highly branched complex N-glycan. ConA has a high affinity with oligo-mannose but not with complex N-glycans. In human cells, unfolded protein response (UPR) increases oligo-mannose and decreases complex N-glycosylation (Wong *et al*., 2018). As phytoplasma infection may induce UPR in a host (Buxa *et al*., 2015), it would be interesting to learn whether plants have a similar mechanism to modulate N-glycosylation. Overall, these findings suggest that phytoplasmas may manipulate the glycosylation machinery of host plants to promote their own survival and colonization. Understanding the specific glycoproteins and glycan structures that are involved in these interactions, and the molecular mechanisms underlying changes in glycosylation patterns, may provide new insights into the phytoplasma pathogenesis and lead to the development of novel strategies for controlling these devastating plant diseases.

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Interaction

Understanding interactions of '*Candidatus* Phytoplasma solani' with grapevine through the lens of complex networks

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Abstract

'*Candidatus* Phytoplasma solani' is the agent associated with the most widespread grapevine yellows disease, the "bois noir", which causes significant economic losses every year. Understanding the interaction between this phytoplasma and the grapevine disease in question remains an intriguing open problem. New approaches developed to improve the understanding of the infection through complex network-based modelling are presented. This computational modelling allows seamless integration of data from multiple sources and provides insights into relationships between genes or other entities of interest. Two approaches and the newly obtained results that further improve the understanding of "bois noir" and other grapevine yellows are reported.

Keywords: '*Candidatus* Phytoplasma solani', grapevine, "bois noir", RNA-seq, sRNA-seq, miRNA, hormones

Introduction

Systems biology often resorts to computational approaches to better understand individual processes and key molecular players that can provide an expert with new insights. Network-based approaches have been shown to perform well when looking at highly correlated and interconnected biological datasets (Chisanga *et al*., 2017; Zakrzewski *et al*., 2017; Skrlj *et al*., 2021; Dermastia *et al*., 2021). One of the main problems in studying such systems is their heterogeneity – rarely are present networks with only one type (even if they are weighted), which can lead to problems in applying naive methods based on homogeneous networks. To address this shortcoming, the field of heterogeneous network analysis was created (Dong *et al*., 2017; Kralj *et al*., 2018). In addition, there are many biological heterogeneous networks that are used by researchers to investigate new associations and connections between molecular entities.

"Bois noir" is an important economic grapevine yellows disease associated with '*Candidatus* Phytoplasma solani', "stolbur", group 16SrXII-A (Quaglino *et al*., 2013). While the main plant processes involving '*Ca*. P. solani' are now known, the underlying molecular mechanisms of these interactions remain poorly understood. To improve the knowledge of these interactions, an information-rich structure analysis methods was applied to reconstruct networks from genome-wide RNA-seq data to model natural events associated with "bois noir" (Dermastia *et al*., 2021; Skrlj *et al*., 2021).

Materials and Methods

The plant material, phytoplasma detection, RNA extraction and sequencing, and analysis of mRNA and sRNA data are described in Dermastia *et al*. (2021) and Skrlj *et al*. (2021). The data obtained were used to investigate whether new cross-talks between signalling pathways involved in infection of grapevine with '*Ca*. P. solani' could be discovered by network-based modelling of changes in the structure of expression profiles over time. At each time point, data from grapevines infected and uninfected with '*Ca*. P. solani' were

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used to reconstruct regulatory networks. The networks were then compared at the level of node communities, dense substructures commonly associated with specific functional groups.

Results and Discussion

By studying complex networks, it was explored how relationships between entities of interest can provide insights into biological phenomena that affect multiple genes simultaneously. There are several important contributions of this research to the understanding of phytoplasma-host interactions.

It was introduced the measure of community dissipation that provides information about how dispersed a particular community is in time (Skrlj *et al.*, 2021). Using this analysis, it was possible to identify the communities that were most dispersed when comparing the different plant states (uninfected and infected). This method was used with experimental temporal expression data from uninfected and from '*Ca.* P. solani' infected plants. These data confirm the presence of several known gene communities involved in this infective process. They also reveal several new gene communities and their potential regulatory networks that were not previously associated with '*Ca.* P. solani'. To confirm the capabilities of the proposed method, selected predictions were also evaluated empirically.

In addition, Dermastia *et al.* (2021) considered several different approaches to understanding phytoplasma infection. The most important innovation related to network analysis is the simultaneous consideration of mRNA and miRNA molecules. Networks formed by considering these different types of nodes allow computational exploration of the relationships between different types of molecules. Here the work was focused on regulatory pathways that contain small RNA molecules. This type of RNA molecule has not been fully explored but is thought to play a key role in regulating the response to disease. Network-based analysis was performed using reconstructed correlation-based networks of normalised expressions.

The networks were used in this study for interactive exploration of the identified relationships. While the early growth phase in symptomless grapevines was very dynamic at the transcriptional level, regulation at the small RNA level was more pronounced later in the season when symptoms developed in infected grapevine plants. Most differentially expressed small RNAs were associated with biotic stress. This study also reveals the less studied role of hormones in disease development, showing that hormone balance was disturbed in infected grapevines before the symptoms developed. Analysis of gene communities and mRNA-microRNA interaction networks revealed several new genes (*e.g.*, expansins and cryptdin) not previously associated with phytoplasma infectious process. These players may provide a new frame of reference for study and detection of phytoplasma diseases in grapevine.

The network-based modelling presented was successfully applied and led to new hypotheses, some of which could also be confirmed empirically. The analysis of complex networks is a promising approach for the growing field of interaction analysis between phytoplasmas and grapevines. Modelling nodal communities as well as the importance of individual nodes is a useful computational approach that serves as a complementary evidence pillar to more conventional methods that neglect the rich interactions otherwise present in real systems.

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Interaction

First insights into the genome of '*Candidatus* Phytoplasma rubi' highlight effector protein repertoire of 16SrV phytoplasmas

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Abstract

The elm yellows phytoplasma group include phytopathogenic strains associated with important insect vector-borne diseases in grapevine, alder, elm, Chinese jujube and *Rubus* spp. such as raspberry and blackberry. The *Rubus* stunt disease is associated with the presence of '*Candidatus* Phytoplasma rubi'. In contrast to other 16SrV phytoplasma taxa "flavescence dorée" and '*Ca.* P. ziziphi', no genome sequence of this phytoplasma is available. The complete genome of '*Ca.* P. rubi' strain RS enabled a comprehensive analysis of the derived protein content. First analyses revealed a group-specific 16SrV metabolism as well as a diversified secretome that includes two conserved effector proteins.

Keywords: 16SrV, *Rubus*, complete genome, effector proteins

Introduction

'*Candidatus* Phytoplasma rubi' belongs to the 16SrV-group, also known as elm yellows group (Malembic-Maher *et al.*, 2011). This insect vector-borne bacterium is associated to the *Rubus* stunt disease in raspberries and blackberries. Beside stunting, cultivars show proliferation, enlarged sepals, phyllody, reduced or impaired fruits, leading to decline and total failure in many varieties and high economic losses. Despite its importance for small and large producers, only a few sequence data on this pathogen have been available so far. The complete genome sequences of "flavescence dorée" and '*Ca.* P. ziziphi', have been published (Debonneville *et al.*, 2022; Wang *et al.*, 2018), and recently '*Ca.* P. rubi' strain RS from Baden-Württemberg (Germany) was added to this collection (Duckeck *et al.*, unpublished). This dataset provides an excellent data basis for a comparative analysis of the metabolic pathways and the proteins involved in the pathogen-host interaction of phytoplasmas from the 16SrV group. First insights into the analysis are presented.

Materials and Methods

The shared and unique genetic repertoire of '*Ca.* P. rubi' strain RS (GenBank accession number CP114006), "flavescence dorée" strain CH (GenBank accession number CP097583), '*Ca.* P. ziziphi' strain jwb-nky (GenBank accession number CP025121) was determined using the Orthologous Matrix Algorithm 2.0 (Altenhoff *et al.*, 2021). Metabolic reconstruction was performed in KEGG (Kaneshisa and Goto, 2000) and Metacyc (Caspi *et al.*, 2014). Prediction of the secretome was achived by analysing the deduced proteins with respect to transmembrane domains and signal peptides in Phobius (Käll *et al.*, 2004). The proteins were classified into three categories comprising peptide sequences with transmembrane helices and/or signal signatures.

Results

The analysis of the three complete genomes of 16SrV-group phytoplasmas illustrates the phylogenetically separate position of this branch in the metabolism and secretome. With 37 proteins carrying only one signal peptide in the genomes studied, the '*Ca.* P. rubi' encodes the highest number of secreted proteins, followed by '*Ca.* P. ziziphi' with 24 and "flavescence dorée" with 12, the fewest predicted proteins. In the category of proteins with signal peptides but no transmembrane domain, a common secretome of 5 proteins was identified between the three phytoplasmas (Figure 1). The highest number of shared proteins of 8 within this group

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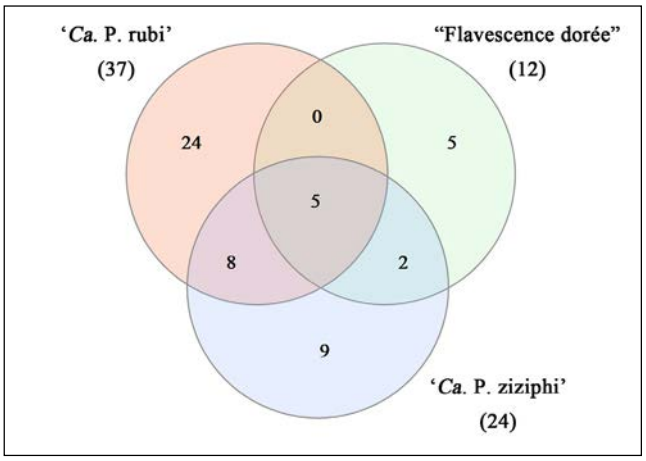


Figure 1. Shared proteins with signal peptide but no transmembrane domain. Encoded proteins with signal signature of '*Ca.* P. rubi' (orange) were compared with those of "flavescence dorée" phytoplasma (green) and '*Ca.* P. ziziphi' (blue) using Orthologous Matrix algorithm 2.0 (Altenhoff *et al.*, 2021) and Phobius signal signature prediction (Käll *et al.*, 2004). The total number of shared proteins between two or three genomes is displayed as a Venn diagram. The total number of predicted proteins is in brackets. Pseudogenes were integrated into the analysis. The Venn diagram was generated with InteractiVenn (Heberle *et al.*, 2015).

is obtained for '*Ca.* P. rubi' and '*Ca.* P. ziziphi'. A few experimentally validated effectors have been described for 16SrV phytoplasmas. SJP1 and SJP2 of '*Ca.* P. ziziphi' (Zhou *et al.*, 2021) could be identified in '*Ca.* P. rubi', while they are absent in the "flavescence dorée" phytoplasma. The two effectors were found to be Sap11-like and cause proliferation, a typical symptom of the shoot and root system of rubus stunt.

Discussion

'*Ca.* P. rubi' shares conserved transport systems, lipid and energy metabolism with other 16SrV phytoplasmas, but differences in proteins released from the cell became apparent. This prediction of proteins processed through the Sec-dependent secretion pathway is of particular interest with respect to their effects on host interactions and disease induction. Several experimentally validated effectors have been described for phytoplasmas (Zhou *et al.*, 2021; MacLean *et al.*, 2011; Sugio *et al.*, 2011). Some are associated with proliferation as SAP11 (Zhou *et al.*, 2021; Sugio *et al.*, 2011) or phyllody as SAP54 (MacLean *et al.*, 2011). The proliferation-associated effectors SJP1 and SJP2 of '*Ca.* P. ziziphi' are inconsistently shared by the 16SrV phytoplasmas, they occur independently in at least two taxa and may therefore have been lost in others. The coding of these proteins in '*Ca.* P. rubi' suggests the origin of proliferation and stunting symptoms in infected *Rubus* spp. and experimental hosts such as *Catharanthus roseus*.

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Interaction

Identification of a "flavescence dorée" phytoplasma VmpA candidate receptor in the experimental insect vector, *Euscelidius variegatus*

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Abstract

The "flavescence dorée" phytoplasma initiates the infection process using the adhesin VmpA to adhere to insect vector cells although the insect receptor for this protein still remains unknown. *In vitro* interaction assays were performed using recombinant VmpA-His_6 and cultured cells of the experimental vector *Euscelidius variegatus*. It was possible to identify thirteen interesting putative VmpA targets that have been tested for their role as VmpA receptors in cultured cells subjected to RNAi. These preliminary results show the implication of an unidentified protein containing several leucine-rich repeat domains in the adhesion of beads coated with recombinant VmpA to vector cells in culture.

Keywords: adhesion, insect cells, "flavescence dorée" phytoplasmas, *Euscelidius variegatus*

Introduction

"Flavescence dorée" (FD) phytoplasmas are transmitted from grapevine to grapevine in vineyards by the leafhopper *Scaphoideus titanus*, but the model system consisting of broad bean and the leafhopper *Euscelidius variegatus* results more convenient for studying this pathosystem in controlled conditions (Caudwell *et al.*, 1972). Phytoplasmas need to adhere to the surface of insect cells, penetrate the cells and go through in order to complete the multiplicative and circulative cycle into their vectoring insects. For the FD phytoplasma, variable membrane protein A (VmpA) was shown to be an adhesin that binds glycoconjugates at the surface of the insect cells (Arricau-Bouvery *et al.*, 2021). Furthermore, ecological and genetic insights into the emergence of the grapevine FD epidemics in Europe have shown that compatibility of FD phytoplasma strains with different subfamilies of insect vectors is correlated with *vmpA* gene sequence (Malembic-Maher *et al.*, 2020). Identifying the receptor for VmpA in the insect vector could not only improve the basic knowledge of the early stages of infection, but can also help as a tool for the prediction of new potential emergent vector species. This study focus on the research and the screening for VmpA putative targets, using an *in cellula* model consisting in *E. variegatus* cultured cells.

Materials and Methods

Protein extracted from the *E. variegatus* cultured cells Euva-11 using RX buffer (Suzuki *et al.*, 2006) have been incubated overnight with recombinant protein VmpA-His_6 of the FD phytoplasma strain FD92. The VmpA-Euva-11 proteins complexes have been then purified in an affinity Nickel column and the fractions containing the retained proteins have been sent to proteomics platform of the University of Bordeaux (France) for mass spectrometry analysis. A database consisting in *in silico* translated transcriptome of *E. variegatus* has been used as reference for peptides identification. The matching putative proteins were screened basing on their VmpA specific retention ratio, the prediction of potential N-glycosylation sites in their sequence (NxS/T) as well as the presence of transmembrane domains (TMHMM 2.0). To increase the number of membrane proteins identified by MS analysis another experiment on protein extracts from Euva cells has been performed. It was implemented the RX buffer with DOC and with an increased concentration of Triton X-100 (RX-T-DOC). A far western blot anti VmpA has been performed on these samples and the interesting bands have been excised on the corresponding Coomassie gel and sent to the proteomics platform for mass spectrometry analysis.

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A selection of putative VmpA targets followed as described previously.

RNA interference experiments have been performed on Euva11 using double strand RNA (dsRNA) of about 500 bp for each putative target and a transfection reagent. dsRNA of the GFP sequence has been used as a control. Ratio of transcripts inhibition has been assessed using RT-qPCR and normalised using the insect reference gene glutathione S-transferase (GST). Adhesion assays on inhibited Euva11 cells have been performed incubating the cells three days post transfection with amine-modified fluorescent beads coated with recombinant VmpA-His_6 for one hour. Twenty fields were randomly observed per condition using a Zeiss AxioImager epifluorescent microscope. The ratio of retained beads per cell has been then calculated and compared to the control condition (Kruskal-Wallis test, p-value 0,05).

Results

The selection process of Euva-11 proteins extracted with RX buffer and interacting with VmpA resulted in the identification of four candidates identified through BLASTn as endoplasmin, epidermal growth factor receptor, tumor necrosis factor wengen and ubiquitin E3 ligase HERC4 –like. Nine putative candidates from Euva-11 proteins extracted with RX-T-DOC buffer and interacting with VmpA were identified through BLASTn as unidentified protein 1 with LRR domains, unidentified protein 2 toll-like receptor like, unidentified protein 3, integrin β, draper, scavenger receptor class B CD36, fasciclin, Na/Ca exchanger, and cueball LDL like protein. The inhibition of the target transcripts in *E. variegatus* cultured cells trough RNAi has been achieved, with variable efficacy depending on the target (fold change values in gene expression ranging from 2 to 80 times). Adhesion assays were performed using fluorescent VmpA-His_6-coated beads on dsRNA treated Euva-11 cells targeting each of the 13 selected genes. Statistically significant differences have been observed between the number of beads adhering to Euva-11 cells treated with dsRNA targeting the gene coding for the unidentified protein 1 with LRR domains when compared to control cells treated with dsRNA targeting green fluorescent protein (GFP).

Discussion

These results show that the unidentified protein 1 probably interacts with VmpA allowing adhesion of coated beads to *E. variegatus* cultured cells. This protein is predicted to be a transmembrane protein exposed to the surface of the cell and anchored in the plasma membrane by a transmembrane portion of 22 amino acids in its C-term part. An homology search (Interpro) has revealed the presence of four LRR domains of 219, 163, 80 and 146 aa (from N-ter to C-ter sequence). Furthermore, structural prediction (Robetta, BakerLab) revealed a horseshoe-shaped molecule consisting of parallel β-strands on the inner (concave) side and helical elements on the outer (convex) sites. Proteins with such a structure are frequently implicated in protein-protein interaction (Kobe and Kajava, 2001) and LRR domains are evolutionarily conserved in many pattern recognition receptors. Several studies have shown how LRR-only proteins are implicated in the binding with pathogen associated molecular patterns (PAMPs), which supports the obtained data suggesting that the unidentified protein 1 can act as a receptor for VmpA, and in the regulation of immune effectors (Zhang *et al*., 2022; Wang *et al*., 2016a, 2016; Sriphaijit *et al*., 2007). Nevertheless, these results show only a partial inhibition of beads adhesion and a simultaneously inhibition of several *E. variegatus* LRR proteins could be interesting to investigate the possible redundancy of VmpA receptors in insect vector cells.

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Interaction

Phenotyping a grapevine population segregating for resistance to "flavescence dorée" disease

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Abstract

A segregating population obtained by crossing Chardonnay and Tocai friulano, two varieties with very different susceptibility to "flavescence dorée" (FD), was studied to dissect the genetic basis of FD resistance. The visual observations in the vineyard of FD-infected F_1 individuals allowed to verify the segregation of the characters and to evaluate and measure traits associated with the low or high susceptibility to the disease. Furthermore, genotyping by sequencing was used to genotype the F_1 offspring and a linkage map was subsequently constructed.

Keywords: "flavescence dorée", genotyping by sequencing, phenotyping, susceptibility

Introduction

"Flavescence dorée" (FD) is a destructive grapevine disease that occurs in many European winegrowing regions. Symptoms include rolling and decolouration of leaves, flower and bunch shrivelling, lack of cane lignification, and progressive decline of the plant. Different levels of susceptibility to FD were observed among grapevine cultivars. Previous evidence have made it possible to identify cultivar Chardonnay as highly susceptible, and Tocai friulano, grown in North-East Italy, as partially resistant (Casarin *et al*., 2023).

In the present work, an F_1 population, obtained by crossing these two cultivars, was studied to dissect the genetic basis of interest. The observations in the vineyard of FD-infected F_1 individuals allowed to evaluate and quantify the parameters associated with the low or high susceptibility to the disease. Furthermore, genotyping by sequencing (GBS) was used for genotyping and to construct a genetic linkage map.

Materials and Methods

In 2011 and 2017, Chardonnay was crossed with Tocai friulano producing an F_1 offspring of over 600 individuals. A subset of 50 individuals and the two parentals were grafted and multiplied, and finally planted in a vineyard in Alba (Piedmont, Italy), with 24 plants per individual using a randomized block design. During 2019 and 2020 growing seasons, more than 6,000 insect vectors (*Scaphoideus titanus*) were used to infect the plants, forced to feed in one net-cage per plant. Phytosanitary surveys were carried out in the vineyard to evaluate symptomatic plants in two moments of the growing season: in July (2020, 2021, 2022) and in September (2021, 2022). Spearman's rank analysis was performed on the data collected with R corrplot package.

Total RNA from cortical scrapings of the trunks of 40 FD-infected plants belonging to seven F1 individuals and the two parentals was extracted and analysed as described in Casarin *et al*. (2023). In the meantime, 184 seedlings and the two parentals were genotyped using a GBS modified protocol (Elshire *et al*., 2011). After SNP (single nucleotide polymorphism) filtering, the SNP set and 10 SSRs (used for cross checking) were converted in cross-population segregating type codes suitable to construct the linkage map with JoinMap 4.0.

Results

Although the low number of individuals observed (50), a high variability of the phenotype traits was observed in the

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F_1 population, which is a very good basis for QTL identification. Surveys conducted in three consecutive years in July focused on eight traits (Figure 1). In all the three years a strong negative correlation between vigour and symptom severity was observed. Vigour was negatively and significatively correlated with the degree of downward rolling of the leaves in 2020 and with the rubber consistence of the canes in 2022; in the remaining years the correlation was always negative, but not statistically significant. The surveys performed in September (2021, 2022) considered the traits mainly linked to the canes (lignification, number of symptomatic and leafless canes, desiccation of cane apexes, symptom severity). A significant positive correlation was observed between symptom severity and the number of symptomatic canes in both years. Two positive relationships were found in 2021 between non-lignified canes and both percentage of symptomatic canes and symptom severity. Furthermore, dried canes were negatively correlated with non-lignified canes in both years.

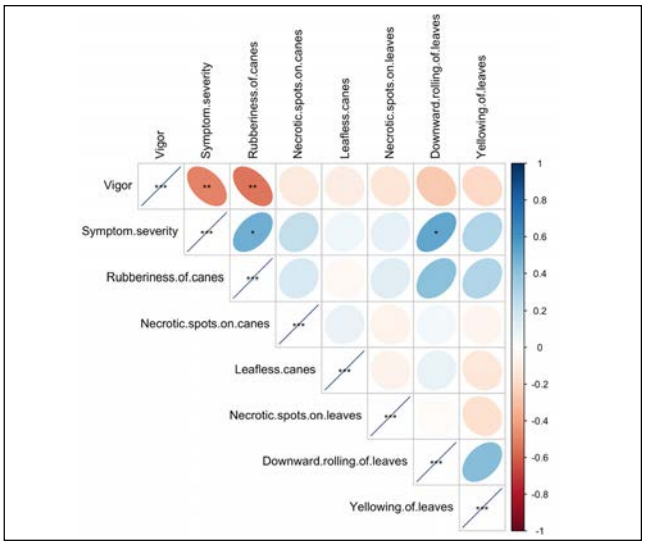


Figure 1. Spearman's rank correlation coefficients between pairs of phenotypes measured in July 2022. Positive and negative correlations are shown; *; **, and *** - significant at p < 0.05, 0.01, and 0.001, respectively.

Seven F_1 individuals showing a medium level of disease severity were selected for measurement of the mean FD phytoplasma (FD) titer in the trunk of infected plants. A clear relationship emerged between the severity of the infection and the titer of FD phytoplasma. Indeed, F_1 individuals showing low disease severity also exhibited the absence or low titer of FD phytoplasma, similarly to Tocai friulano. As FD severity increased, a higher FD phytoplasma titer was found, albeit in the absence of a linear correlation. The GBS analysis produced 472 million reads, with an average read pair count per sample of 2.4 million and 122,049 polymorphisms were found. After filtering, 8,556 SNPs and ten SSRs were used for genetic map construction. The *consensus* linkage map displayed 2,923 markers, 201 being co-localized. The total length of the map was 1336.05 cM.

Discussion

Up to now, the evaluation of the different levels of susceptibility between *V. vinifera* cultivars has involved the use of a few parameters, such as the development of symptoms, the titre of the phytoplasma, and the number of infected plants (Eveillard *et al.*, 2019; Ripamonti *et al.*, 2021). In the present work, several traits were evaluated, which could be combined with those available, to find out an efficient and reliable method to be applied in the vineyard to track FD susceptibility in a large population.

Vigour, necrotic spots on the leaves, differential yellowing of the leaves, rubberiness of canes and downward rolling of the leaves could be interesting traits to measure in the full growing season, while one-year-old cane should be evaluated in September. Furthermore, the quantification of FD phytoplasma titre in the trunk of infected plants seems to be an additional parameter related to the disease severity, as less susceptible individuals harboured lower FD phytoplasma titre.

Genetic features responsible for the low susceptibility to FD could be found combining phenotyping and genotyping of a segregant population. The map obtained in this work showed comparable results with the densest maps published so far (Sapkota *et al.*, 2019), suggesting successful clustering and sorting of the SNP data points. Collectively, the reported results will be used to evaluate susceptibility to FD in a larger number of F_1 individuals and, finally, to achieve QTL identification.

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Omics

Towards a metabolomic characterization of the grapevine response to "flavescence dorée" infection by NMR and LC-MS profiling

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Abstract

"Flavescence dorée" (FD) is a major threat to vineyard sustainability in different European grape-growing areas. This quarantine disease involves interactions between plants, leaf hopper vectors and FD phytoplasma. It was developed an innovative protocol for the inoculation of FD phytoplasma under controlled conditions and it was shown that there are differences in susceptibility between grapevine varieties. The present study aims at determining the primary and secondary or specialized metabolites whose contents are modified in the grapevine following infection by FD phytoplasmas under controlled conditions to identify metabolites or pathways that are associated with a better resistance to FD. Preliminary results are presented.

Keywords: "flavescence dorée", metabolome, plant susceptibility, ^{1}H-NMR, LC-MS

Introduction

"Flavescence dorée", a severe epidemic quarantine disease of grapevine present in different European grape-growing areas, is associated with phytoplasmas (Boudon-Padieu, 2002). Experiments under controlled conditions have revealed differences in susceptibility between grapevine varieties (Eveillard *et al.*, 2016). Thus, Cabernet Sauvignon (CS) is highly susceptible to FD with many infected plants and high FD phytoplasma titers, while Merlot (M) is poorly susceptible to FD with a few infected plants and low FD phytoplasma titers. The objective of the study is to determine the primary and secondary or specialized metabolites whose contents are modified in the grapevine following infection by FD phytoplasmas under controlled conditions. The comparison between healthy and infected plants on one hand, highly susceptible and poorly susceptible plants on the other hand, will make it possible to identify metabolites or pathways associated with resistance to FD.

Materials and Methods

Phytoplasma strains, plants and insects

Scaphoideus titanus were infected with the FD phytoplasma strain PEY as described in Eveillard *et al.* (2016). In a high confinement greenhouse, six healthy and six FD phytoplasma-infected *S. titanus* were deposited for one week on the fifth leaf from the apex of grapevine (*Vitis vinifera*) varieties CS and M. Negative controls were without insects. The leaves which were in contact with insects were collected one-week post-inoculation (1 wpi) on half of the plants and 7 weeks post-inoculation (7 wpi) on the other half of the plants and immediately frozen in liquid nitrogen. At 7 wpi, the fifth leaves from the apex were also collected.

Nucleic acid extraction and phytoplasma quantification

Plant DNA extraction and phytoplasma quantification by qPCR were done using a described protocol (Eveillard *et al.*, 2016).

Metabolomic analyses of leaf extracts

Metabolomic analyses of methanolic extracts were performed on a selection of the leaf-sample methanolic extracts by ^{1}H-NMR at 500 MHz (adapted from Deborde *et al.*, 2019), LC-LTQ/Orbitrap-HRMS (adapted from Musseau *et al.*, 2020), and LC-QqQ-MS/MS (adapted from Loupit *et al.*, 2020).

Data management

The phytoplasma titer, metabolite quantification or metabolomic signature data of leaf samples were managed with ODAM tool (Jacob *et al.*, 2020; https://pmb-bordeaux.fr/dataexplorer/?ds=metabofla1). Multivariate and univariate statistical analyses were performed to mine the metabolomic profile data.

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Results

Phytoplasma titer determination showed as expected that at 1 wpi, all leaves in contact with FD phytoplasma-infected *S. titanus* were positive for the presence of the phytoplasma for the two grapevine varieties with no statistical differences between CS and M. At 7 wpi, the leaves in contact with FD phytoplasma-infected *S. titanus* showed higher phytoplasma titers in CS than in M. The fifth leaves collected at 7 wpi were also tested but none was positive for the presence of FD phytoplasma in M and only 9% of these leaves were positive in CS with a titer similar to the one observed at 1 wpi, showing FD phytoplasma diffusion along the plant.

Protocols for metabolomic analyses, based on ^{1}H-NMR, LC- HRMS and LC- MS, for extraction, chemical analysis, spectra processing, data and metadata management were tested and adapted. They allowed the identification of 21 primary metabolites (amino acids, soluble sugars, organic acids) and 29 specialized metabolites (flavonoids, stilbenes and phenolic acids) using in-house NMR and LC-MS databases. Visual observations of the NMR spectra and a principal component analysis (PCA) revealed several tendencies, for example grapevine variety effects and infection effects in M (Figure 1). These effects were reflected in a variation in the content of certain metabolites.

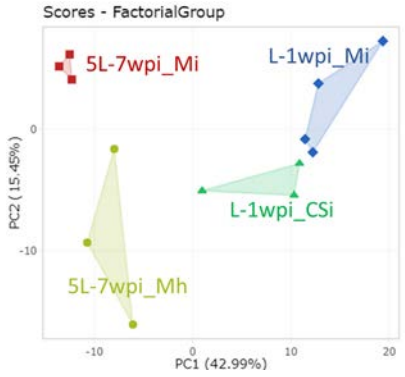


Figure 1. PCA scores plot of primary metabolites data for the leaves sampled 1 wpi for FD phytoplasma-infected CS and M (L-1wpi_CSi and Mi respectively), and for the fifth leaves from apex sampled 7 wpi for healthy and FD phytoplasma-infected M (5L-7wpi_Mh and Mi respectively) (PCA on 284 ^{1}H-NMR variables after constant sum normalisation and unit-variance scaling).

Discussion

Phytoplasma infection induces transcriptomic and metabolomic changes in plants (Bertazzon *et al.*, 2019; Dermastia, 2019; Margaria *et al.*, 2014; Prezelj *et al.*, 2016).

Here, the metabolome of healthy and infected grapevine on one hand, and highly susceptible and poorly susceptible grapevine on the other hand were compared. The preliminary results are encouraging as 36 metabolites could be identified and some differences were observed. This finding will make it possible to identify metabolites or pathways associated with a resistance to FD. The latter could be used as biomarkers of response to infection. For the annotation of still unknown signals, further NMR and LC-MS acquisitions will be performed. The use of one M close relative genotype, poorly susceptible to FD, will be useful for the comparison. These data will allow a better understanding of grapevine-FD phytoplasma interactions and will be useful in the context of varietal selection.

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Omics

Insights into grapevine gene responses to ''flavescence dorée" phytoplasma

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Abstract

"Flavescence dorée" (FD) is a severe disease affecting grapevines associated with the presence of "flavescence dorée" phytoplasmas. After extensive surveys in vineyards and experiments in controlled conditions, it was shown that differential susceptibilities to FD exist among grapevine varieties, such as Cabernet Sauvignon (CS) (highly susceptible) and Merlot (M) (less susceptible). The objective of this study is to investigate the relative gene expression in response to FD phytoplasma infection in CS and M under controlled conditions, and to identify genes or pathways associated with higher resistance to FD. Using high-throughput qPCR targeting defence genes, the study showed significant differences in gene expression between infected and healthy plants, and between highly and poorly susceptible plants, revealing the role of specific genes in plant defence. These preliminary results provide new insights into the mechanisms underlying the contrasting FD susceptibility in grapevines, and lay the foundation for further research.

Key words: transcriptome, plant susceptibility, gene expression, plant defence, Cabernet Sauvignon, Merlot

Introduction

"Flavescence dorée" (FD) is a severe and widespread quarantine disease affecting grapevines in various European grape-growing regions (Boudon-Padieu *et al.*, 2002; Chuche and Thiéry, 2014). This disease is associated with "flavescence dorée" phytoplasmas and differential susceptibility exists among grapevine varieties, with Cabernet Sauvignon (CS) being highly susceptible and Merlot (M) being poorly susceptible (Eveillard *et al.*, 2016). Previous studies have demonstrated transcriptomic and metabolomic changes in plants infected by phytoplasmas (Bertazzon *et al.*, 2019; Dermastia, 2019; Margaria *et al.*, 2014; Prezelj *et al.*, 2016). The purpose of this study is to investigate the relative gene expression in response to FD phytoplasma infection in highly (CS) and poorly (M) susceptible grapevine varieties under controlled conditions. A high-throughput qPCR (Fluidigm©) and the NeoViGen chip allowing the expression profiling of 93 genes related to grapevine defence pathways were used (Dufour *et al.*, 2016).

Materials and Methods

Phytoplasma strains, plants and insects

In this study, 7 CS and 10 M grapevine plants were inoculated on one leaf by the FD phytoplasma strain PEY using *Scaphoidus titanus* as described in Eveillard *et al.* (2016). In a high confinement greenhouse, for each grapevine variety, healthy or FD phytoplasma-infected *S. titanus* were placed on the fifth leaf from the apex for one week. Six weeks post-inoculation these leaves were collected from each plant and immediately frozen in liquid nitrogen for later analysis. The other parts of the plant were cut into sections and stored for phytoplasma quantification.

Nucleic acid extraction and phytoplasma quantification

Plant DNA extractions and phytoplasma quantifications by qPCR were done using the protocol described in Eveillard *et al.* (2016).

Transcriptomic analyses of leaf extracts

The RNA extraction and reverse transcription steps were performed as described in Dufour *et al.* (2016). Four housekeeping genes were used as internal standards (*EF1g*, *THIORYLS8*, *TIP41* and *TubA*) and the final qPCR was realized by the GenoToul platform of INRA Toulouse with the FLUIDIGM BIOMARK HD System using Dynamic Array IFCs.

Data analysis

RQ (relative quantification or fold change) was calculated in an Excel table. Nparcomp t.test was used to assess the significance of the 3 biological replicates. Principal component analysis (PCA) was done using R-studio (R-Studio, 2015).

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Results

No phytoplasma was detected in the leaves of the plants that were in contact with healthy insects. Three CS out of the 7 infected and 3 M out of the 10 infected were selected. They had similar FD phytoplasma concentrations in the leaf in contact with insects with means of 1.40E+04 ±1 FD phytoplasma cells/mg FW in CS and 7.61E+03 ±2 FD phytoplasma cells /mg FW in M.

Among the 88 tested genes involved in defence pathways, 24 are commonly modulated in CS-I, M-I and M-H, compared to CS-H (Figure 1a). Venn diagram also highlights specific gene expression for each plant condition. For example, infected-Merlot shows an overexpression of genes involved in JA pathways (*JAR, JAR2, Lox3*) whereas healthy-Merlot modulates genes involved in salicylic acid and ethylene pathways (*SAPB2a, EDS1c*).

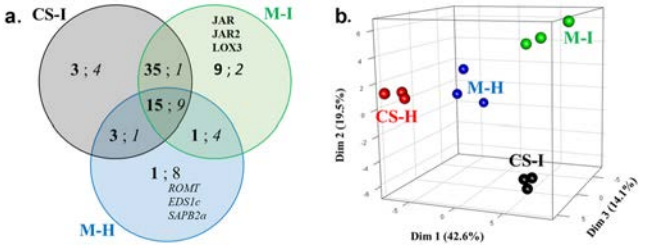


Figure 1. (a) Venn diagram of significant relative expression of genes (log 2) in leaves of infected CS (grey), healthy M (blue) and infected M (green), compared to healthy CS leaves. Overexpression: bold. Repression: italics. (b) 3D representation of Principal Component Analysis (PCA).

The first three dimensions of the principal component analysis (PCA) represent 76.2% of explained variances (Figure 1b). It allows to discriminate the varieties (CS or M), as well as the infectious status (infected or healthy). Indeed, healthy and infected plants are separated on the first dimension according to differential expression of genes coding PR protein (*CHIT3/PR8, PR4, PR7, PR10),* or involved in the flavonoid pathway (*CHORM, CHI, CHS*). The second dimension highlights varieties difference for FD phytoplasma-infected plants, with differential expression of genes such as PR protein (*PR3, PR14*), and genes involved in lignin synthesis (*CAD CAGT, PECT2, APOX*), or ethylene pathway (*EDS1, ACC, ACo1*). Finally, the third dimension highlights varieties difference for the healthy condition, with genes like *PR1bis*, *CAGT* and *NrT2* explaining these differences. Hierarchical clustering analyses confirm these results (data not shown).

Discussion

The preliminary results highlight the role of specific genes in plant defence against FD phytoplasma infection and could lead to the identification of markers associated with resistance. These results are in agreement with RNAseq data originating from the same plants (not presented) and with data already described (Bertazzon *et al.*, 2019; Margaria *et al.*, 2014) showing that the transcriptomic profiles of grapevines show significant differences between healthy and phytoplasma-infected plants, as well as between highly susceptible and poorly susceptible varieties. Other high-throughput qPCR (Fluidigm ©) will be done with more sample replicates from experimental inoculation and with CS and M samples collected in vineyard to confirm the involvement of the identified genes in the specific response to FD. The transcriptomic aspect will be further investigated by incorporating the study of primary metabolism (Bodin *et al.*, 2020).

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Omics

Overexpressing a molecular target of SAP11$_{CaPM}$ in apple

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Abstract

The bacterial effector SAP11$_{CaPM}$ can bind several members of the TCP transcription factor gene family. To investigate the role of the interaction in the infection process, apple plants stably overexpressing the *MdTCP4a* gene were generated and infected with a '*Candidatus* Phytoplasma mali' strain. Preliminary results show a statistically significant lower concentration of the phytoplasma in the aerial parts of the *in vitro* transgenic lines than in the non-transformed "Gala" plants, suggesting that the overexpression of *MdTCP4a* gene could limit phytoplasma multiplication. Interestingly, soil-acclimatized transgenic plants displayed phenotypic characteristics similar to the symptoms of apple proliferation.

Keywords: '*Candidatus* Phytoplasma mali', effector proteins, TCP transcription factor, *Malus domestica*

Introduction

In the last years, research focused on effector proteins allowed the discovery of several new effectors and shed light on their mechanisms of action, providing insight into the complex interplay between phytoplasmas and their plant hosts. Effector proteins secreted by phytoplasmas are diverse and multifunctional, and their effects appear to range from suppressing plant immune responses, altering plant hormone levels, disrupting cell division, and inducing abnormal development. SAP11 is among the first phytoplasma effectors discovered and characterized. Its homolog in '*Candidatus* Phytoplasma mali', often referred to as SAP11$_{CaPM}$, was shown to bind several members of the TCP transcription factor gene family (Janik *et al.*, 2017; Strohmayer *et al.*, 2021; Mittelberger *et al.*, 2022). These genes play a crucial role in regulating plant morphogenesis, flowering, leaf development, and responses to abiotic and biotic stresses (Danisman, 2016). However, there is limited knowledge of their specific functions in apple since most of the data derive from studies on model plant species. In the context of a better understanding of the role of *TCP4a* gene in *Malus domestica* and its deactivation in apple proliferation, transgenic plants stably overexpressing the gene have been generated and characterized. Here it is presented the preliminary results of the infection of the generated transgenic plants with '*Ca.* P. mali', to test the hypothesis that the deactivation of *TCP* gene transcription factors by the bacterial effector SAP11$_{CaPM}$ is crucial for a successful infection.

Materials and Methods

The full-length coding sequence of the *MdTCP4a* gene of *M. domestica* cultivar Golden Delicious under control of the *Cauliflower Mosaic Virus* 35S promoter was transformed into *M. domestica* cultivar Gala via *Agrobacterium*-mediated transformation. The relative expression of *MdTCP4a* gene in both *in vitro* and soil-acclimatized plants was estimated by a quantitative PCR assay (qPCR). Three transgenic lines and "Gala" for comparison were infected with '*Ca.* P. mali' by *in vitro* micrografting with infected material of *M. domestica* cultivar Golden delicious plants as described by Jarausch *et al.* (1999). A minimum of ten replicates per line was performed. After 40 days of graft contact, scions were separated from the rootstocks and partitioned into two/three subgroups each, and subcultured to generate a final set of 25 single infected plants per line. After 30 additional days of culture DNA was extracted from the aerial parts of the plants and used in a qPCR assay to detect the presence of the phytoplasma as described by Baric and Dalla Via (2004) by amplifying the phytoplasma 16S rRNA gene and the *M. domestica* chloroplast gene coding the tRNA leucine. The Cq values of target and reference, calculated on technical triplicates, were combined to calculate the ΔCq value for each plant and these values were used to estimate the relative phytoplasma titer with the formula $x=2\Delta Cq$ (Silver *et al.*, 2006). The values thus obtained were multiplied by 100,000 to facilitate data visualization.

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Results

The relative expression of *MdTCP4a* gene of the transgenic lines varies between *in vitro* and *ex vitro*: the first show a moderate increase or slight decrease compared to non-transformed shoots, while the in soil-acclimatized ones display a 6- to 10-fold increase of the *MdTCP4a* gene transcript. These results reflect on the plants' phenotype as well: the 35S::*MdTCP4a* plants did not display phenotype changes after more than a year of micropropagation while kept *in vitro*, shortly after the soil acclimatisation the transgenic lines displayed loss of apical dominance, smaller leaves and shorter stems compared to the non-transformed (Figure 1). Interestingly, these phenotypic characteristics disappeared approximately six months after the appearance.



Figure 1. Phenotype characteristics of transgenic apple plants (cv Gala) overexpressing the *MdTCP4a* gene two months after the soil acclimatisation, compared to a non-transformed "Gala" plant. Line IA.2 shows smaller and crinkled leaves compared to non-transformed, while lines IB.3 and IIIA show a loss of apical dominance and are shorter than the non-transformed.

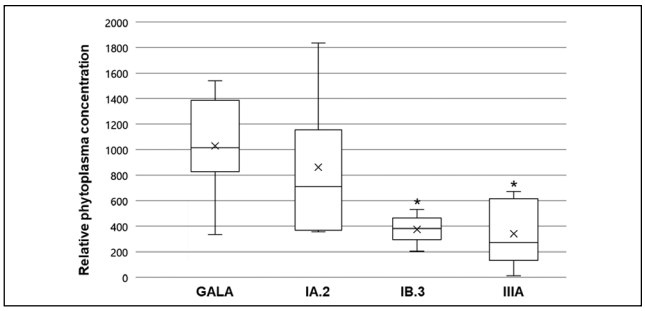


Figure 2. Box plot illustrating the '*Ca*. P. mali' titer measured for three 35S::*MdTCP4a* transgenic lines (IA.2; IB.3; IIIA) and the control (non-transformed "Gala") via qPCR. Boxes represent the distribution of first and third quartiles, while the error bars refer to the total distribution of non-outliers' samples. "X" and the horizontal lines inside the boxes indicate mean and median values, respectively. Asterisk indicates a statistically significant difference ($p < .05$).

Results of the micrografting infection tests, displayed as a box plot in Figure 2, indicate that the transgenic line IA.2 does not show a significant difference in the phytoplasma concentration compared to non-transformed, while lines IB.3 and IIIA display a significantly lower quantity ($p < .05$ per one-way ANOVA analysis with Tukey's Honest Significant Difference post hoc test).

Discussion

Soil-acclimatized transgenic plants showed dramatic phenotypic changes during the first three months including smaller stems, loss of apical dominance, and small, crinkled leaves. Interestingly, these phenotype characteristics resemble some of the typical symptoms of apple proliferation, which include small leaves, development of shoots from axillary buds, which give rise to secondary shoots that originate witches' brooms and, often, stunting (Schmid, 1975; Seemüller, 1990). The phenotype characteristics disappeared three months after the acclimatization, indicating the establishment of a physiological condition after the disequilibrium induced by the acclimatisation process. This phenomenon is consistent with the hypothesis of post-transcriptional tight regulation of *TCPs* operated by miR319 (Palatnik *et al*., 2003).

Interestingly, two out of three *in vitro* transgenic lines infected with '*Ca*. P. mali' showed a statistically significant lower concentration of the phytoplasma in the plant's aerial parts than non-transformed "Gala". The concentration of phytoplasma in the aerial parts of the plant is strongly correlated with the severity of the symptoms displayed by infected plants (Carraro *et al*., 2004). Nonetheless, these promising results need to be confirmed by increasing the sample size and performing *ex vitro* infection screening, thus allowing the phenotype characterization of infected plants.

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Omics

Complete genome sequences of phytoplasma strains in group 16SrII associated with *Parthenium* phyllody in India

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Abstract

It was performed the whole-genome sequencing of two phytoplasma strains in the 16SrII group, PR34 and PR08, associated with phyllody and witches' broom disease in the common weed, *Parthenium hysterophorus*. These phytoplasmas are associated with diseases in numerous pulse crops in India. Complete circular genomes were obtained after multiple sequencing attempts, employing DNA pre-processing and hybrid assemblies that combined short and long-read sequences. These genomes, deposited under GenBank accession numbers CP097206 and CP097207, offer valuable insights into the pathogenicity and evolutionary molecular characteristics of phytoplasma enclosed in the 16SrII group.

Keywords: Peanut witches' broom phytoplasma, 16SrII group, phyllody, genome sequence, *Parthenium*

Introduction

Phytoplasmas are phloem-inhabiting obligate plant pathogens linked with different diseases in legumes, horticultural crops, and weed species across the globe (Duduk *et al*., 2018). In India *'Candidatus* Phytoplasma aurantifolia' and *'Ca*. P. australasia', are associated with phyllody and witches' broom diseases in various economically important crops and their associated weeds (Thorat *et al*., 2016; 2017; Kirdat *et al*., 2020b). Weeds, in particular, serve as secondary hosts for phytoplasmas (Rao *et al*., 2017; Duduk *et al*., 2018). Given the significant economic impact of these phytoplasmas, the genomes of two strains (PR08 and PR34) detected in the common weed *Parthenium hysterophorus* were sequenced. The goal was to gain a better understanding of the genome structure of this pathogen and to shed light on the factors responsible for its pathogenicity. These newly obtained genome sequences are expected to enhance comprehension of the biology of 16SrII phytoplasmas and aid in developing effective strategies for managing the diseases associated with them.

Materials and Methods

P. hysterophorus plants exhibiting typical phyllody and witches' broom symptoms were collected from the Pune region of Maharashtra state of India. The presence of phytoplasmas was confirmed by PCR amplification followed by Sanger sequencing of the 16S rRNA gene from all samples (Deng and Hiruki, 1991; Kirdat *et al*., 2022). The identity of phytoplasmas strains PR34 and PR08 was confirmed using the EzBioCloud database (Yoon *et al*., 2017). The genomic DNA was extracted from infected leaf tissue using the CTAB method and enriched for prokaryotic DNA selection using the NEBNext microbiome enrichment kit (New England BioLabs, USA). The enriched DNA was sequenced on the Illumina NovaSeq 6000 and Oxford Nanopore Technology (ONT) MinION platform following the manufacturer's instructions and described previously (Ranebennur *et al*., 2022).

The bioinformatics pipelines described by Kirdat *et al*. (2020a) was used to generate hybrid genome assemblies of strains PR08 and PR34. Briefly, all QC-passed Illumina reads were subjected to metagenomic assembly using MEGAHIT v1.1.3 (Li *et al*., 2016), followed by taxonomic binning using MetaBAT2 v2.12.1 (Kang *et al*., 2015). The raw reads obtained from both platforms were mapped to all 16SrII genomes available on NCBI and phytoplasma-specific bin using Bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012) and minimap2 v2.22-r110 (Li, 2018). These mapped reads were used to generate hybrid assembly in Unicycler v0.4.8 (Wick *et al*., 2017). The order and orientation of contigs were determined, and scaffolding was done by MeDuSa v1.6 (Bosi *et al*., 2015). The assemblies were curated for low coverage bases, submitted to the DDBJ/ENA/GenBank database, and underwent annotation with PGAP (Zhao *et al*., 2012). The OGRI values were obtained using the EzBioCloud orthoANI calculator (Yoon *et al*., 2017) and GGDC (Auch *et al*., 2010). The obtained genomes were characterized for the presence of various genome features, especially related to pathogenicity.

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Results and Discussion

The phytoplasma-specific reads were assigned to generate Unicycler assemblies for strains PR34 and PR08. The 16S rRNA gene fetched from these genomes found their closest match with reference sequences of *'Ca.* P. aurantifolia' (strain WBDL, GenBank accession number U15442) and *'Ca.* P. australasia' (strain *Carica papaya*, GenBank accession number Y10097), respectively. For PR34, six contigs spanning 614,946 bp were obtained in the first attempt, while PR08 generated a single circular genome sequence of 588,746 bp. All shorter contigs lacking significant similarity to phytoplasma sequences were removed. The PR34 and PR08 genomes were inspected manually and curated for low coverage bases. The annotated protein sequences of both genomes were verified for their association with phytoplasma using BLASTx searches. The MeDuSa oriented and scaffolded contigs of the strains PR34 and PR08 yielded a single circular chromosome of size 614,574 bp and 588,746 bp (GenBank accession numbers CP097206 and CP097207), respectively.

Table 1. Genome statistics of sequenced strains.

Strain ID	PR34	PR08
Contig	1	1
Genome length	614,574	588,746
Proteins	474	468
rRNAs	6	6
tRNAs	28	27
Coding density	70.61	72.74
%GC	24.65	24.36

The genome of strain PR34 has the smallest reported size among phytoplasmas in the 16SrII group, with a genome coverage of 5700X for Illumina reads and 180X for ONT. PR34 assembly had two rRNA operons, 474 protein-coding genes, 28 tRNA genes, 18 pseudogenes, and 24.65% G+C content. The genome coverage of strain PR08 for Illumina reads was 2750X, and for ONT, it was 70X. The final assembly showed 24.36% G+C content and included two rRNA operons, 27 tRNA genes, 468 protein-coding genes, and 15 pseudogenes (Table 1). The genome features of both 16SrII strains include the presence of a wide range of homologs of effector proteins, the absence of potential mobile units (PMUs), the presence of superoxide dismutase (SOD) gene, multiple copies of truncated hemolysin genes, and the presence of characteristic full-length and truncated group II introns.

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Omics

Investigating the microbial composition of "flavescence dorée"- infected grapevine plants

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Abstract

Plants are inhabited by a wide variety of microorganisms that interact and influence the ability of pathogens to colonize the hosts. Phytoplasmas are part of this network and compete with the rest of the microorganisms for nutrients and space. To investigate the microbial community composition of grapevine plants infected with "flavescence dorée" phytoplasmas, next generation sequencing analyses, using the Illumina method, were performed on total DNA. The percentage of phytoplasma OTUs identified in the infected plants was high and consistent with the results obtained by PCR/RFLP analyses. The identification of bacteria in families *Micrococcaceae*, *Sphingomonadaceae* and *Beijerinckiaceae* should help the search of possible microorganisms helpful for "flavescence dorée" disease control strategies.

Keywords: phytoplasma, endophytic bacteria, next generation sequencing, OTU

Introduction

Plants are inhabited by both beneficial and harmful microorganisms, collectively defined microbiome, having different interactions extending from mutualism to parasitism. When pathogens colonize a host, they share the space with diverse complex microbial communities. Phytoplasmas become part of this network and compete with other endophyte microorganisms for nutrients and space in both plants and insect vectors. This interaction may play an important role for the success of infections and for the ability of these pathogens to be spread from plant to plant by insect vectors. Phytoplasmas are wall-less intracellular bacteria with close relationships with plants and insect vectors. These associations have several peculiar traits, related also to the fact that these bacteria are colonizing hosts belonging to two different kingdoms. "Flavescence dorée" (FD) is a threatening grapevine disease associated to phytoplasmas enclosed in 16SrV-C/D subgroups (Martini *et al.*, 1999) that are quarantine organisms in the EU. The disease is distributed within the most important wine-producing areas in Europe and has severe effects on both vineyard productivity and landscape management and, despite the efforts toward its containment, it is actively spreading. The aim of this study was to investigate the microbial composition in grapevine FD-infected plants to verify the presence of possible useful relationships among detected bacterial genera.

Materials and Methods

During summer 2018 and 2019 a survey for phytoplasma presence was conducted in four vineyards located in Massa Carrara province, Tuscany, Italy. A total of 28 grapevine plants showing leaf reddening and shorter internodes (Figure 1) were collected, together with 13 weed plants (mainly bindweed) located in the same vineyards.



Figure 1. Reddening symptoms on grapevine "flavescence dorée" infected leaves.

Total nucleic acids were extracted from leaf midribs of grapevines and weeds using a phenol-chloroform method (Prince *et al.*, 1993). Nested-PCR/RFLP analyses were carried out to identify the phytoplasmas as described (Zambon *et al.*, 2018). Extracted DNAs were further purified and used

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for library preparation following the "16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System" instructions. Variants of the 799F and 1193R primers (Beckers *et al.*, 2016) optimized for phytoplasma amplification based on nucleotide alignments of their 16S ribosomal gene DNA sequences were used for amplification (Nicolaisen *et al.*, 2022). All the reads were aligned to the pertinent reference sequences using USEARCH v. 10 and OTUs were then compared to the Genbank sequences using the BLAST tool.

Results

Almost all the grapevine samples (25 out of 28) collected during the survey conducted on 2018 and 2019 seasons resulted positive for "flavescence dorée" phytoplasmas enclosed in 16SrV-C subgroup after nested-PCR/RFLP analyses. On the other hand, the molecular analyses carried out on weeds collected in the same vineyards highlighted the presence of phytoplasmas belonging to the ribosomal groups 16SrII, 16SrVI, 16SrX, 16SrXII-A and 16SrXII-H, in 8 out of the 13 samples tested. The main positive species was bindweed mostly infected with phytoplasmas of ribosomal subgroup 16SrXII-H ('*Candidatus* Phytoplasma convolvuli') (Martini *et al.*, 2012). After Illumina sequencing overall 431 bacterial OTUs (organism taxonomic unit) were identified in the samples monitored and the bioinformatic analyses allowed the identification of the detected genera. In the infected grapevine plants the percentage of sequences related to *Acholeplasmataceae* family ranged from 94% and 95% in the two years considered (Figure 2).

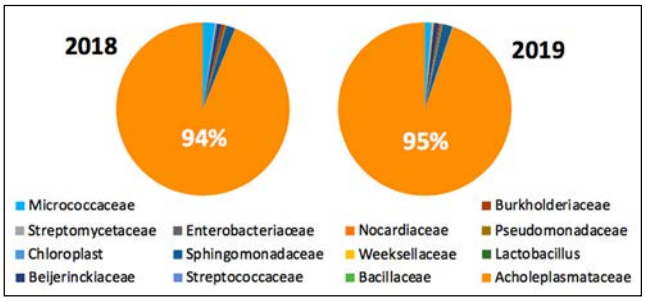


Figure 2. Bacterial family composition in FD infected grapevines.

After BLAST analyses the prevalent OTUs identified were 100% identical to the sequences of phytoplasmas enclosed in the 16SrV group. After the bioinformatic analysis on weeds, only in bindweed it was obtained a suitable detection of phytoplasma reads, with OTUs 100% identical to sequences of '*Ca.* P. convolvuli'. The preliminary analysis on bacterial communities showed the prevalence of bacteria from *Micrococcaceae, Sphingomonadaceae* and *Beijerinckiaceae* families in both, grapevine and bindweed phytoplasma infected plants. Moreover, in grapevines in which the lowest phytoplasma concentration was detected, bacteria from *Enterobacteriaceae* family were the most present, while *Sphingomonadaceae* were the most abundant in the weed plants resulted negative to phytoplasma presence (Table 1).

Table 1. Results of NGS analyses on grapevines and weeds (phytoplasma OTUs and other bacterial family abundance).

Plant species	Year of sampling	Phytoplasma OTUs	Most abundant bacterial families
Grapevine	2018/ 2019	Abundant	*Micrococcaceae*
Bindweed	2019	Good	*Micrococcaceae*
Other weeds	2019	None	*Sphingomonadaceae*

Discussion

The preliminary results obtained after bacterial microbiome analyses on grapevine plants infected with FD phytoplasmas could help in the understanding details on phytoplasma infection processes and will made it possible to verify the presence of positive and negative relationships among microorganisms living the same host species. The knowledge on microbial communities in phytoplasma infected grapevine plants could support research on control strategies based on field application of specific bacteria or microorganism to contain phytoplasma-associated diseases reducing at the same time the dangerous application of pesticides against insect vectors.

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Omics

Microbiomes of soil and roots of two palm species infected with '*Candidatus* Phytoplasma palmae' in two different ecosystems: single-strand conformation polymorphism analysis

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Abstract

During last 5 years for the first time phytoplasmas have been detected in the two native palm species *Brahea brandegeei* and *Washingtonia robusta*, in two different ecosystems, urban (El Centenario) and their natural habitat (Sierra de las Cacachilas) in Mexico. Single-strand conformation polymorphism analysis technique was implemented for preliminary analysis of mixed bacterial microbiomes in soil and roots of these two palm species and preliminary data are presented.

Keywords: microbiomes, phytoplasma, SSCP, palms, urban and native ecosystems

Introduction

Plants live in complex associations with diverse microorganisms in the rhizosphere, nearby soil around the roots, in the roots and in the phyllosphere. Divers and dynamic interactions occur between the plant and its microbiota, as well as among the diverse microbial communities. The microorganisms can have beneficial, neutral or detrimental effects on plant health and development, acting as antagonists, commensalists, symbionts, or pathogens. Single-strand conformation polymorphism (SSCP) analysis is a sensitive and rapid method for detecting mutations in PCR amplified fragments (Orita *et al*., 1989). Due to its technical simplicity, SSCP is used in various investigations, ranging from the clinical diagnosis of human hereditary diseases (Papp *et al*., 2007), to the characterization of microbial communities (Hori *et al*., 2006). SSCP was also used also as a tool for the detection of molecular variability of phytoplasmas (Seruga-Music *et al*., 2008; Valasevich and Schneider, 2016).

Materials and Methods

The root samples from palms *Brahea brandegeei* and *Washingtonia robusta* from two urban and native ecosystems were analysed preliminary with scanning electron microscopy (SEM) (Hitachi S-3000N) (Poghosyan *et al*., 2019). For microbiome analysis of roots and soil near the palms roots, DNA from 23 roots and soil samples was extracted using the FastDNA™ Spin Kit for Soil (Biomedicals, LLC) in combination with the FastPrep® cell disruptor (MP Biomedicals, LLC) (Figure 1). For SSCP analysis the 23 samples were also processed according to SSCP technique reported for environmental microbiology (Johnston-Monje and Lopez Mejia, 2020). DNA extracts were purified with the Wizard DNA Clean-Up System (Promega) for humic acids removal, and subjected to PCR amplification using universal primers for bacterial detection (Schwieger and Tebbe, 1998). Bioinformatic analysis was carried out with GelComparII software (Applied Maths) for dendrogram construction. Bands of interest were selected for DNA sequencing (Macrogen, Corea).

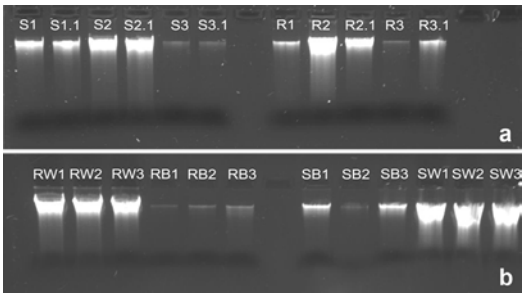


Figure 1. Electrophoresis of DNA extracted from root and soil samples. a) Sierra de las Cacachilas; b) El Centenario.

Results

Analysis of root samples of *B. brandegeei* and *W. robusta* collected in both ecosystems using SEM technique, clearly revealed the presence of phytoplasmas in the phloem of the roots (Figure 2).

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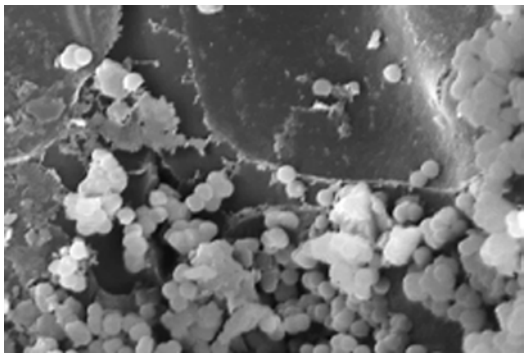


Figure 2. Phytoplasmas in phloem tissue: *Washingtonia robusta* (native ecosystem).

Analysis in the BLAST program revealed the presence of endophytic bacteria of different taxa, beneficial and pathogenic, some known as human pathogens, which reside in the soil (Figure 3 and 4).

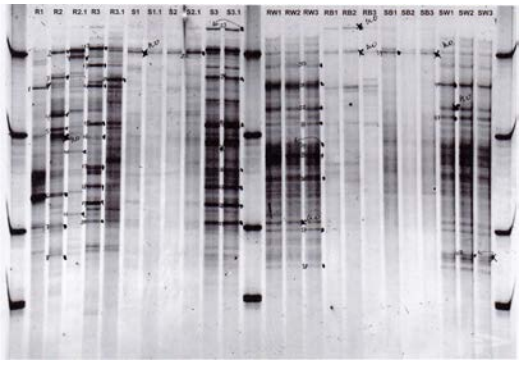


Figure 3. Bacterial populations profile from soil and roots communities, obtained by SSCP. Bands are labeled for sequencing.

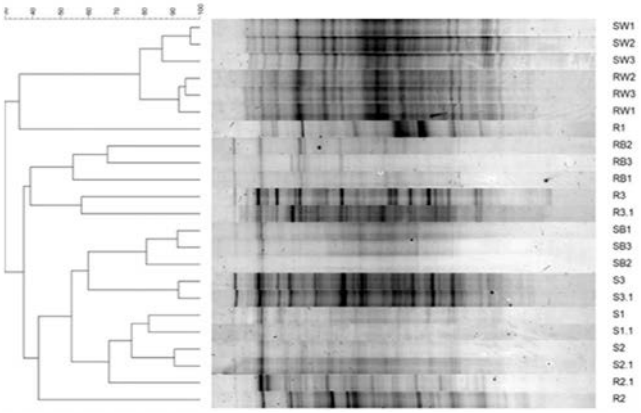


Figure 4. Dendrogram of clusters of SSCP bands derived from soil and root samples from the two sites.

The SSCP allowed visualizing the different populations of each sample in an acrylamide gel from which 42 bands of interest were selected for DNA recovery. The results of the analysis of these sequences showed the presence of 22 genera of bacteria: *Rhizobium*, *Sinorhizobium*, *Terribacillus*, *Bacillus*, *Bordetella*, *Ralstonia*, *Pseudomonas*, *Azotobacter*, *Bartonella*, *Methylobacterium*, *Enterobacter*, *Streptomyces*, *Devosia*, *Burkholderia*, *Massilia*, *Pontibacter*, *Serratia*, *Nocardioides*, *Luteimonas*, *Lysobacter*, *Marmoricola*, *Sphyngopyxis*. The main phyla were Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes.

Discussion

The results obtained by SSCP technique allow the understanding of ecology of the microorganisms, and how microbial interactions may impact on plant growth in different environmental conditions, causing phytosanitary problems, in this case, lethal yellowing of palms. There are other impacts also, as in the case of various bacterial genera, including *Burkholderia*, *Enterobacter*, *Ralstonia*, *Staphylococcus* and *Stenotrophomonas*, which contain root associated strains that may have bivalent interactions with plants and human hosts (Berg *et al*., 2005). Here are evidence that phytoplasmas and other mollicutes may be also part of this rhizosphere community.

Acknowledgements

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Insect vectors

Presence and distribution of known, alternative and putative insect vectors of phytoplasmas associated with "flavescence dorée" disease in Northeast Italy

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Abstract

Since 2020 grapevine "flavescence dorée" (FD) epidemic outbreaks have been reported in the northern Italy regions even where a vector control was carried out. In 40 severely FD infected vineyards the presence and distribution of known, alternative and putative vectors and of symptomatic grapevines were monitored during 2021-2022 vegetative season. Results confirmed that *Scaphoideus titanus* is the main vector of "flavescence dorée" and highlighted the possible role in the FD epidemiology of *Orientus ishidae* and *Phlogotettix cyclops*. Moreover, *Neoaliturus fenestratus* tested positive to the "flavescence dorée" phytoplasma with high captures and partially associated with symptomatic grapevines distribution.

Keywords: grapevine, phytoplasma disease, *Scaphoideus titanus*, leaf hoppers

Introduction

"Flavescence dorée" (FD) is a quarantine grapevine disease associated with phytoplasmas enclosed in 16SrV-C and D subgroups efficiently transmitted by the leafhopper *Scaphoideus titanus* Ball, (Hemiptera, Cicadellidae), (Chuche and Thiery, 2014). Additionally, other insects such as *Dictyophara europaea* (Linnaeus) and *Orientus ishidae* (Matsumura,), are able to transmit FD phytoplasmas to grapevine (Filippin *et al*., 2009; Lessio *et al*., 2016). Recent surveys highlighted that *Allygus* spp. (Malembic *et al*., 2020), *Phlogotettix cyclops* (Strauss *et al*., 2018) and *Hishimonus hamatus* (Belgeri *et al*., 2022) are positive to FD phytoplasmas, making them putative alternative vectors. Since 2020 epidemic outbreaks have been reported in the northern Italy regions, even where *S. titanus* control is carried out. The aim of the present work was to investigate the spatial distribution of known and putative alternative insect vectors in FD infected vineyards in Veneto region and at what extent they harbour FD phytoplasmas, to understand if they could have an active role in epidemics.

Materials and Methods

In 2021-2022, 40 severely FD infected vineyards located in different grapevine growing areas in the Veneto region (northern-eastern Italy) were monitored. In each site, yellow sticky traps were positioned according to line transect, sampling from the surrounding wooded areas to the center of the vineyard. In each vineyard from one to three transects were designed. The traps were placed from July to the first decade of October, weekly replaced and stored at -20°C for morphological identification and genetic analysis. The presence and distribution of symptomatic grapevines was visually evaluated in August observing at least 1,000 grapevines along the line transect considered for the traps. The total nucleic acids of the known, alternative and putative FD phytoplasma vectors and of the symptomatic grapevines were extracted by a CTAB method (Angelini *et al*., 2001). Subsequently, each insect and plant sample was analyzed with TaqMan quantitative PCR (Angelini *et al*., 2007) for screening FD phytoplasma presence.

Results

The presence and the FD phytoplasma positivity of known and putative alternative FD vectors is reported in Table 1, while the insect distribution and the spatial correlation between insect vector and symptomatic grapevines is described in Table 2. *S. titanus* was found in all the investigated sites, with high population density and captures located mainly in the borders of the vineyards, probably due to the compulsory insecticide applications. *D. europaea* and *O. ishidae* were widespread in the wooded areas,

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particularly the mosaic leafhopper showed very high captures especially in hilly conditions. About the putative alternative insect vectors, the prevalent were *H. hamatus* and *P. cyclops* (with high-level captures), while the presence of *Allygus modestus* Scott was lower. According with their ecology, these leafhoppers were found mainly in the surroundings. Additionally, *Allygidius atomarius* (Fabricius), *Euscelidius variegatus* (Kirschbaum,) and *Neoaliturus fenestratus* (Herrich-Schäffer) tested positive to FD phytoplasma. *N. fenestratus* was very common, with high captures in almost all sites and especially within the vineyards. Comparing the spatial distribution of FD phytoplasma vector captures and FD symptomatic grapevines within the vineyards, *S. titanus* was positively correlated, while *O. ishidae, P. cyclops* and *N. fenestratus* presented partial association. The restricted presence or the low captures do not allow to underline a positive correlation between the other leafhoppers (*D. europaea, A. modestus, H. hamatus, A. atomarius* and *E. variegatus*) and the distribution of symptomatic grapevine.

Table 1. Presence and FD phytoplasma in known, alternative and potential FD insect vector in the investigated vineyards.

	Catches 2021-2022			FD Positivity
	Incidence (%)	Max (n°)	Mean (n°)	2021 (%)
Scaphoideus titanus	100	826	100	64.1
Dictyophara europaea	65	80	8	2.5
Orientus ishidae	85	1635	94	7.8
Allygus modestus	28	8	3	12.5
Hishimonus hamatus	98	81	9	2.4
Phlogotettix cyclops	80	612	52	7.6
Allygidius atomarius	45	130	14	20.0
Euscelidius variegatus	55	52	4	2.5
Neoaliturus fenestratus	95	693	88	5.5

Table 2. Distribution of known, alternative and potential FD insect vectors in the investigated sites and spatial correlation between insect vectors and symptomatic grapevine plants within vineyards.

	Catches distribution 2021-2022			Spatial correlation
	Vineyard (%)		Woods (%)	vector/symptomatic
	Center	Border		grapevine 2021*
Scaphoideus titanus	38.2	51.7	10.1	20
Dictyophara europaea	9.3	17.4	73.3	8
Orientus ishidae	1.4	24.9	73.7	15
Allygus modestus	14.7	16.9	68.4	4
Hishimonus hamatus	8.8	19.7	71.5	9
Phlogotettix cyclops	2.7	7.1	90.2	12
Allygidius atomarius	9.4	17.7	72.9	6
Euscelidius variegatus	21.5	30.8	47.7	7
Neoaliturus fenestratus	60.7	35.7	3.6	12

* = (n°/out of 40)

Discussion

Serious FD outbreaks detected in the recent years in northern Italy could be related to the use of less effective insecticides against *S. titanus* due to the pesticide limitation by the European Commission [Regulation (EC) No 1107/2009]. Indeed, the result showed that *S. titanus* is the most widespread species with high population density and its FD phytoplasma infectivity and spatial correlation between captures and the FD symptomatic grapevines, confirm its main role in the FD spreading. Moreover, the limited application of broad-spectrum insecticides may have influenced the presence of occasional leafhoppers in the vineyard, some of which able to acquire/transmit FD phytoplasmas. The research highlighted the possible role in FD epidemiology of *O. ishidae* and *P. cyclops*. Results also indicated that *N. fenestratus* were found positive to FD phytoplasmas with high capture numbers and partially associated with symptomatic grapevines distribution. Considering that *N. fenestratus* was positive to 16SrI, 16SrII, 16SrXII-A (Trivellone, 2019) further investigation on its ability to transmit FD phytoplasma is necessary.

Acknowledgements

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Insect vectors

Potential insect vectors of phytoplasmas in wheat and maize crops in Poland

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Abstract

Plant diseases associated with phytoplasmas are of growing concern for their rapid spread and potential devastating impact on cultivated plants. Early warnings of pathogens and pests outbreaks demand frequent monitoring of the crops and the whole agricultural ecosystem. Leafhoppers and planthoppers collected from wheat and maize orchards were tested for phytoplasma presence. The most abundant leafhoppers were *Zyginidia scutellaris* and *Psammotettix alienus*. In these two species phytoplasmas of subgroups 16SrI-C and 16SrI-B were detected. In the less abundant *Laodelphax striatellus* insects, the 16SrI-C strain was identified. The 3.4% of the tested insects resulted positive for phytoplasma presence.

Keywords: leafhoppers, planthoppers, phytoplasma detection, 16SrI-C, 16SrI-B

Introduction

Phytoplasmas are wall-less bacterial pathogens which affect hundreds of plant species worldwide (Bertaccini *et al*., 2014). These plant pathogens have a great spread potential due to being transmitted by hemipteran insects such as leafhoppers, planthoppers and psyllids (Weintraub and Beanland, 2006). There is a general concern that the presence of phytoplasmas in temperate regions of the world will increase soon as a result of climate warming, which can also help the survival of insect vectors and lead to their increased numbers. Wheat (*Triticum aestivum*) and maize (*Zea mays*) are globally important food crops used in the human diet and animal nutrition (Zörb *et al*., 2018). European Union is the global leader in wheat grain production (135 Mt produced in 2022) and the fourth world's largest producer of maize (56 Mt) (www.indexmundi.com). The study aimed to assess the percentage of phytoplasma infections in leafhoppers and planthoppers feeding on wheat and maize cultivated in Central Europe and thus help to predict potential disease outbreaks and identify potential insect vectors.

Materials and Methods

Adult leafhoppers and planthoppers (Hemiptera, Fulgoromorpha and Cicadomorpha) were collected from winter wheat and summer maize plantations in southwest Poland in 2019. Collections were conducted twice, from plants in two developmental phases (wheat BBCH 24 and 39 weeks; maize BBCH 17 and 39 weeks). Insects were identified based on morphological features. The phytoplasma detection was carried out by total nucleic acids extractions from individual insects using kit NucleoSpin Tissue (Macherey-Nagel) followed by nested PCR assay with phytoplasma 16S rDNA specific primers P1/P7 and fU5/rU3 (Deng and Hiruki, 1991; Schneider *et al*., 1995, Lorenz *et al*., 1995). Obtained amplicons were Sanger sequenced on both strands. Raw chromatograms were assembled into consensus sequences by BioEdit. Based on comparative pairwise sequence analysis conducted with selected reference strains, phytoplasmas were identified at the ribosomal subgroup level. Multiple alignments were performed with ClustalW and the phylogenetic relationships were analysed using MEGA 7.

Results and Discussion

Among 748 insects collected from the wheat fields, 7 species were identified, all classified in the family Cicadellidae (Hemiptera). The most abundant were *Zyginidia scutellaris* (47.9%), *Psammotettix alienus* (27.7%) and *Empoasca pteridis* (22.9%) (Figure 1). A total of 899 insects were collected from maize fields, including Cicadellidae (9 species) and Delphacidae (2 species) families.

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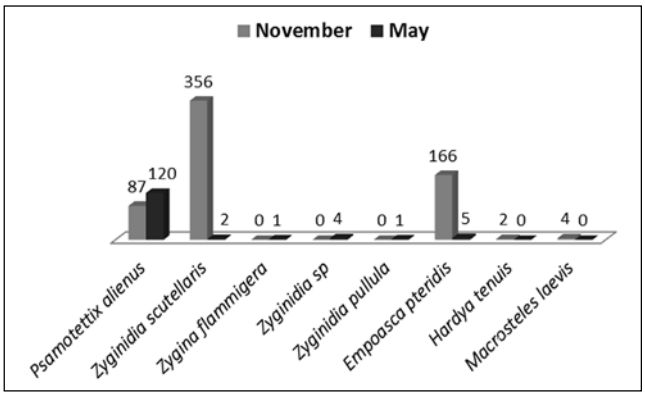


Figure 1. Number of leafhoppers caught from winter wheat crops in November (tillering phase - early plant development) and in May (late stem elongation phase).

The most numerous were *Z. scutellaris* (79.2%), *P. alienus* (14%) and *Laodelphax striatellus* (3.2%) (Figure 2). In total, 4.9% of insects captured in maize and 1.74% of insects captured in wheat tested positive to phytoplasmas. The group of insects that were carriers of phytoplasmas included *Z. scutellaris*, *P. alienus* and *L. striatellus* (Figure 3).

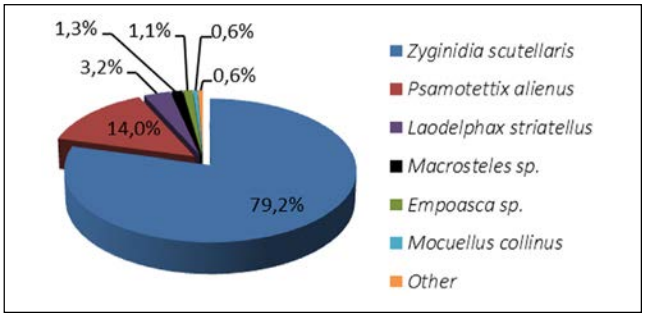


Figure 2. Summary chart of insects collected from maize in the stage of seven unfolded leaves (BBCH 17) and in the stage of stem elongated to nine nodes (BBCH 39).

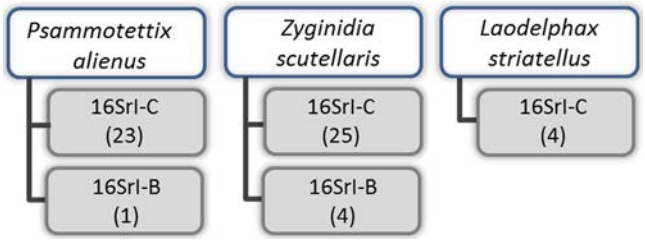


Figure 3. Phytoplasmas were detected in three insect species. The number of phytoplasma-positive insects is given in parentheses.

The comparison of 16S rRNA gene sequences showed that most of the phytoplasma strains detected in insects were identical to '*Candidatus* Phytoplasma tritici' strain NZ_AVAO01000003 (Zhao *et al.*, 2021) enclosed in subgroup 16SrI-C. The other five sequences were identical to the rapeseed phyllody phytoplasma RP166 strain (CP055264) and therefore were '*Ca.* P. asteris' strains (Cho *et al.*, 2020). Phylogenetic analysis positioned phytoplasmas clustering in agreement with the two taxa, with high confidence values (data now shown).

In this study, new possible threats to wheat and maize production were monitored by testing potential insect vectors of phytoplasmas. The results indicate the presence of group 16SrI phytoplasmas, mostly 16SrI-C, less often 16SrI-B in wheat and maize fields. Insect infection rates of around 5% and 2% respectively, suggest a moderate risk of phytoplasma disease outbreaks. However, it should be pointed out, that the emergence of vectors and the acquisition of phytoplasmas from other host plants by them are very dynamic processes, depending on weather conditions and food availability. Here, it was observed that both *Z. scutellaris* and *P. alienus* can feed in large numbers on wheat and maize, which means that they can migrate between these crops and potentially transmit the phytoplasmas. A large contribution of *Z. scutellaris* to the leafhopper populations of agricultural areas in Poland is a relatively new phenomenon, observed only since 2018. Therefore, there is a risk that new leafhopper species will bring new phytoplasma strains. The results show that *Z. scutellaris* carry phytoplasma strains already reported in Europe, however, it remains to be tested whether they are vectoring these strains to healthy plants.

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Insect vectors

Assessment of proportion of populations of *Haplaxius crudus* and *Oecleus mackaspringi* carrying '*Candidatus* Phytoplasma palmae' in Jamaica

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Abstract

Lethal yellowing, associated with the presence of '*Candidatus* Phytoplasma palmae' is a devastating disease of coconut palms in the Caribbean basin. The disease was originally described from Jamaica and continues to threaten sustainable production there to date. In Florida, the insect vector was determined to be the planthopper *Haplaxius crudus* in the late 1970s, however attempts to replicate this in Jamaica have failed. Two surveys were done in Jamaica in October 2019 and in February 2022. Specimens of *H. crudus* and another common planthopper, *Oecleus mackaspringi*, were collected and screened for the presence of phytoplasmas. A total of 79 *O. mackaspringi* have been collected in two surveys in two different sites with documented spread of lethal yellowing. All 79 specimens of *O. mackaspringi* resulted negative for lethal yellowing. For *H. crudus*, 44 specimens (31 from site one and 13 from site two) were collected with only one specimen from site two testing positive. These results indicate that the percentage of the population of insect vector carrying lethal yellowing is low, which is consistent with field studies in Mexico and Florida. Future efforts in Jamaica need to sample during months with peak of adult insect activity to increase probability of detecting the phytoplasma from *H. crudus* and verify if *O. mackaspringi* is also a vector.

Keywords: insect vector, survey, palms, Caribbean

Introduction

Lethal yellowing disease (LY) is a fatal phytoplasma infection of coconut palms associated with the presence of '*Candidatus* Phytoplasma palmae' (Bertaccini *et al*., 2022) that is widespread in the Caribbean basin. While LY was discovered in Jamaica (Fawcett, 1891), it has been reported from Mexico, Florida – USA, Antigua, Cuba, and Guadeloupe. In the 1970s, the vector of LY was determined to be the cixiid planthopper *Haplaxius crudus* (Howard and Thomas, 1980). Since this discovery, little progress has been made on assessing the insect vector(s) of LY in other regions of the Caribbean. While it is presumed that *H. crudus* is also the vector of LY in Jamaica, it has not been experimentally demonstrated.

Recent survey work in Jamaica resulted in the discovery of a new insect species, *Oecleus mackaspringi* (Myrie *et al*., 2019), that is in the same family as *H. crudus* (Cixiidae) and was found closely associated with coconut palms declining from LY in various sites in Jamaica. Because of this close association, speculation arose that *O. mackspringi* may be contributing to the spread of LY, stimulating research efforts to evaluate its capacity as a vector of the LY phytoplasma. The primary objective of this study was to survey populations of *H. crudus* and *O. mackspringi* (Figure 1) at two field sites with documented cases and spread of LY to determine what percentage of the insect populations is carrying the phytoplasma to determine whether both species are vectors or if one or the other has a higher rate of infection.

Materials and Methods

In October 2019 and February 2022, two sites were selected (Spring Garden – SG and Hart Hill – HH) that had previously documented spread of LY. At each site all coconut palms that were accessible by sweep net were sampled and all individuals of *H. crudus* and *O. mackaspringi* were aspirated and transferred directly to 95% ethanol until further processing.

All specimens had total DNA extracted using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's protocol. Final eluate was used as template for quantitative PCR (qPCR) analysis and high resolution melt curve analysis (HRMA) as per the parameters by Bahder *et al*. (2017). Confirmation of insect species identity was done by examination of genitalia and comparing to illustrations by Kramer (1979) for *H. crudus* and

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comparison to holotype material of *O. mackaspringi* held at the University of Florida's Fort Lauderdale Research and Education Center.

Results

A total of 79 *O. mackaspringi* have been collected between the two trips from the two sites (68 from SG and 11 from HH) with documented LY spread. All 79 specimens of *O. mackaspringi* tested negative for LY (Table 1). For *H. crudus*, 44 specimens (31 from SG and 13 from HH) were collected with only one specimen from site two testing positive.

Table 1. Proportion of insects testing positive for LY based on qPCR and HRMA analyses.

	H. crudus		*O. mackaspringi*	
	SG	HH	SG	HH
No. Positive/Total Tested	0/31	1/13	0/68	0/11
Avg. Ct	No Ct	25.4	No Ct	No Ct
Tm of amplicon	64.2°C	78.2°C	64.4°C	64.4°C

Discussion

These data, while preliminary, confirm that at least *H. crudus* is capable of carrying the LY phytoplasma under field conditions in Jamaica, further supporting its role as a possible vector of LY. While '*Ca.* P. palmae' was not detected in *O. mackaspringi*, it is possible that this is due to a low sample size. It has been documented that insect vectors carrying palm phytoplasmas have very low rates of phytoplasma presence under field conditions (Mou *et al*., 2020). Future research efforts need to establish when the peak abundance of *H. crudus* and *O. mackaspringi* is in Jamaica and survey must be done during this time period to maximize likelihood of collecting LY-positive insects, allowing better understanding of the capacity of each species to contribute to the epidemiology of LY in Jamaica.

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Insect vectors

Study on epidemiology of streak yellows disease of date palm in Iran

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Abstract

A disease with symptoms similar to palm lethal yellowing was observed in Iran in date palm. Symptomatic trees showed a variable incidence and severity of streak yellows. Molecular analyses detected the presence of two phytoplasmas in groups 16SrVI and 16SrVII, respectively. Possible insect vectors were collected from infected plants and identified. Through the collected insect samples, the specimens of 4 species were positive to phytoplasma presence. The sequencing analysis showed the presence of '*Candidatus* Phytoplasma trifolii' in *Tropidocephala prasina* that can be considered a putative vector for one of the palm streak yellows associated phytoplasmas.

Keywords: bug, date palm, phytoplasma, insect putative vector

Introduction

The phytoplasmas implicated in diseases of date palms have been identified as group 16SrIV (America; Harrison and Jones, 2004), 16SrI (Egypt and Saudi Arabia; Al Khazindar, 2014), 16SrII (Saudi Arabia; Alhudaib *et al*., 2008, 2014) and 16SrXIV (Sudan; Cronjé *et al*., 2000). A streak yellows and decline disease affecting date palms was first recognized in Ahvaz, Khuzestan province of Iran in different cultivars such as Estameran and Berhi in 2013. Date palm is the most important crop cultivated in this province in south of Iran. Due to the detection of some phytoplasma infected samples (Ghayeb Zamharir and Eslahi, 2018) possible insect vectors were collected and identified in this area. The detection of 16SrVII and 16SrVI group phytoplasmas in the symptomatic date palms and *Tropidocephala prasina* as putative vector for phytoplasmas associated with this disease are reported.

Materials and Methods

A total of 53 samples were collected in 2015 and 2016 from symptomatic and asymptomatic date palm trees in infected orchards located in Khuzestan, Bushehr and Kerman provinces of Iran.

Amplifications were performed in 25 µl final reaction volumes each containing 50 ng of DNA template, 50 ng of each primer P1/Tint (Deng and Hiruki, 1991; Smart *et al*., 1996), 125 µM of each dNTP, 1 U of Taq DNA polymerase and buffer with 1.5 mM $MgCl_2$. PCR was performed for 35 cycles. Insects were collected by sweep net shaking the plants, yellow sticky traps and a water traps inside infected palm groves. Among the different methods of sampling, the most successful methods were shaking insects off plants and using a sweeping net.

The collected insects, which comprised Heteroptera and Auchenorrhyncha, were sorted and identified. The identification of Heteroptera species was performed by A. Carapezza, University of Palermo, Italy. Kollar *et al.* (1990) method was used for extraction of DNA from and the detection of phytoplasmas was done by nested PCR based on16S rRNA genes using the universal phytoplasma primer set P1/Tint, followed by the primer set R16F2n/R2 (Gundersen and Lee, 1996) in the DNA extracted from putative insect vectors collected from date palm groves.

Results

Nested PCR using universal primers revealed that 40 out of 53 trees were positive for phytoplasma presence while asymptomatic date palms (controls) tested negative. RFLP analyses and DNA sequencing of 16S rDNA indicated the presence of phytoplasmas in groups 16SrVI and 16SrVII, respectively. Sequence analysis confirmed that palm streak yellows phytoplasmas in each group were uniform and are phylogenetically clustering with '*Candidatus* Phytoplasma fraxini' and '*Ca*. P. trifolii'.

Through the collected insect samples, the specimens of the following species all collected in Shadegan resulted positive for phytoplasmas *Eysarcoris ventralis* (Westwood, 1837), (Figure 1), *Nysius graminicola* (Kolenati, 1845), *Nysius* sp. and *Omanocoris versicolor* (Herrich-Schaeffer,

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1841), moreover specimens of *Tropidocephala prasina* (Melichar, 1902) (Figure 2) collected in Darkhovin were also positive to phytoplasma presence.



Figure 1. *Eysarcoris ventralis* (Westwood, 1837) collected in Shadegan.



Figure 2. *Tropidocephala prasina* (Melichar, 1902), collected in Darkhovin.

After sequencing and RFLP analyses *T. prasiana* resulted infected with 16SrVI-A, '*Ca.* P. trifolii', while the phytoplasmas in the other positive species are still under identification.

Discussion

Until now, phytoplasmas of the 16SrIV, 16SrI, 16SrII and 16SrXIV have been reported as associated with diseases in date palm such as white tip dieback, slow decline and yellowing in America, North Africa and Kuwait, respectively (Al Awadhi *et al.*, 2002; Cronjé *et al.*, 2000). The RFLP and DNA sequencing data indicate that the palm leaf streak disease associated phytoplasmas in Iran are '*Ca.* P. fraxini' (16SrVII-A) and '*Ca.* P. trifolii' (16SrVI-A) (Ghayeb Zamharir and Eslahi, 2018). The planthopper, *T. prasina* known as an endemic species in Iran can be considered as a probable vector for palm leaf streak disease since it was found to harbour one of the two phytoplasmas associated with the disease.

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Insect vectors

Study on soybean bud proliferation phytoplasma insect vector

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Abstract

A '*Candidatus* Phytoplasma trifolii' strain was detected in soybean samples with symptoms of bud proliferation and aborted seed pods. Experimental infection of healthy soybean plants with symptomatic soybean shoots resulted in bud proliferation and seed pods abortion symptoms 8 weeks after grafting. The Hemiptera *Creontiades pallidus*, known also as cotton shredder bug, transmitted '*Ca.* P. trifolii' to healthy soybean plants under insect proof conditions. The identification of insect vector of this disease will help to develop of effective strategies to manage this disease under the Iranian field conditions.

Keywords: phytoplasma, insect transmission, *Creontiades pallidus*

Introduction

Soybean bud proliferation and seed pod abortion (SbpSpa) disease was first observed in soybean in Iran in 1985 and tobacco streak virus, tobacco and tomato ringspot viruses were reported as possible etiological agents (Rahimian and Hamdollahzadeh 1985, Rahimian *et al*., 1995). In other studies the bean yellow mosaic, mosaic, cucumber mosaic, black-eyed cowpea mosaic, peanut mottle, tobacco mosaic, soybean mosaic, tomato spotted wilt and pea enation mosaic viruses were detected (Golnaraghi *et al*., 2004). However, biological analysis couldn't demonstrate their etiological role in SbpSpa (Golnaraghi *et al*., 2004; Shameli *et al*., 2019) and did not result correlated with the disease symptoms. After 35 years' research, it was verified the consistent presence of '*Ca.* P. trifolii' (16SrVI-A) in the symptomatic soybean samples. This phytoplasma was transmitted by grafting (Ghayeb Zamarir *et al*., 2022).

In some cases, SbpSpa symptoms were associated with the presence of the cotton shredder bug, *Creontiades pallidus* (Hemiptera: Miridae) and its control was able to prevent the disease spreading (Faraji and Reisi, 2018). The reported identification of an insect vector in the field conditions in presented.

Materials and Methods

About 50 *C. pallidus* mirids were collected from a SbpSpa-affected field showing the symptoms every year, using an insect net. The species was identified at the Rasht University as described (Khan and Quade, 2008). The collected insects fed on healthy 3-month-old seed-grown soybean plants 50 cm high in separate cages in a growth chamber with 23°C for day, 18°C for night, and 14 hours light per day. The mirids were split in two groups, group A was used for nucleic acid extraction and PCR and group B was used for transmission experiments. The offspring were reared in separate cages maintained under the same conditions.

The phytoplasma presence was verified in field collected insect specimens by specific nested PCR assays. Mirid bugs without phytoplasma infection were obtained from hatched egg produced by insects raised on uninfected soybean in greenhouse conditions. Ten offspring were randomly collected from these cages and used as negative controls feeding them on healthy plants. Uninfected mirids nymphs were inserted in homemade cages of 2 meters x 2 meters x 1 meter on average containing 40 symptomatic soybean plants in the field for 20 days acquisition (Figure 1). The insects were then transferred to healthy soybean plants under greenhouse conditions for phytoplasma transmission. After two weeks the plants were treated with imidacloprid (SC 35%) to kill the insects that were then PCR tested after DNA extraction to verify phytoplasma presence. The development of symptoms was then monitored. These transmission experiments were also repeated three times under field conditions (Gorgan, Iran) under net cages.

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Figure 1. Insect acquisition condition in soybean field.

Results

A total of 10 *C. pallidus* specimens among those used for the transmission were tested and showed 100% 16S ribosomal sequence identity to that of SbpSpa phytoplasma (data not shown). Soybean plants that were experimentally infested with mirids that had fed upon SbpSpa symptomatic plants confined in field cages showed SbpSpa symptoms 45 to 60 days after inoculation with mirid bugs (Figure 2).



Figure 2. Soybean plant showing proliferation symptoms after infection with insects.

Nested PCR analysis confirmed the phytoplasma presence and the identification by sequencing indicated the presence of 16S ribosomal sequences 100% identical to those of SbpSpa phytoplasmas.

Discussion

The SbpSpa phytoplasma was transmitted by grafting and experimental infestation by *C. pallidus* to healthy soybean plants after feeding on symptomatic soybean plants. Moreover, the control of this mirid resulted effective in controlling the SbpSpa disease spreading (Faraji and Reisi, 2018) indirectly confirming its role in the phytoplasma transmission. This is quite a new finding since it was mostly reported that an insect must feed in the phloem to be able to transmit phytoplasmas in a non-destructive manner, but reports of insect vectors having different feeding habits support a mirid role in phytoplasma transmission (Mitchell, 2004). Two families of hemipterans, including Tingidae and Pentatomidae, were demonstrated as phytoplasma vectors and adults and nymphs of the brown marmorated stink bug *Halyomorpha halys* Stål (*H. mista* Uhler) were shown to vector the paulownia witches' broom phytoplasma and aster yellows phytoplasma to paulownia and periwinkle in Japan, Korea, China and in Italy, respectively (Okuda *et al*., 1998; Hiruki, 1999; Paltrinieri *et al*., 2016).

In conclusion, SbpSpa disease in Iran is consistently associated with '*Ca*. P. trifolii' transmitted by *C. pallidus*. These findings are helpful for developing strategies for the management of this disease in soybean in Iran.

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Insect vectors

Molecular characterization of a phytoplasma associated with sesame phyllody diseases and identification of its insect vector in Kerman province of Iran

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Abstract

Phyllody is one of the most important sesame diseases in the Kerman province of Iran with 5 to 100% incidence. Molecular detection and multilocus sequence characterization of the associated phytoplasma was conducted on 16S rRNA, *rp*, *secY* and *secA* genes sequences. BLAST searches, phylogenetic and *i*Phyclassifier analyses revealed that the phytoplasma associated with sesame phyllody in the region belongs to subgroup 16SrII-D. Biological and molecular experiments showed that the leafhoppers *Orosius albicinctus* and *Circulifer haematoceps* could transmit this sesame phyllody phytoplasma to sesame seedlings.

Keywords: oilseed crop, MLSA, *Orosius albicinctus*, *Circulifer haematoceps*

Introduction

Sesame (*Sesamum indicum* L.) is the main oilseed crop in the tropical regions of the Kerman province of Iran with over 8,500 ha cultivation area (Ahmadi *et al*., 2022). Phyllody is one of the most important diseases of sesame in this region. Phytoplasmas belonging to groups 16SrI, 16SrII, 16SrVI and 16SrIX has been reported in association with sesame phyllody disease in Pakistan, Thailand, Taiwan, Oman, India, Myanmar, Turkey and Iran (Salehi and Izadpanah, 1992; Al-Sakeiti *et al*., 2005; Khan *et al*., 2007; Esmailzadeh-Hosseini *et al*., 2007; Sertkaya *et al*., 2007; Akhtar *et al*., 2009; Win *et al*., 2010; Kumar *et al*., 2011; Manjunatha *et al*., 2012; Catal *et al*., 2013; Tseng *et al*., 2014). The leafhoppers *Orosius albicinctus* and/or *Circulifer haematoceps* have been reported as insect vectors of this disease in Turkey, India, Thailand and central provinces of Iran (Salehi and Izadpanah, 1992; Sertkaya *et al*., 2007; Esmailzadeh-Hosseini *et al*., 2007).

Materials and Methods

Plant samples were collected from sesame fields of Kerman province, Iran during the growing season. DNA was extracted from leaf midribs using DNeasy plant mini kit (QIAGEN Gmbh, Hilden) and PCR assays were performed to amplify the 16S rRNA, *secA*, *secY* and *rp* genes of the associated phytoplasma by specific primer sets (Table 1) and the amplicons were directly sequenced. The obtained sequences were searched in NCBI using BLASTn online search (http://www.ncbi.nlm.nih.gov/blast), and the phytoplasma ribosomal group was determined using *i*PhyClassifier (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) after sequence editing by BioEdit (Hall, 1999). Phylogenetic trees were constructed using MEGA X software based on the Neighbor-Joining (Kumar *et al*., 2018). The leafhoppers *Orosius albicinctus*, *Circulifer haematoceps* and *Empoasca decipiens* were captured, checked for phytoplasma presence by PCR tests and released in groups of 30 on pots containing 3-4 leaf stage sesame seedlings. The pots were covered by an insect-proof net until symptom expression. The seedlings were visually checked for symptoms and tested for phytoplasma presence by PCR (Bosco and Tedeschi, 2013).

Table 1. Primer sets used for MLSA analysis of the sesame phyllody and leafhoppers phytoplasmas in Kerman province of Iran.

Primer set	Target gene	Amplicon size	Reference
P1/P7	16S rRNA	1.8 kb	Deng and Hiruki, 1991 Schneider *et al*., 1995
R16F2n/R16R2	16S rRNA	1.25 kb	Gundersen and Lee, 1996
SecYF1(II)/SecYR1(II)	*secY*	1.7 kb	Foissac *et al*., 2013
rp(II)F1/(II)R1rp	*rp*	1.2 kb	Martini and Lee, 2013
SecAFor1/SecArev3	*secA*	800 bp	Dickinson and Hodgetts, 2013

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Results

Incidence of sesame phyllody disease varied from 5 to 100%. The highest incidence was observed in Rudbar Jonoub. The expected PCR products were amplified from the symptomatic plants and *O. albicinctus* and *C. tenellus* leafhoppers using the 16S rRNA, *rp*, *secY* and *secA* genes specific primers but not from the asymptomatic plants and *E. decipiens* insects. BLAST and *i*PhyClassifier analyses of the sequences showed that the phytoplasmas in symptomatic plants, *O. albicinctus* and *C. tenellus* were identical and belong to 16SrII-D subgroup. The identification was confirmed using phylogenetic analysis of the 16S rRNA and other housekeeping genes described above (data not shown). The sesame seedling inoculated by *O. albicinctus* and *C. tenellus* showed little leaf and stunting symptoms, two weeks after inoculation. The same phytoplasma could be detected in the infected seedlings by sequence data.

Discussion

In addition to sesame, some of the vegetables, oilseeds, ornamentals and alfalfa have been reported as alternative hosts for 16SrII-D subgroup phytoplasma in Iran (Amirmijani *et al*., 2020). Wide host range of 16SrII-D subgroup phytoplasmas and high populations of *O. albicinctus* and *C. tenellus* leafhoppers, as its insect vectors, makes this pathogen a great threat to horticultural and agricultural plants in Kerman province and the country as well.

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Insect vectors

Insect vectors of phytoplasmas in soybean fields: discovery of *Recilia dorsalis* and *Exitianus indicus* through feeding medium assay

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Abstract

The inoculative potential ability of *Recilia dorsalis* and *Exitianus indicus* in soybean fields was detected through a feeding medium assay and TaqMan-quantitative PCR. The study inferred a seasonal pattern in detecting the phytoplasmas in the feeding medium. Phytoplasmas were consistently detected in the insect bodies throughout the season, suggesting also a possible role of alternate weed hosts as reservoirs. The PCR test of artificial diets provides an easy and reliable way to assess insect potential inoculative ability and enables large-scale testing.

Keywords: qPCR, insect transmission, seasonal phytoplasma pattern, artificial diet

Introduction

Phytoplasmas are associated with numerous plant diseases, affecting a wide range of plants worldwide (Lee and Davis, 2000) and are transmitted by phloem-feeding insect vectors belonging primarily to *Cicadellidae*, *Derbidae*, and *Cixiidae*, commonly known as leafhoppers, planthoppers, and psyllids (Weintraub and Beanland, 2006). The impact of phytoplasma diseases and their distribution is essentially dependent on the feeding behaviour of the insect vectors (Bosco *et al*., 1997).

In India *'Candidatus* Phytoplasma aurantifolia' (16SrII-B) and *'Ca.* P. australasia' (16SrII-D) are commonly associated with phyllody and witches' broom diseases in various pulse crops (Thorat *et al*., 2017). The no pod disease incidence in these crops resulting from phytoplasmas infection has led to significant crop yield losses (Thorat *et al*., 2016). In addition to crops, 16SrII phytoplasmas had also been reported from weeds (Duduk *et al*., 2018; Kirdat *et al*., 2020). The large distribution of phytoplasmas in crops and weeds underscores the contribution of insect vectors that allow pathogen transmission (Duduk *et al*., 2018; Rao *et al*., 2017). While several studies have detected phytoplasmas in insect DNA using PCR and suggested potential insect vectors for 16SrII phytoplasmas (Yadav *et al*., 2015; Salehi *et al*., 2007), only a few studies have confirmed the vectoring ability of insects through transmission assays (Phookan *et al*., 2019).

This study aimed to investigate the diversity of *Cicadellidae* insect vectors in soybean fields and determine their vectoring ability using feeding medium assays. The assay used in this study was a PCR-based method for detecting phytoplasma in insect feeding medium.

Materials and Methods

Insects belonging primarily to the *Cicadellidae* family were collected using a white light trap from soybean fields in western Maharashtra, India. The samples were collected in September and October 2017 (n=212) and 2018 (n=260). They were divided into 55 groups based on morphological characters, and representatives of each group were DNA barcoded using the cytochrome c oxidase (COI) gene (Folmer *et al*., 1994). All 472 samples were screened for phytoplasma presence using a TaqMan-qPCR assay (Christensen *et al*., 2004). *Recilia dorsalis* (Motschulsky) and *Exitianus indicus* (Distant) were selected for transmission studies owing their abundance and percentage of positivity to phytoplasma presence.

A feeding medium assay was used to assess the vectoring ability of *R. dorsalis* (n=318) and *E. indicus* (n=341), collected periodically from July to October 2019 and 2021, following the protocol of Tanne *et al.* (2001). In brief, each insect was placed in a sterile 1.5 ml Eppendorf tube filled with 200 µl of sterile TE sucrose (5%) in their caps and sealed with parafilm. The field-collected insects were placed directly in these tubes and allowed to feed on the medium for 4 to 5 days. The TaqMan-qPCR assays was used to detect the presence of phytoplasmas in both insect body and feeding medium.

Results and Discussion

The insect samples (n = 472) were identified morphologically and confirmed with DNA barcoding (GenBank accession numbers MW221037 to MW228467). *R. dorsalis* (n = 162)

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and *E. indicus* (n = 86) were found predominantly infected with phytoplasmas in the soybean fields. The qPCR analysis revealed that 50.41% of the insects (n = 472) carried phytoplasmas. Moreover, this study identified known phytoplasma vectors, such as *Hishimonus phycitis*, *Nephotettix virescens*, and *Cofana unimaculata*, in soybean fields carrying phytoplasmas. Additionally, this study reports *Balclutha incisa* and *Yamatotettix sexnotatus* as potential phytoplasma vectors.

This study confirmed the inoculative ability of *E. indicus* and *R. dorsalis* through feeding medium assay. The qPCR assays confirmed the presence of phytoplasmas in the feeding medium and insect bodies with an average positivity rate of 15.03% and 27.35%, respectively. The detection of phytoplasmas in the feeding medium showed a seasonal pattern (Figure 1).

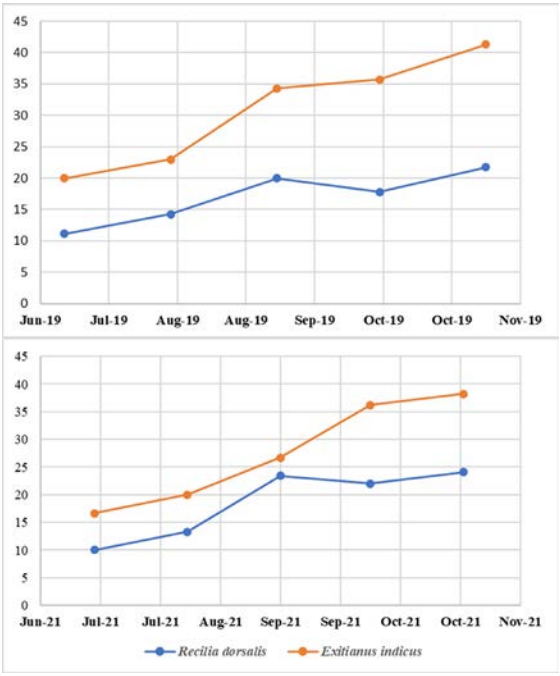


Figure 1. Inoculative ability of *E. indicus* and *R. dorsalis* through feeding medium assay assessed using qPCR assays from July to October 2019 (top) and 2021.

In contrast, phytoplasma detection in the insects' bodies did not indicate a season-related pattern. Phytoplasmas were found in the insect bodies throughout the season, suggesting a role of alternate weed hosts as phytoplasma reservoirs. Identification of insect vectors and their transmission parameters is crucial for controlling vector-transmitted diseases. However, large-scale biological assays for vector identification can be impractical, making the PCR test of artificial diets a reliable and easy tool for assessing insect inoculative ability.

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Epidemiology

Jujube witches' broom disease: bacteria that drive the plants crazy

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Abstract

The jujube witches' broom (JWB) also called "zaofeng" disease in Chinese, associated with '*Candidatus* Phytoplasma jujube', originates heavy losses to the jujube industry. This review intends to provide an up to date of JWB disease studies and describes the present situation in China with a look at perspectives for its management.

Keywords: *Ziziphus jujuba*, phytoplasma, plant disease, pathogenesis

Introduction

Chinese jujube (*Ziziphus jujuba*), also called common jujube, Chinese date, red date, or "zao", is priced for its delicious and nutrient fruit (Liu *et al*., 2020). The most destructive disease of jujube is the witches' broom (JWB) associated with the presence of '*Candidatus* Phytoplasma ziziphi' (Jung *et al*., 2003). JWB-infected jujube trees show the modification of fundamental plant development processes, resulting in yield loss, decrease of fruit quality, and death of the plants in a few years, therefore bringing heavy losses to the jujube industry (Ye *et al*., 2017).

The JWB disease is named "zaofeng" disease by local farmers in northern China and it was first reported by Ji in 1951. In Chinese, "zao" is jujube, and "feng" means uncontrolled or crazy, these words are describing the uncontrolled symptoms of witches' broom, proliferation of shoots, phyllody (leafy flower), yellowing, and growth stunning of the diseased trees (Liu *et al*., 2009). Nowadays, the JWB disease has spread all over Chinese main jujube cultivation areas. The JWB disease was first observed in middle part of Korea in the 1930s, and spread out by the 1950s. Nowadays, '*Ca*. P. ziziphi' associated diseases are distributed in every province throughout Korea. Occurrence of JWB has also been reported in Japan and India.

Witches' broom and phyllody are the most characteristic JWB disease symptom. Diseased trees show the precocious development of proliferating secondary shoots, which have an over-abundance of abnormally small and sometimes chlorotic leaves. The flower sepal and pedal turn to leaf like structures, petiole and pistil show elongation, sometimes the pistil turn to two small leaves (Figure 1).

Transmission experiments confirmed as insect vectors of JWB phytoplasma *Hishimonus sellatus*, *Hishimonides chinensis*, *Hishimonoides aurifascialis*, *Typhlocyba sp.*and *Hishimonus lamellatus*. Other than *Z. jujuba*, '*Ca*. P. ziziphi' infects a few other plants in the *Ziziphus* genus, some common fruit trees, garden trees, grass, and crops as well.



Figure 1. Jujube small plant infected by '*Candidatus* Phytoplasma ziziphi' (courtesy A. Bertaccini).

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Pathogenesis

The photosynthesis reduction is a major physiological function impaired by the JWB phytoplasma. The mineral elements content of healthy, JWB diseased and tetracycline treated JWB diseased jujube trees leaves show significant variation. Phytoplasma infection also change the anatomic structures of jujube trees and resulted in jasmonic acid content variation (Ye *et al*., 2017). The infection and colonization from this phytoplasma modulate the gene expression pattern of host plants. To reveal the key gene and pathway responding to phytoplasma infection, suppressive subtraction hybridization (SSH), qRT-PCR, transcriptomics, proteomics, and metabolomics analysis were performed in health and diseased jujube plants. Some transcription factors, and functional genes involved in the plants-phytoplasma interaction were identified. Most of them were involved in the biotic stress, phytohormone biosynthesis, or metabolism pathways (Chen *et al*., 2019; Wang *et al*., 2019; Li *et al*., 2022). The jujube witches' broom phytoplasma, strain nky, genome was sequenced. The jwb-nky genome consisting of one circular chromosome of 750,803 bp with a G+C content of 23.3%, encoding 694 protein-coding genes, two operons for rRNA genes and 31 tRNA genes. Four potential mobile units (PMUs) containing clusters of DNA repeats were also identified (Wang *et al*., 2018). A few JWB phytoplasma effectors have been identified. Secreted JWB phytoplasma protein, SJP1 and SJP2, were determined to target the TCP transcription factor ZjBRC1, induce witches' broom with increased lateral branches, promote the accumulation of endogenous auxin indole-3-acetic acid in jujube *calli*. SJP3 disrupts the expression of MADS-box transcription factors associated with floral organ identity and flowering time, inducing phyllody in both jujube and *Arabidopsis* (Deng *et al*., 2021). It was also identified a JWB phytoplasma effector, named Zaofeng6, that interacts with ZjTCP7, homologue of *Arabidopsis* BRC1 by its first two α-helix domains in the cell nuclei, down-regulate gene expression in strigolactone signalling pathway, and induce shoot proliferation (Chen *et al*., 2022).

Detection and Control

The JWB disease symptoms, especially witches' broom and phyllody, are quite typical, allowing to recognize JWB, and distinguish it from other jujube disorders (Liu *et al*., 2009).

JWB phytoplasmas could be detected by 16S rDNA amplification with either phytoplasma universal primers or JWB specific primers (Jung *et al*., 2003). Recently loop mediated isothermal amplification of DNA (LAMP) and CRISPR/Cas12-based visual assay, have been applied for pathogen detection. An all-in-one dual CRISPR assay technique for phytoplasma detection has been developed, which could also be used for JWB phytoplasma detection. This technique is sensitive, accurate, visual and efficient, do not rely on costly equipment, and can be used in the field.

Comprehensive control of JWB disease include efficient measures in seedling cultivation, symptom grading, orchard management, antibiotics application and sanitary measures (Liu *et al*., 2009). Phytoplasma-free seedlings may be obtained by breeding for resistance, phytoplasma-free tissue culture, tetracycline treatment, heat treatment, or cryopreservation. The comprehensive control measures may not eliminate phytoplasmas completely but control the phytoplasma presence at a relatively economic level.

Future study on JWB disease should focus on properties of JWB phytoplasma and interaction of JWB phytoplasma with plant/insect hosts. Culture in artificial media and genome sequencing are the key to reveal the diversity, evolution and molecular properties of these bacteria. More JWB phytoplasma effectors and the target protein degradation mechanism induced by JWB phytoplasma effectors still need further study.

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Epidemiology

Origin of isolated cases of "flavescence dorée" in North-East of France: search for reservoir plants and insect vectors in semi-natural habitats near vineyards

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Abstract

The origin of grapevine "flavescence dorée" (FD) isolated cases detected in Champagne and Alsace was studied. Grapevines, woody plants and auchenorrhynchan insects were sampled in the environments of vineyards. The only perennial plants found infected by 16SrV-C phytoplasmas were alders. *Scaphoideus titanus* and the known alternative vectors *Allygus* spp. and *Orientus ishidae* were infected by FD phytoplasma, map M38 genotype. They are good candidates for the transfer of M38 from alders to grapevine. *Lamprotettix nitidulus* was also infected by the M38 genotype in Alsace. The origin of M50 and M50 variant detected in some vineyards remains unknown since both plants and insects tested were not infected by these FD phytoplasma genotypes.

Keywords: epidemiology, riparian habitat, *Vitis vinifera*, Auchenorrhyncha

Introduction

"Flavescence dorée" (FD) is a grapevine disease in Europe, epidemically transmitted by the monovoltine grapevine leafhopper *Scaphoideus titanus*. FD originally emerged due to the conjunction of the introduction of *S. titanus* into Europe and the two spillovers of subgroup 16SrV-C phytoplasmas from alders to grapevine in Western Europe and from *Clematis vitalba* to grapevine in Eastern Europe.

The auchenorrhynchan species *Allygus* spp., *Dictyophara europaea* and *Orientus ishidae* were demonstrated as alternative vectors of FD phytoplasma in environments of vineyards with occasional transfer to grapevine (Filippin *et al*., 2009; Lessio *et al*., 2016; Malembic-Maher *et al*., 2020). Recent detection of FD phytoplasmas in refuge plant and leafhopper species could potentially complexify the epidemiologic cycle of FD (Casati *et al*., 2017).

The objective of this study was to investigate the origin of FD isolated cases detected in grapevine in 2019 and 2020 in Champagne and Alsace (France). A first step consisted in searching FD phytoplasmas in perennial plants and Auchenorrhyncha from semi-natural habitats near vineyards.

Materials and Methods

The sampling was carried out in 6 sites: in Alsace in Bergholtzzell (site B) and in Champagne in Arrentières (A), Chouilly-South (Cs), Chouilly-North (Cn), Reuil (R) and Saudoy (S). In each site, ligneous trees, bushes and grapevines present in semi-natural habitats surrounding the vineyards were inventoried, identified with the Plantnet Application (https://plantnet.org) and wood samples were collected in autumns 2020 and 2021. Auchenorrhynchan insects were captured on a total of 28 yellow sticky traps placed on riverbanks, forest edges, hedges and inside grapevine plots. Traps were renewed every 2 weeks from June to October 2021. Insects were also collected by nets beating the vegetation. Each captured Auchenorrhyncha insect (except Typhlocybinae) was identified with a taxonomic key (Biedermann and Niedringhaus, 2009). Total DNA from plants was extracted with CTAB and TNES protocols from plants and insects, respectively. Plants were first screened by universal quantitative PCR (Christensen *et al*., 2004) and sequencing of 16S rDNA for phytoplasma identification. Phytoplasmas of 16SrV group were further characterised by sequencing the *map* gene as described in Arnaud *et al*. (2007).

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Table 1. Detection of 16SrV phytoplasmas in insect samples, by site.

Insect species	Number of 16SrV positive samples / Number of samples tested, map genotype by site					
	B (M38)	A (M50 var)	Cn (M50)	Cs (M50 var)	R (M38)	S (M50 var)
S. titanus[1]	-	0/20[2], 0/12[3]	0/80[2]	NT	1/347[2] 8/82[3] M38, ND	0/30
O. ishidae	88/157 M38, ND	-	0/2	-	-	-
Allygus spp.	55/83 M38, M47, ND	11/32 M43, M45, M53, ND	5/31 ND	0/3	8/18 M53, ND	2/9 M38, ND
L. nitidulus	41/52 M38, ND	0/20	-	0/2	1/3 ND	0/3
Allygidius spp.	NT	3/12 '*Ca.* P. ulmi', ND	9/22 '*Ca.* P. ulmi', ND	5/11 '*Ca.* P. ulmi', ND	5/8 '*Ca.* P. ulmi', ND	-
Euscelidius spp.	-	0/12	0/10	NT	1/22 ND	1/7 M38
Fieberiella sp.	NT	2/9 ND	0/5	1/9 ND	0/2	0/4
Thamnotettix sp.	-	0/1	-	3/9 ND	-	0/1

[1]ST (*S. titanus*) tested by pools of 1 to 4 individuals. [2]ST collected near grapevine in 2020. [3]ST collected near alders in 2021. NT, Not tested. ND, Not determined. var, genotype variant.

Grapevine samples giving FD phytoplasma detection were map genotyped at LDA33 and IFV Grau-du-Roi accredited laboratories. Insects were directly screened by nested PCR and sequencing of the *map* gene.

Results and Discussion

In Alsace, where *S. titanus* is at the introduction stage but absent at site B, the genotype map M38 was detected on a single grapevine stock.

In Champagne, where *S. titanus* is established from long time, the situation was more complex with sporadic FD cases: M38 genotype in site R, M50 in Cn, and M50 variant (1 SNP) in Cs, A and S. A total of 1,094 wild plants belonging to 45 genera were inventoried and 346 samples were collected and tested. *Alnus glutinosa*, only present in sites B, A and R, were infected by 16SrV-C phytoplasmas (18/21) with the alder yellows phytoplasma (AldY) M52 genotype or with unresolved mixed infections. The other plants, *i.e.*, 49 *Clematis vitalba*, 28 *Corylus avellana* and 16 *Salix* spp. were not infected by 16SrV-C or -D phytoplasmas. '*Candidatus* Phytoplasma ulmi' was detected in some *Ulmus* sp. samples (5/9) and in *Crataegus* sp. (1/15), '*Ca.* P. pyri' in *Crataegus* sp. (2/15), '*Ca.* P. prunorum' in *Prunus* spp. (3/39), '*Ca.* P. asteris' in *Populus* sp. (1/8) and '*Ca.* P. spartii' in *Cytisus scoparius* (1/6). A total of 28,376 insects belonging to 70 species were collected and identified. High numbers of *S. titanus* were captured, except in site B. Only *S. titanus* collected in R in grapevine near alders were infected by FD phytoplasmas, M38 genotype (Table 1). *O. ishidae* was mainly captured in site B, with a high FD phytoplasma infection rate (56%) and M38 detected. *Allygus* spp. captured in sites B and S were infected by M38 FD phytoplasma genotype and by AldY genotypes M43, M45, M47 and M53 in other sites. *Lamprotettix nitidulus* was also infected by FD phytoplasma with a high infection rate in B (79%) with M38 detected. *Euscelidius* spp., *Fieberiella* sp. and *Thamnotettix* sp. were also sporadically detected infected by 16SrV phytoplasmas. Finally, *Allygidius* spp. were detected infected by '*Ca.* P. ulmi'.

In sites Cs, Cn and S, the reservoir plants and alternative insect vectors of the M50 and M50 variant genotypes, are still unknown and need further investigations. In B, the alternative vectors *Allygus* spp. and *O. ishidae* are suspected to be responsible for the transfer of M38 from reservoir alders to grapevine. The role of *L. nitidulus* as an alternative vector of M38 is under study by transmission trials (B. Jarausch and M. Maixner, personal communication). In R, the main suspect insect vector for M38 transmission to grapevine is *S. titanus*.

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Epidemiology

Epidemiology of pear decline in orchards without vector control in Southwest Germany

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Abstract

In the past decade, pear decline is spreading in Southwest Germany due to climate change and reduced insect vector control. Monitoring of nine old pear orchards revealed infection rates between 40% and 80%. The presence of '*Candidatus* Phytoplasma pyri' was confirmed by PCR. In 2021-2023, all three known pear psyllid vectors were captured in various population densities. Winter forms of *Cacopsylla pyri* were infected between 0.4 – 4.4% while winter forms of *C. pyricola* had infection rates of 2.9 – 9.4%. The univoltine species *C. pyrisuga* was scarcely captured with only one infected individual. *C. pyri* and *C. pyricola* transmitted the phytoplasma to healthy test plants, but quantitative PCR data indicated that only about 40% of infected individuals of both species had sufficient high phytoplasma loads to be regarded as infectious. As both species are very abundant in orchards without insect vector control, spread of pear decline is high.

Keywords: '*Candidatus* Phytoplasma pyri', *Cacopsylla pyri*, *Cacopsylla pyricola*, *Cacopsylla pyrisuga*, qPCR

Introduction

Pear decline (PD) is a devastating phytoplasma disease of pear in Europe and North America. '*Candidatus* Phytoplasma pyri' is spread by the polyvoltine pear psyllids *Cacopsylla pyri* and *C. pyricola* (Jarausch *et al.*, 2019). Recently, the vector capability of *C. pyrisuga* has also been demonstrated (Riedle-Bauer *et al.*, 2022). *C. pyri* and *C. pyricola* overwinter as winter forms on *Pyrus* spp. whereas the univoltine species *C. pyrisuga* remigrates from conifers to *Pyrus* spp. by middle of March (Jarausch *et al.*, 2019). These winter forms and remigrants, respectively, had the highest transmission efficiencies (Riedle-Bauer *et al.*, 2022) and thus represent the major risk for PD spread in the orchards. Highly varying data have been published for the natural phytoplasma infection rate of *C. pyri* ranging from a mean of 0.3% in Bulgaria (Etropolska *et al.*, 2015) and 5% in Austria (Riedle-Bauer *et al.*, 2022) to 50% (Italy) and up to almost 100% in Spain (Jarausch *et al.*, 2019). Respective data for Germany are almost missing. Herzog *et al.* (2012) reported for *C. pyri* an overall infection rate of 3.5% in Sachsen but did not capture and test *C. pyricola*. *C. pyrisuga* specimens were not infected. *C. pyricola* seems to be more abundant in the northern part of Germany and has been reported as vector in Great Britain and the Unites States of America (Jarausch *et al.*, 2019).

Since insect vector control has been reduced and hot summers with drought stress the trees, the impact of PD has increased in Germany and adjacent regions. Therefore, the epidemiology of PD in Southwest Germany was revised by monitoring older pear orchards and analysing the winter forms of the pear psyllids which are considered the most efficient spreader of PD.

Materials and Methods

Nine more than 10 year's old pear orchards were monitored for PD symptoms in the Palatinate region in Southwest Germany. '*Ca.* P. pyri' presence was confirmed by PCR in a substantial number of trees per orchard (Jarausch *et al.*, 2023) using primers fO1/rO1 as described in Jarausch *et al.* (2011). No or only limited (one per season) treatments against pear psyllids were performed in these orchards. Pear psyllids were captured by the beating tray method in February-March and November-December 2021 and 2022 as well as in February 2023. Population densities were estimated as psyllids per branch. Species were identified and total nucleic acid was extracted from individuals using the TNES protocol described by Sauvion (2012). Phytoplasma detection was achieved by PCR with primers fO1/rO1. For phytoplasma quantification in 14 infected *C. pyri* and 45 *C. pyricola* individuals forward and reverse primers of Nikolic *et al.* (2010) were used in quantitative PCR with SYBR Green as described by Jarausch *et al.* (2011). Normalisation was done by quantification of a *wg* gene fragment of the *Cacopsylla* species (Jarausch *et al.*, 2010).

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Table 1. '*Ca.* P. pyri'-infection rate of pear psyllids at different periods (number PCR-positive/total number tested).

	February/March 2021	November/December 2021	February/March 2022	November/December 2022	February 2023
C. pyri	4 / 997 (0.40%)	5 / 429 (1.17%)	11 / 425 (2.59)	18 / 409 (4.40%)	4 / 641 (0.62%)
C. pyricola	7 / 240 (2.92%)	27 / 554 (4.87%)	23 / 445 (5.17%)	11 / 127 (8.66%)	38 / 403 (9.43%)
C. pyrisuga	0 / 103	-	1 / 33 (3.03%)	-	-

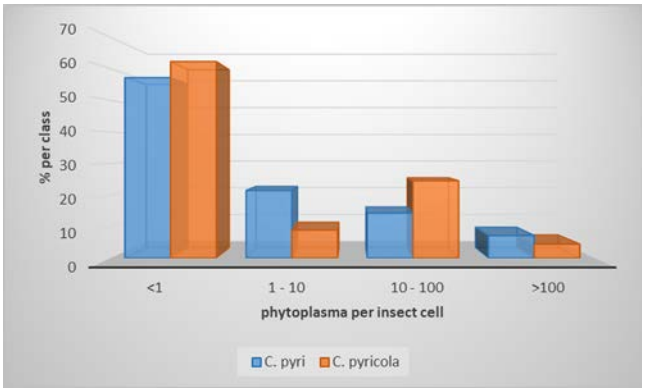


Figure 1. Quantification of '*Ca.* P. pyri' in pear psyllids expressed as phytoplasma per insect cell and data distribution within 4 classes.

In parallel, 10 - 20 field captured pear psyllids were caged with healthy *ex vitro* plants of pear to verify the transmission capacity. Test plants were firstly tested by PCR as described above one year after the transmission experiment.

Results and Discussion

All three pear psyllids were found to be infected with '*Ca.* P. pyri' in varying amount according to year, season and species (Table 1). The mean infection rate of *C. pyricola* was always significantly higher than in *C. pyri*. The data obtained for *C. pyri* were in the range of data reported from Bulgaria and Austria. However, it is surprising that low infection rates were found in *C. pyri* populations captured in highly PD-infected orchards. On the contrary, infection rates of *C. pyricola* increased from year to year. Population densities of both species were high (1-2 psyllids per branch in late winter), but the distribution of both species was not homogenous: in a given orchard only one species was dominant, either *C. pyri* or *C. pyricola*. *C. pyrisuga* was rare, but widespread.

As PCR is not providing indication for infectivity, and thus for the risk of disease spread, quantitative PCR was performed to analyse the phytoplasma loads in the PCR-positive samples. The DNA was extracted from individual psyllids with a crude extraction protocol, and the phytoplasma data were normalized with the quantification of a genomic fragment of the wingless gene of psyllids. The result shown in Figure 1 expresses the phytoplasma load of *C. pyri* and *C. pyricola* specimens as phytoplasmas per insect cell. As the data varied considerably, they were grouped them into 4 classes. It was estimated that individuals with less than one phytoplasma per insect cell just acquired the phytoplasma but are not yet infectious. Thus, about 60% of infected individuals of both species represent no risk for the disease spread even after overwintering. Individuals of *C. pyricola* with a phytoplasma load of more than 100 per insect cell had a phytoplasma concentration of about $5x10^6$ which is in the range reported for *C. pyricola* for California (Jarausch *et al.*, 2019). The transmission experiments confirmed these results: transmission rates for both species were low, but the inoculated plants need to be tested a second time for final results. The data collected indicate that the increase of PD is due to a spread of *C. pyricola* which is a more efficient vector of '*Ca.* P. pyri' than *C. pyri*. *C. pyrisuga* presents no risk in Germany.

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Epidemiology

'*Candidatus* Phytoplasma trifolii' associated with faba bean phyllody in Jordan

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Abstract

Faba bean phyllody associated with a high population of leafhoppers was observed in fields in Jordan Valley. Samples of affected plants were used to transmit the phyllody agent to faba bean by grafting. *Empoasca decipiens* was detected as potential insect vector of the disease. The phytoplasma presence was detected by nested PCR targeting the 16S rDNA using the primer pairs P1/P7 followed by R16F2n/R16R2. Nucleotide sequences of phytoplasmas identified in faba bean, some weeds and insects shared 99.6% sequence identity with '*Ca*. P. trifolii', subgroup 16SrVI-A.

Keywords: phytoplasma, faba bean phyllody, insect vector, 16SrVI group

Introduction

Faba bean (*Vicia faba* L.) ranks among the most globally important legume crops. It has a high content of macro and micronutrients, besides its important role in nitrogen fixation (Etemadi *et al*., 2019). Plant pathogenic phytoplasmas have been reported to infect *V. faba* in several countries including Cuba, Egypt, India (Bihar), Saudi Arabia, Spain, Sudan, Iran, and Peru (Alfaro-Fernandez *et al*., 2012; Al-Saleh and Amer, 2014; Arocha *et al*., 2007; Castro and Romero, 2004; Hamed *et al*., 2014; Jones *et al*., 1984; Omar, 2017; Salehi *et al*., 2016; Singh *et al*., 2013; Torres Suarez *et al*., 2021).

In February 2021, symptoms including abnormal growth, stunted plants, witches' broom, leaf yellowing, phyllody, and shoe-stringed leaves were observed in the faba bean fields located in the Jordan Valley, Jordan. Disease incidence ranged from 10 to 30%, and affected plants were invaded by dense leafhopper populations. Since no report are available on the occurrence of phytoplasmas in faba bean plants in Jordan, the objectives of the current investigation were to detect and identify the phytoplasma present in the faba bean symptomatic plants and to identify its insect vector.

Materials and Methods

During fields survey in Jordan Valley faba bean plants with symptoms of phyllody (FBP), asymptomatic faba bean, some weeds, and leafhoppers were collected and used for further molecular and biological studies. For transmission of the FBP agent, collected symptomatic faba bean plants were used for grafting of two-weeks old healthy 20 faba bean plants. The graft-inoculated plants were kept in the greenhouse. Leafhoppers collected from the same field were directly caged on 18 healthy faba bean plants (10-15 insects/plant) for 14 days of inoculation access time. The plants were sprayed with insecticide and kept in the greenhouse for disease development. Healthy faba bean plants were kept as controls in the same greenhouse. All plants were tested 6 weeks after inoculation by PCR. Total DNA was extracted by DNeasy Plant Mini Kit (Qiagen) following the manufacturer instructions, from fields collected plants, experimentally inoculated plants, and leafhoppers collected from affected faba bean fields. DNA extracts were analyzed by PCR using the phytoplasma universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by nested PCR with primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). PCR products from selected samples were purified and ligated into pGEM T-Easy Vector (Promega), and two clones for each PCR product were sequenced and deposited in NCBI GenBank (accession numbers OQ435606 to OQ435613). Leafhopper identification was done following the key of Le Quesne and Payne (1981) and the interactive key at http://dmitriev.speciesfile.org, which included relevant literature about Jordan or other adjacent areas. Furthermore, the total DNA extracts from the leafhoppers were tested by PCR using two sets of primers covering regions of the mitochondrial cytochrome oxidase subunit I (COI) gene and the 28S rRNA (Farrokhzadeh *et al*., 2014). Amplicons were purified, cloned, and sequenced as described above. The R16F2n/R2 nucleotide sequences were:

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compared with those of the 49 '*Candidatus* Phytoplasma' species described (Bertaccini *et al*., 2022) and analyzed by *i*PhyClassifier online tool for ribosomal group/subgroup assigment (Zhao *et al*., 2009).

Results

Characteristic symptoms of FBP were observed in 4 faba bean fields out of the 7 fields visited during the survey carried out in 2021/2022 growing seasons in Jordan Valley. Nested PCR produced a band of the expected size (1,250 nt) in the collected symptomatic faba bean samples (33), *Chenopodium murale* (5), *Convulvulus arvensis* (2), and *Malva parviflora* (1). No phytoplasma was detected in asymptomatic faba bean samples (6) and one *C. arvensis* sample. Phytoplasmas were detected in the experimentally grafted faba bean plants. Symptoms developed on faba bean inoculated plants were similar to those observed in naturally infected plants. The leafhoppers collected from infected faba bean fields resulted positive for phytoplasma presence and were identified as *Empoasca decipiens* based on the microscopic examination of male genitalia and further confirmed by BLASTn search using nucleotide sequencing of the COI and 28S rRNA genes. The R16F2n/R2 nucleotide sequences of phytoplasmas detected in faba bean, *C. murale*, *C. arvensis*, *M. parviflora*, and *E. decipiens* were identical with an identity percentage of 99.6% with '*Ca*. P. trifolii'. The *i*PhyClassifier analyses assigned '*Ca*. P. trifolii' strains identified in this study to the ribosomal subgroup 16SrVI-A.

Discussion

In this study FBP disease was associated with phytoplasmas based on grafting and insect vector transmission, amplification by PCR and sequence analysis. *E. decipiens* transmitted the FBP to faba bean healthy plants. According to a recent survey conducted by Nabas and Katbeh-Bader (2020), *E. decipiens* was found widely distributed in Jordan and it was confirmed as a potential vector of phytoplasmas in different crops. This is the first report of a 16SrVI group phytoplasma associated with faba bean phyllody disease. Further study should be carried out to monitor the FBP diffusion in Jordan, in depth characterize the '*Ca*. P. trifolii' strain associated with FBP, and to determine the role of reservoir plants (*C. murale*, *C. arvensis*, and *M. parviflora*) in the FBP epidemiological pathway(s).

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Epidemiology

Soybean bud proliferation phytoplasma transmission by seed

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Abstract

Soybean bud proliferation (Sbp) disease is associated with a phytoplasma of the 16SrVI-A subgroup. Because of the widespread occurrence of this disease, the possibility of its seed transmission was studied. Seeds collected from Sbp infected, or symptomatic plants were examined and compared with those collected from asymptomatic soybean plants. The results showed that none of the plants grown from seeds collected from asymptomatic plants were positive for the presence of phytoplasmas. Five months after germination, soybean plants grown from the seeds collected from symptomatic plants showed typical symptoms of Sbp disease under greenhouse conditions and also tested positive for phytoplasmas in nested PCR. Sbp is a new phytoplasma disease that could be transmitted through seeds in Iran.

Keywords: phytoplasma, seed transmission, soybean bud proliferation

Introduction

One of the important soybean diseases that were in epidemic proportion during 2013 and 2014 in Iran is the seed pods abortion (Sbp). Sometimes, these symptoms were found to be associated with insect damage (*Creontiades pallidus*) too. In 2018, a phytoplasma of the clover proliferation group (16SrVI) was reported to be associated with seed pod abortion symptoms (Ghayeb Zamharir and Aldaghi, 2018, Ghayeb Zamharir *et al*., 2022). Seed transmission of phytoplasmas was first reported in alfalfa in Oman (Khan *et al*., 2004). A similar result was found for phytoplasma disease transmission in *Dianthus* spp. (Seruga-Music *et al*., 2004) and tomato (Botti and Bertaccini, 2006). In 2010, a "stolbur" phytoplasma was detected in pea seedlings (Zwoliñska *et al*., 2010). However, no phytoplasmas were detected in chickpea seeds collected from plants infected by a 16SrII-D phytoplasma (Akhtar *et al*., 2009). To study the possibility of seed transmission of the Sbp phytoplasma, experiments were carried out under insect proof greenhouse conditions using seeds collected from field phytoplasma-infected soybean plants.

Materials and Methods

Soybean seeds were collected in Gorgan (36.7941° N, 54.1103° E) Golestan province, Iran from infected soybean plants. Altogether 1,000 seeds were analyzed, 500 from asymptomatic plants and 500 from symptomatic plants in the fields where phytoplasma infection was detected using nested PCR analysis and in which no ToRV, TRSV and ArMV presence was found after serological testing. Surface sterilized seeds were planted in pots in an insect proof greenhouse. Seedlings were sampled for nested PCR analysis 40 days after germination and observed for symptom development until maturity.

Leaf samples were subjected to DNA extraction using a CTAB method. The universal phytoplasma nested primer sets P1/Tint (Deng and Hiruki, 1991; Smart *et al*., 1996) and R16F2n/R16R2 (Gundersen and Lee, 1996) were used as the outside primer set and the inside primer set, respectively, for the amplification of a phytoplasma 16Sr DNA fragment of 1,250 bp. Selected P1/Tint (1,500 bp) and R16F2n/R2 (1,248 bp) amplified fragments from the phytoplasma detected in soybean plants with Sbp symptoms were directly sequenced. Sequence alignments were performed by using Clustal X (Thompson *et al*., 1997). Phylogenetic analyses were performed with maximum parsimony (MP) analysis using the close-neighbor-interchange algorithm.

Results

Results showed that germination percentage in the soybean seeds collected from infected plants was 76.2% which was significantly different from the seeds collected from

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asymptomatic ones (98.4%) by t-test analysis ($P < 0.01$). Only 9 out of 500 (1.8%) seeds collected from infected plants were positive for phytoplasma presence 40 days after germination by nested PCR analysis. No seedlings from seeds collected from asymptomatic samples were positive to phytoplasma presence. All the nine plants from seeds of phytoplasma positive plants showed typical Sbp in mature plants (Figure 1). The expected fragments of 1.25 kb were amplified from 9 out of 381 germinating seeds.



Figure 1. Mature plants germinated from Sbp phytoplasma infected soybean seeds (left and centre) compared with an asymptomatic plant (right).

The molecular analysis of aligned phytoplasma sequences obtained from soybean seedlings from infected plants, showed that the phytoplasma detected is in the 16SrVI-A subgroup.

Discussion

Recent studies in some herbaceous crops have shown that phytoplasmas could be transmitted through the seeds (Olivier and Galka, 2008; Calari *et al*., 2011; Satta *et al*., 2020). Seed-borne inoculum serves as an initial source of infection and may favour insect vector transmission causing widespread disease epidemic. Results of the present work confirm the hypothesis of the presence of Sbp phytoplasma in soybean seedlings producing Sbp symptoms in mature plants. The data suggest the presence of Sbp phytoplasma seed transmission in Iran. The present findings would be useful for developing strategies to control this phytoplasma disease in soybean in Iran.

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Epidemiology

Phytoplasma presence and inoculum sources in carrot fields in Turkey

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Abstract

Intensive surveys were conducted in major carrot cultivation areas of Turkey in 2018-2020 and 272 plant samples showing severe symptoms of chlorosis and reddening were collected in Konya and Ankara provinces, After molecular testing and by *in silico* RFLP analysis on sequences obtained 16SrI-B (*'Candidatus* Phytoplasma asteris') and 16SrVI-A ('*Ca.* P. trifolii') were identified. Furthermore, the inoculum sources of those phytoplasmas in same provinces were investigated. Weeds were collected from surroundings and *Daucus carota* wild, *Medicago sativa*, *Conium maculatum* and *Sinapis arvensis* were detected as infected be phytoplasmas in 16SrVI group. In addition, both phytoplasma groups were present in seedlings germinated from seeds of seven carrot cultivars, six were commercially produced and one was a local red carrot cultivar.

Keywords: phytoplasma epidemiology, inoculum sources, carrot, weeds

Introduction

Carrot (*Daucus carota* L.), a member of the family Apiaceae, is grown and consumed in almost every geographical region of the world. Turkey produces annually an average of 588,778 tons of carrot from 10,989 hectares, of which about 84% of the production is located in Ankara and Konya provinces in central Anatolia (TUIK, 2021). Phytoplasmas are Gram-positive bacteria without cell walls and are colonizing plant phloem tissues. In general, phytoplasma infections in carrot plants are associated with symptoms such as reddening/yellowing of the leaves, shoot proliferation, and decreased root quality (Satta *et al*., 2020). Phytoplasmas belonging to 16SrI and 16SrXII groups in Europe (Duduk *et al*., 2008), to 16SrV in Israel (Weintraub and Orenstein, 2004), to 16SrII in Saudi Arabia (Omar, 2017) and to 16SrVI in the USA (Lee *et al*., 2006) have been reported to infect carrot in the fields. In addition *Convolvulus arvensis* and wild *Daucus carota* were found to be infected with the 16SrXII group phytoplasma in Hatay province located in the Eastern Mediterranean Region of Turkey (Sertkaya, 2014). Several studies showed that phytoplasmas can also be transmitted via seeds in various plant species, including carrots (Calari *et al*., 2011, Satta *et al*., 2020). Moveover, weeds and wild plants are of high importance to understand the epidemiology of phytoplasma associated diseases (Banzato *et al*., 2021). In this study, the potential of carrot seeds and reservoir weed species as inoculum sources of phytoplasmas that occurred in carrot fields in the Ankara and Konya provinces was investigated.

Materials and Methods

Symptomatic and asymptomatic carrot plants and seven weed species in five botanical families were sampled from phytoplasma infected carrot fields and their surrounding areas in Beypazar and Nallhan districts of Ankara and Meram district of Konya, Turkey between April 2018 and September, 2020. Nineteen weed samples were collected, including 5 *Amaranthus retroflexus*, 7 *Sinapis arvensis*, 3 *Conium maculatum*, 1 *Bifora radians*, 1 *Daucus carota* wild, 1 *Medicago sativa* and 1 *Fumaria officinalis*. Symptoms such as reddening of leaf tips, mosaic, and vein clearing were noted on a few of them while most were symptomless. Seeds of eight widely grown carrot cultivars were also obtained from farmers during the surveys. Since the number of carrot seeds was quite limited, only approximately 200-300 seeds per cultivar were sown on pots containing sterilized soil. Total nucleic acid was extracted from the shoot tissues of 19 weed samples and 32 freshly harvested seedlings of eight weed samples and 1 g tissue from seedlings of each cultivar was used. Nested-PCR experiments were done using R16mF2/R16mR2 or R16F2n/R2 primer pairs (Gundersen and Lee, 1996) followed by fU5/rU3 (Lorenz *et al*., 1995) primer pair. The PCR products were separated in 1.2% agarose gel stained with ethidium bromide and visualized under a UV

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imaging system (Genegenius, UK). If there was more than one positive amplification result from the same cultivar in the seedling experiments, two results were chosen for nucleotide sequencing, and one sample was chosen when there was only one positive result. Bidirectional Sanger sequencing of 16 PCR products from four weed samples and seedlings was performed at BM laboratory, Ankara, Turkey. BLAST analysis at NCBI was used to compare the sequences with those of other phytoplasmas in the GenBank. The sequences were then submitted to the NCBI database under accession numbers MZ463005-MZ463020 and used for alignment with other phytoplasma sequences using ClustalW in MEGA X software. Computer-simulated RFLP analyses were performed with Snapgene (GSL Biotech; http://www.snapgene.com) digesting 16 sequences with *Rsa*I.

Results

Vein clearing, reddening at the leaf tips, yellowing, and severe reddening/purpling were observed in one of each *S. arvensis*, *C. maculatum*, *M. sativa*, and *D. carota* wild respectively. There was no phytoplasma symptom in all seedlings at the cotyledon stage. The main symptom in carrot plants was reddenning and chlorosis of leaves. Amplifications were produced in nested-PCR and the expected 883 bp amplicons were obtained. Positive results were obtained from most of the carrot seedlings and from 4 out of 19 weed samples. The sequence of CS8c strain obtained from a commercial carrot cultivar was 99.65% identical to an Iranian strain of '*Candidatus* Phytoplasma asteris' (GenBank accession number DQ266089). The remaining strains showed 99.78-99.89% sequence identities with some '*Ca.* P. trifolii' strains (GenBank accession numbers MT240537, MK392485, and MT071396). In the phylogenetic tree constructed on a 883 bp fragment using sequences of different phytoplasma groups/subgroups the phytoplasmas from four weeds and eleven seedlings strains clustered within '*Ca.* P. trifolii', only the CS8c strain clustered with '*Ca.* P. asteris'. After comparing computer-simulated RFLP analysis with *Rsa*I enzyme 15 strains were enclosed in 16SrVI and 16SrI groups.

Discussion

This study aimed to reveal seed and weeds as inoculum sources that play crucial roles in the spread of phytoplasma diseases associated with reddening, chlorosis, and reduced root quality widely observed in the largest carrot growing areas of Turkey (Ankara and Konya). Transmission of phytoplasmas through seeds was reported in tomato, corn and winter oilseed rape (Calari *et al.*, 2011). The presence of phytoplasmas in carrot seeds was first reported by Carminati *et al.* (2019) and confirmed by Satta *et al.* (2020) reporting that seedlings were infected at the cotyledon stage with phytoplasmas in groups 16SrI and 16SrXII. This study confirms the presence of 16SrI group and detect the 16SrVI group in the seedlings of carrot cultivars for the first time. The two phytoplasmas groups were not found together in any infected seedlings grown from the same cultivar.

This work also revealed that symptomatic *M. sativa*, *S. arvensis*, *D. carota* wild, and *C. maculatum* weeds were infected by phytoplasmas in the 16SrVI group. Phytoplasmas in 16SrIX-C, and 16SrXII-A subgroups have been reported in *S. arvensis*, *C. maculatum*, and *D. carota* wild, respectively (Casati *et al.*, 2016; Sertkaya, 2014). Therefore, this is the first report of 16SrVI group phytoplasmas in these wild species.

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Epidemiology

Seed transmission of phytoplasmas in tomato and chilli varieties commonly grown in Mauritius

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Abstract

Tomato and chilli leaf samples were collected from plants showing stunting, reduced leaf size, leaf yellowing and leaf and fruit distortions in different localities across Mauritius. Disease incidence at field level ranged from 5 to 60% in tomato and from 10 to 50% in chilli. The presence of 16SrI phytoplasmas was detected in 80% symptomatic tomato and 75% symptomatic chilli plants. Seeds from mature tomato and chilli fruits collected from phytoplasma positive mother plants were sown under insect proof conditions. After 4 to 6 weeks, germinated seedlings were checked for the presence of phytoplasmas. A total of 1% tomato and 4.2% chilli seedlings were positive to 16SrI phytoplasmas. This is the first report of phytoplasmas in chilli and also of phytoplasma transmission through seeds in both tomato and chilli in Mauritius.

Keywords: Phytoplasmas, PCR, RFLP, disease, seed transmission

Introduction

Tomato (*Solanum lycopersicum* L.) and chilli (*Capsicum annum* L.) are solanaceous crops of major social and economic importance in Mauritius. Both crops are affected by a number of important diseases, pests and abiotic disorders, wherever they are commercially grown. There are reports on the occurrence of phytoplasma diseases in tomato in Mauritius (Dookun *et al*., 1999; Gungoosingh-Bunwaree *et al*., 2007, 2013); however so far there are no records of phytoplasma diseases in chilli. Moreover, phytoplasma transmission was mostly attributed to insect vectors, after leafhoppers *mpoasca*, *Balclutha*, *Amrasca* and *Afrolestes* spp. were collected from infected tomato plantations (Gungoosingh *et al*., 2013). However, since phytoplasma infections were observed in tomato plants grown under strict insect proof conditions, the possibility of seed transmission was investigated. The survey was extended to chilli, since leaf distortions and yellowing were observed in chilli plants in the vicinity of phytoplasma infected tomato fields.

Materials and Methods

Twenty tomato plantations (including both protected and open field plantations) of 5 varieties: Swaraksha, MST 32/1, Pêche Rose, Topinas and Francesca were surveyed in different regions of Mauritius. Survey in chilli was carried out across 12 open field plantations on varieties Piment Cipaye, Petit Piment and Piment carri. To check the phytoplasma presence in tomato and chilli mother plants, one gram of leaf midribs was used for DNA extraction (Prince *et al*., 1993). Phytoplasma detection was performed by nested PCR in a total volume of 25 µl using as PCR mix 16.3 µl H_2O, 2.5 µl 10X PCR buffer, 2.0 µl dNTPs, 2.0 µl $MgCl_2$, 0.5 µl each of 20 µM forward and reverse primers, 0.2 µl of 5 U/µl *Taq* polymerase and 20 ng template DNA. A tube with reaction mixture devoid of DNA template was included as negative control. Phytoplasma specific universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) were used in PCR followed by nested PCR on amplicons diluted 1:30 with primers R16F2n/R2 (Gundersen and Lee, 1996). For further confirmation, primer pairs R16(I)F1/R1 (Lee *et al*., 1994) were used. The PCR was run as follows: an initial denaturation step at 94°C for 2 minutes followed by 35 cycles at 94°C for 1 minute, 50°C for 2 minutes, 72°C for 3 minutes and a final extension step at 72°C for 10 minutes. PCR products were electrophoresed on 1.0% agarose gel. Crotalaria phyllody (CrP, 16SrII-C) and "stolbur" (STOL, 16SrXII-A) phytoplasmas (Bertaccini, 2023) were used as positive controls. The gel was stained with ethidium bromide and visualised under UV light.

Mature fruits from plants tested phytoplasma positive were dried at ambient conditions and the seeds collected were sown in trays under insect proof conditions. Then, 1 g of leaf sample was used to check for presence of phytoplasmas after being processed as above described. Identification of phytoplasmas from seedlings was carried

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out by RFLP analyses with restriction enzymes *Tru*I, *Rsa*I and *Alu*I (Fermentas, Vilnius, Lithuania) under conditions provided by the manufacturer. The products were separated by electrophoresis in 6.7% polyacrylamide gel, and visualised as described above.

Results

Disease incidence in tomato ranged from 5% to 60%. The disease was prevalent in tomato variety Topinas grown under greenhouse in the south of Mauritius. Three mature fruits were collected from each symptomatic plant tagged, for a total of 120 fruits. Predominant symptoms observed in suspected phytoplasma infected tomato plants were stunting, leaf distortions, apical bushiness and purple leaf colouration (Figure 1). In chilli, the disease incidence ranged between 10% to 50%. A total of 25 chilli plant samples were collected. Three mature fruits were collected from each symptomatic plant amounting to a total of 75 fruits. Main symptoms in chilli were stunting, reduced leaf size, leaf and fruit distortions and general yellowing.

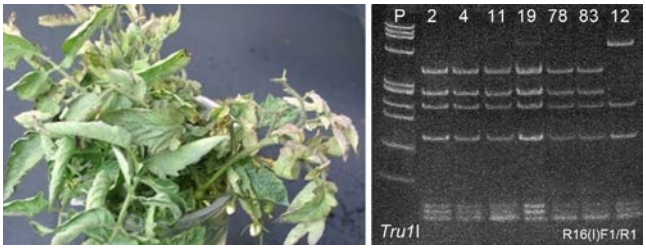


Figure 1. Apical bushiness, leaf distortions and purple colouration on phytoplasma infected tomato plants (left). RFLP results in tomato and chilli seedling amplicons obtained with R16(I)F1/R1 with *Tru1*I restriction enzyme. Samples 2, 4, 11, 19, chilli; samples 78, 83, tomato; 12, control strain CrP (16SrII-C); P: marker PhiX174 *Hae*III digested (right).

Phytoplasmas in 16SrI group were detected in 80% of chilli symptomatic mother plants irrespective of the varieties surveyed and in 75% of tomato symptomatic mother plants collected from varieties MST 32/1, Swaraksha and Topinas. Only a 20% germination rate was recorded in chilli compared to 85% in tomato. Around 4.2% chilli leaf samples tested (10 out of 240 seedlings from variety Piment Cipaye) were positive to 16SrI phytoplasmas (Figure 1). In tomato, 2 out of 200 seedlings from "Swaraksha" (1%) were positive to 16SrI group phytoplasmas.

Discussion

Based on molecular tests conducted, the presence of phytoplasmas in chilli was confirmed. As for the 20% symptomatic chilli samples and the 25% tomato mother plant samples tested negative to phytoplasmas, leaf distortions observed on mother plants could be due to insect infestation or to viruses. This justifies the need for molecular diagnosis since field samples can at times be confusing and can lead to wrong diagnostics if laboratory tests are not done. The adverse effect of phytoplasma infection was seen to be more pronounced in the case of chilli than in tomato. This was reflected by a reduced germination rate of seedlings obtained from infected chilli mother plants. Moreover, given that 16SrI group (aster yellows phytoplasmas) was detected from 1% of tomato seedlings only in variety Swaraksha and from the 4.2% of chilli seedlings of variety Piment Cipaye, it confirms phytoplasma seed transmission in both crops and varieties mentioned. The present findings are in line with earlier records of phytoplasma seed transmission in tomatoes and carrots in Europe (Calari *et al*., 2011; Satta *et al*., 2020; Mateeti *et al*., 2022).

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Epidemiology

Seed transmission of phytoplasmas infecting eggplants in India

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Abstract

Eggplant is one of the important solanaceous vegetable crops consumed in India and worldwide. Seeds from symptomatic eggplants showing little leaf disease were planted in an insect-proof greenhouse. The DNA of these seedlings and those of their progeny were tested by nested PCR on phytoplasma 16S rRNA gene. RFLP analysis of obtained amplicons allowed the identification of phytoplasmas enclosed in five ribosomal groups in the seedlings. Moreover, 16SrI and 16SrXII groups were found in both seedlings and their progeny, confirming seed transmission of phytoplasmas infecting eggplants. Phytoplasmas belonging to 16SrIII and 16SrVI ribosomal groups were detected in symptomatic eggplants collected in the same field in the next season, confirming transmission of phytoplasmas also through the presence of insect vectors.

Keywords: eggplant little leaf, brinjal, molecular identification, bacteria, mollicutes

Introduction

Eggplant is a significant and important solanaceous vegetable crop, and it is considered the king of vegetables in the Indian subcontinent, where it is more commonly referred to as brinjal, a name derived from Arabic and Sanskrit languages. Among the different diseases reported in eggplants, the little leaf is a phytoplasma-associated disease widespread in India, causing yield loss of up to 100% in the disease-infected plants (Rao and Kumar, 2017). Infected plants exhibit thin, soft, smooth, and yellow leaves and reduced length of stem internodes. Subsequently, there has been a crowding of small leaves at the axils of plants that shows a bushy appearance followed by the presence of phyllody and virescence (Majumdar and Das, 2020). So far, phytoplasmas enclosed in six ribosomal groups have been reported to infect brinjal worldwide that are 16SrI, 16SrII-D, 16SrIII-J, 16SrIII-U, 16SrVI-A and -D, 16SrIX-C and 16SrXII-A. Among these 16SrVI-D, 16SrII-D and 16SrI were detected mainly in symptomatic brinjal plants in eight states of India (Kumar and Rao, 2017). This study aimed to verify the phytoplasma seed transmission from infected eggplants to their progeny.

Materials and Methods

Seeds from symptomatic eggplant exhibiting symptoms of little leaf were collected in May 2021 from Garga village, Dharwad district in Karnataka state (India). In addition, eggplant samples were collected from the same field in November 2021. After seed germination in a climatized chamber the seedlings were transplanted in potting mix in an insect-proof greenhouse. To verify the presence of phytoplasmas, DNA was extracted from eggplant samples field collected and from the seedlings and their progeny by a CTAB method (Angelini *et al*., 2001). As the seedling number was high, DNA extractions were performed on selected plant samples based on their growth at about 50, 60, and 70 days after transplanting plus at fruiting stage. All the seedlings or embryos from first-generation plant fruits were also tested. PCR amplification on the 16S rRNA gene was carried out using 1 µl of the extracted DNA diluted 1: 30 with R16F2n/R16R2 primer pair. Nested-PCR assays on 1: 30 diluted PCR products was performed with R16(I)F1/R1, fU5/rU3 and 16R758f (=M1)/16S1232r (=M2) primer pairs (Bertaccini *et al*., 2019). Amplification of isoleucine and *secA* genes was carried out to verify the identity of one of the phytoplasma strains detected in field-collected symptomatic eggplant using published protocols (Abeysinghe *et al*., 2016; Hodgetts *et al*., 2008). The amplicons were subjected to RFLP analysis with *Tru*1I and *Tsp509*I enzymes (Fermentas, Vilnius, Lithuania). The PCR and RFLP products were analyzed by electrophoresis on 1% agarose gel and in 6.7% polyacrylamide gel stained with ethidium bromide, respectively and documented with KODAK EDAS 290 digital camera with a bench-top UV transilluminator at 312 nm. Selected gene amplicons were directly sequenced in both senses using the primers of nested PCR, and obtained sequences were assembled, aligned, and compared with nucleotide sequences available at NCBI database using BLAST (www.blast.ncbi.nlm.gov) function.

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Table 1. Results of phytoplasma detection and identification by nested PCR/RFLP analyses in eggplant seedling.

Ribosomal groups of phytoplasmas detected in eggplant seedlings				Ribosomal groups of phytoplasmas detected in second-generation eggplant seedlings
50 days	60 days	70 days	Fruiting	
16SrI	16SrII	16SrII	16SrI	16SrI
16SrXII	16SrV, 16SrVI	-	16SrXII	16SrXII



Figure 1. From left eggplants with fruits under greenhouse, cut fruit with seeds, close up of seed germination inside the fruit.

The molecular analysis detected phytoplasmas of five ribosomal groups (Table 1). The plants were substantially asymptomatic but seed pregermination in fruits was observed (Figure 1). Among the 16S rRNA gene sequences of phytoplasmas detected in seedlings the 16SrXII-A phytoplasma (802 nt) had 99.63% identity to '*Ca.* P. solani' (3 SNPs); the 16SrII phytoplasma (1,016 nt) had 99.02% identity to '*Ca.* P. aurantifolia' (6 SNPs and 4 GAPs); the 16SrI phytoplasma (938 nt) had 98.83% identity (8 SNPs and 3 GAPs) to '*Ca.* P. asteris'. The phytoplasmas in 16SrIII and 16SrVI groups detected in the eggplant field-collected samples had 16S rRNA gene sequences of group 16SrIII (840 nt) 99.76% identical to '*Ca.* P. pruni' (2 SNPs), and group 16SrVI (506 nt) 99.80% identical to '*Ca.* P. trifolii' (1 SNP). The *secA* (339 nt) and *leu* (955 nt) genes of the latter phytoplasma were 100% and 99.76 to 100%, respectively, identical to '*Ca.* P. trifolii' sequences from Indian brinjal little leaf strains.

Discussion

Phytoplasmas in eggplants were transmitted to progeny until second-generation seeds. Their presence in early growth stages was most abundant confirming results in other plant species (Calari *et al.*, 2011; Satta *et al.*, 2020). The overall transmission rate of phytoplasmas is low, in agreement with data reported for other plant species (Satta *et al.*, 2019). The detected phytoplasma groups, especially 16SrVI-D and 16SrII-D subgroups were reported in India. The 16SrI and 16SrXII groups were found in both first-generation plants and their seedlings while the 16SrII, 16SrV and 16SrVI groups were found only in the first-generation plants. The presence of phytoplasmas affects both quality and quantity of production and transmission by seeds and helps in spreading phytoplasma diseases worldwide.

Studies are needed to understand how much phytoplasmas can spread from seedlings to mature plants and also their ability to induce symptoms. The presence of germinated seeds positive to phytoplasmas in fruits under insect-proof conditions is a confirmation of phytoplasma presence. Phytoplasmas of groups 16SrIII and 16SrVI were found in the plants grown in the same field in a different season, indicating that these pathogens are also transmitted further through insect vectors. More research is needed to acquire appropriate knowledge about phytoplasma seed transmission in eggplants for appropriate and focused management strategies to reduce the disease impact in this crop.

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Control

Adaptive management trials for the control of *Scaphoideus titanus*, main vector of "flavescence dorée" phytoplasmas

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Abstract

The first detection of grapevine's "flavescence dorée" (FD) in Switzerland and the application of the mandatory control measures date back to 2004. The use of buprofezin allowed an optimal control of the insect vector *Scaphoideus titanus*. However, buprofezin has been banned since 2019 raising the need for an alternative control strategy. Assuming a slow *S. titanus* population growth in the first post-treatment years, the Swiss Canton of Ticino received the permission to suspend the mandatory insecticide treatments for two consecutive years, before resuming the insecticide sprayings with pyrethrins in the third year. This study aimed at assessing the impact of the suspension and resumption of treatments on the population dynamics of *S. titanus* and the consequences on the FD incidence in vineyards. The mean captures of *S. titanus* specimens increased eleven fold over the two-year period of treatment suspension, while the subsequent pyrethrins applications did not bring the populations back to the pre-suspension level. Nevertheless, the overall increments were not statistically significant ($p > 0.05$) mostly due to the variability in population density among vineyards. However, and most importantly, the incidence of infected grapevines remained stable. Under specific frame conditions, such as low initial vector populations and cultivation of less susceptible cultivars, adaptive management options may represent a possible alternative control strategy for an extensive use of insecticide applications in the vineyard agroecosystem.

Keywords: disease management, grapevine yellows, insect vector, Switzerland

Introduction

"Flavescence dorée" (FD) is a detrimental grapevine disease associated with phytoplasmas acquired and transmitted by the Nearctic leafhopper *Scaphoideus titanus* Ball (Chuche and Thiéry, 2014). In Switzerland, FD was observed for the first time in 2004 in Canton Ticino (TI, Southern Switzerland) and later in Vaud (2015) and Valais (2016). In TI, the mandatory control measures of the disease were systematically applied, and the insecticide treatments based on buprofezin were very successful in containing the insect vector population. Nevertheless, FD kept on spreading to almost the whole winegrowing area, even though the incidence of FD-infected grapevines remained low (Jermini *et al.*, 2014). This pushed for the adoption of an adaptive strategy based on containment instead of eradication with the aim of lowering insecticide inputs into the vineyard agroecosystem. In a pilot phase started with three vineyards in 2016 and later applied to the whole TI in 2019, insecticide applications were suspended for two consecutive years. A survey was carried out with the aims of monitoring population reconstitution of *S. titanus* after treatment suspension; testing if pyrethrins, since 2019 the only authorized active ingredient in Switzerland, were effective enough to lower the populations after two years without sprayings; and assessing the percentage of FD-infected grapevines.

Materials and Methods

Up to nine representative vineyards (depending on the treatment type) located in TI were included in the analysis, of which seven are cultivated with the less susceptible cultivar Merlot, while two with the highly susceptible cultivar Chardonnay (Eveillard *et al.*, 2016). All vineyard plots were cultivated following good agronomic practices and FD-infected grapevines were systematically rogued. *S. titanus* populations were monitored with yellow sticky traps, while grapevines expressing symptoms linked to grapevine yellows were systematically sampled and subjected to molecular analysis by quantitative PCR.

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Results

Data were grouped in five treatment types and consecutive years, namely, buprofezin, suspension year 1 and 2, and pyrethrins year 1 and 2 (Figure 1). The insecticide suspension led to increases of *S. titanus* captures by a factor of 4.52 and 2.46 in the first and second year, respectively. Pyrethrins were able to lower the populations only partially over two years but were still 7.22 times higher than with buprofezin. Moreover, the trend on the second year of application was practically flat (factor 1.07) (Figure 1). Nevertheless, no significant differences were found between treatment types when considering all plots ($p > 0.05$). Despite the general increase of insect vector population, the incidence of FD-infected grapevines did not change significantly over this interval of time, nor showed differences with respect to the FD susceptibility of the cultivar ($p > 0.05$) (Figure 2).

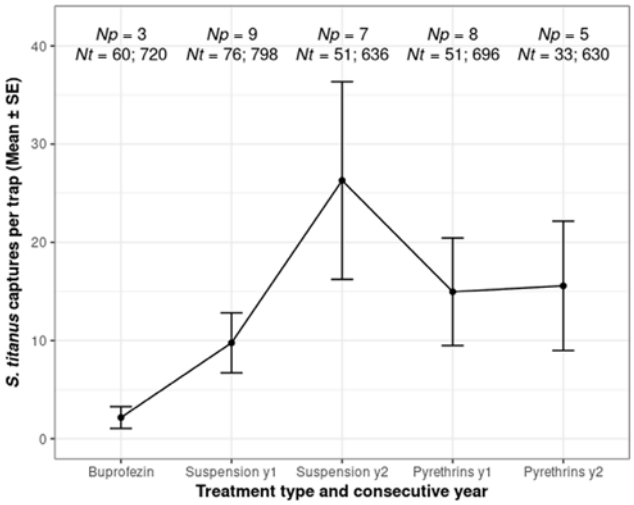


Figure 1. *S. titanus* captures per trap (mean ± standard error) according to treatment type and consecutive year (y). Np = number of plots, Nt = number of trap positions per treatment; number of considered traps.

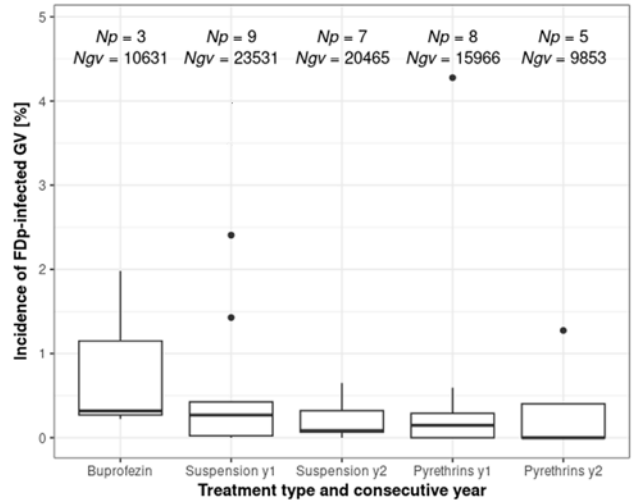


Figure 2. Incidence of FD-infected grapevines (GV) according to treatment type and consecutive year (y). Np = number of plots, Ngv = number of total grapevines.

Discussion

The increase of *S. titanus* populations due to two years of treatment's suspension may still be deemed acceptable in terms of risk of FD spread in vineyards with very low initial population densities and low percentage of FD-infected grapevines. However, it poses a threat in vineyards where the population is not correctly regulated and, in general, where the intrinsic risk linked to cultivar susceptibility is higher. Buprofezin proved to be a good active ingredient for the control of *S. titanus* over the long-term reaching 2.16 ± 1.93 captures per trap (mean ± standard error) after several years of mandatory sprayings. Two years of pyrethrins applications were not sufficient to lower the populations to the level pre-suspension and showed important limitations when comparing the two years of application. Moreover, the use of pyrethrins is strictly prohibited in proximity to waterways. Thus, considering the particular geomorphology of TI, up to 20% of the winegrowing surface cannot be treated, although the treatment is mandatory. This issue may thus contribute to the survival of important vector population pockets, which may then recolonize treated vineyards. Nevertheless, it is assumable that two years of treatment's suspension followed by the use of a more efficient and less restrictive active ingredient may allow for a strategy which includes the application of insecticides every third year only. The feasibility of an adaptive strategy is further supported by the fact that the incidence of FD-infected grapevines did not increase significantly over a three-year period. In the specific case of TI, where 85% of the winegrowing area is cultivated with the less susceptible cultivar Merlot, such strategy may be an additional support to lower insecticide inputs, while ensuring a relatively low risk of uncontrolled spread of FD at vineyard level. In other regions, a preliminary screening should be conducted to assess the initial *S. titanus* population densities, as well as the share of highly FD susceptible cultivars. Nevertheless, the constant restrictions on the authorized active ingredients may pre-empt the adoption of such adaptive strategies.

Acknowledgements

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Control

Detailed assessment of control measures against "flavescence dorée" allows reduction of pesticide use

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Abstract

The wine industry plays important social and economic roles in the region of canton Vaud in Switzerland. Since 2015, "flavescence dorée" (FD), a disease associated with a phloem-limited bacterium, has been present in the vineyards and represents a major threat to viticulture. The disease is propagated epidemically by its principal vector, the leafhopper *Scaphoideus titanus*. The "flavescence dorée" phytoplasma is a quarantine pest, and is therefore under a national surveillance program. Control measures such as roguing infected stocks and insecticide treatments must be applied in affected perimeters. Regional phytosanitary services are responsible to organize the inspection of these areas. Each year, a systematic survey of *S. titanus* populations and symptomatic grapevines is conducted to assess that the actions are properly implemented. Here, a few examples from representative outbreaks are presented. The compulsory measures are efficient and, as expected, the pathway to disease eradication strongly depends on the initial severity of the outbreak. An adapted management strategy, based on individual risk analysis of each affected perimeter, to reduce the surface treated with insecticides was proposed.

Keywords: grapevine disease outbreaks, Vaud, Switzerland

Introduction

"Flavescence dorée" (FD), one of the most devastating grapevine yellows, is associated with the presence of "flavescence dorée" phytoplasma, belonging to the 16SrV ribosomal group. The propagation of the disease is epidemic only in the presence of the leafhopper *Scaphoideus titanus* Ball (Chuche and Thiéry, 2014). FD is a quarantine disease in the EU. The disease appeared in southern Switzerland in 2004 (Schaerer *et al*., 2007) and has been present north of the Alps since 2015 in the vineyards of canton Vaud. Since then, the prospection programme organized yearly by the cantonal authorities to screen the about 3,800 ha of vineyards of the canton allowed the discovery of several FD outbreaks. Multiple introductions of infected planting material were most likely the original sources of these different outbreaks detected between 2015 and 2022.

At the time of announcement, the density of diseased plants in these perimeters varied from a few to several hundred per plot. Mandatory control measures (Federal Office for Agriculture, Directive No 9, May 2021) including insecticide treatments and roguing infected plants were consistently applied in infected areas. Each year after the discovery of the outbreak, a monitoring of the insect populations as well as an intensive survey of the symptomatic grapevines within the control perimeters was performed by the regional Plant Protection Service and Agroscope.

Materials and Methods

Four representative vineyards (according to the initial number of infected plants) were included in the study. All vineyard plots were cultivated following standard farming practices. Until 2018, plots were treated with buprofezin, and from 2019 and later with natural pyrethrins. During the month of August, at the peak of flight, 20 yellow sticky traps were used to monitor the populations of *S. titanus* in the outbreak perimeters. Traps were collected every two weeks. Grapevines expressing typical yellows symptoms were sampled for molecular analysis. The presence of FD phytoplasma was assessed by quantitative PCR according to Pelletier *et al.* (2009).

Results

Severe outbreaks were detected in the area of Blonay/La Tour-de-Peilz and Chardonne/St.-Saphorin in 2015 and 2017, respectively. Initially, several hundred of grapevines were

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affected by FD (Table 1) and entire plots were uprooted (those with a disease incidence of more than 10%). Since then, the systematic application of control measures allowed a drastic decrease in the number of symptomatic grapevines. However,

Table 1. Number of FD-infected grapevines in four selected outbreak perimeters.

	Blonay / La Tour-de-Peilz	Chardonne / St-Saphorin	Mont-sur-Rolle	Yvorne
2015	733*	n/a	n/a	n/a
2016	76	n/a	n/a	n/a
2017	37	698*	n/a	n/a
2018	24	653*	n/a	n/a
2019	19	83	4	n/a
2020	1	67	0	2
2021	2	12	0	0
2022	2	9	0	0

*Estimated number from uprooted surfaces, only a fraction of grapevines underwent molecular analysis to confirm FD presence. n/a=not available.

the complete eradication of the disease in these two perimeters has not yet been achieved after several years. In contrast, the early discovery of a few infected stocks in Mont-sur-Rolle (in 2019) and Yvorne (in 2020) allowed the rapid elimination of the disease corresponding to the two years of mandatory fight. Monitoring *S. titanus* populations showed that insecticide treatments reduced the captures by more than 90% in both severely affected areas compared to an untreated plot in the region (Figure 1). In the perimeter of Blonay / La Tour-de-Peilz, treatments were suspended in 2021 and 2022, but the insect populations remained stable at the same low level as in the previous years.

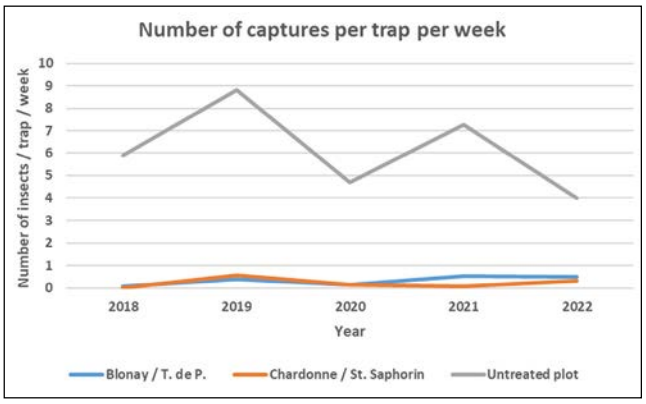


Figure 1. *S. titanus* captures per trap and per week during the month of August in two selected outbreak perimeters. An untreated plot is shown as control. No treatment was applied in Blonay / La Tour-de-Peilz in 2021 and 2022.

Discussion

The efficiency of mandatory control measures applied in FD outbreak perimeters is controlled by systematic monitoring of symptomatic grapevines and *S. titanus* populations. As seen for the severe outbreaks of Blonay/ La Tour-de-Peilz and Chardonne/ Saint-Saphorin, although the number of infected grapevines has been drastically reduced over the years of intensive control measures, the disease is still present after years of fighting. On the other hand, in Mont-sur-Rolle and Yvorne, where the initial number of infected plants was low, FD could be rapidly eradicated and control measures stopped. It is therefore essential to detect cases of FD as early as possible to achieve rapid control. The prospection programme conducted by the cantonal phytosanitary service is of crucial importance, particularly in those regions where the disease is absent. Other prophylactic measures such as awareness-raising among winegrowers, training phytosanitary inspectors, planting healthy, certified and hot water treated material, are critical as well in the battle against the pest.

Continuous monitoring of diseased grapevines in each outbreak also allows risk to be assessed in order to implement control measures appropriate to each local situation. For example, in Blonay / La Tour-de-Peilz, only one FD positive sample was found in 2020, and the insect population was very low for the third consecutive year. Therefore, an adaptive management strategy to minimise the use of phytosanitary products has been tried in this zone, suspending insecticide sprayings in 2021 and 2022 in agreement with the federal phytosanitary authorities. During these two years, the incidence of infected grapevines remained very low and the number of insects did not increase significantly. These facts support the feasibility of individualized strategy. The use of insecticides is extremely costly and has a strong negative impact on the environment. Such alternative control strategy may represent a good option to reduce the area of vineyards to be treated.

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Control

Plasma activated water and phytoplasma interactions in *Catharanthus roseus* alkaloid pathway

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Abstract

Catharanthus roseus is used as model plant for studies on phytoplasmas. In the recent years it has been proved that PAW treatment activates the plant resistance, with an overexpression of genes involved in the phytoalexins pathway. In this work the interaction of PAW and phytoplasmas in the alkaloid pathway in phytoplasma-infected and healthy periwinkle micropropagated shoots was investigated by quantitative PCR (qPCR) to evaluate gene expression changes. The results indicated that PAW induced an overexpression of the investigated genes and in particular of *CrDAT* in infected shoots. More studies will allow to clarify the role of phytoplasmas infected PAW-treated plants for alkaloid production.

Keywords: periwinkles, phytoplasmas, PAW, induced resistance, alkaloids, gene expression

Introduction

Catharanthus roseus (L.) G. Don, or periwinkle, is a tropical plant that is very susceptible to phytoplasma infections, and it is therefore used as model plant in studies related to these bacteria. Phytoplasmas are associated with diseases in more than a thousand plant species worldwide and are transmitted by phloem-feeding homopteran insects, mainly leafhoppers and planthoppers (Weintraub and Beanland, 2006). Living and multiplying in the phloem sieve tubes, these prokaryotes alter physiological functions, including photosynthesis, secondary metabolism and plant hormone balance (Lepka *et al*., 1999; Bertamini *et al*., 2002a, 2002b; Hoshi *et al*., 2009; Hren *et al*., 2009). Management of phytoplasma diseases has been mainly focused on the use of healthy plant material, removal of infected plants, and control of insect vectors and alternative host species. In the recent years a novel approach based on the use of plasma activated water (PAW) has been investigated and it has been proved that PAW treatment enhances plant growth and induces plant resistance to bacterial infections (Perez *et al*., 2019; Zambon *et al*., 2020; Laurita *et al*., 2021). Since it is well known that in plants several synthesis pathways can be influenced by diseases or stress factors, in this work the interaction of PAW and phytoplasmas in the alkaloid pathway in periwinkle phytoplasma-infected shoots was studied.

Materials and Methods

A total of 80 ml of sterile distilled deionized water (SDW) was exposed to a nanosecond pulsed dielectric barrier discharge, as described (Zambon *et al*., 2020). The PAW aliquots produced were then used to treat '*Candidatus* Phytoplasma asteris'-infected and healthy micropropagated periwinkle shoots (Bertaccini, 2023) maintained under controlled conditions (24 ± 2°C and 16 hours day light). The shoots (5 replicates/treatment) were submersed in the micropropagation glass tube for 25 minutes with 20 ml PAW, SDW (negative control) and a solution of 2.5 g/l fosetyl aluminium (Aliette, Bayer Crop Science, Italy), positive control. The whole shoots were collected from each replicate/treatment at six time points: 0 hour, 7 hours, 24 hours, 48 hours, 96 hours, and 120 hours. Total RNA was extracted from 100 mg of plant tissue following a described procedure (Zambon *et al*., 2020) and activity testing was performed on *CrCalS11*, *CrPAL1*, *CrSGD* and *CrDAT* genes involved in the phytoalexin pathway (Dixon *et al*., 1995). For all genes of interest, about 1.5 ng of cDNA template was used for qPCR, with the expression normalized to the

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ubiquitin gene. The qRT-PCR reactions were performed using SYBR Green master mix (Applied Biosystems, USA) performing three technical replicates, and a minimum of three biological replicates per experiment with primers and amplification protocols described by Zambon *et al.* (2020).

Results

Micropropagated healthy and '*Ca.* P. asteris'-infected periwinkle shoots treated with PAW and collected at different times points were examined by qRT-PCR for gene expression changes. The comparative analysis carried out on *CrPAL1* and *CrSGD* genes showed overexpression after PAW treatment, slightly more evident in healthy shoots than in the infected ones (data not shown). Furthermore, the *CrCalS11* gene expression in shoots treated with both, PAW and fosetyl aluminium (FoAll), was significantly increased compared to the control, but the latter was more effective in both, healthy and infected shoots (data not shown). On the other hand, the *CrDAT* gene expression analysis highlighted a clear difference between healthy and infected treated shoots (Figure 1). PAW was more efficient in stimulating the overexpression of this gene in the '*Ca.* P. asteris'-infected shoots after 48 h (T3), while in the healthy shoots, the higher overexpression was induced by FoAll after 72 h (T4).

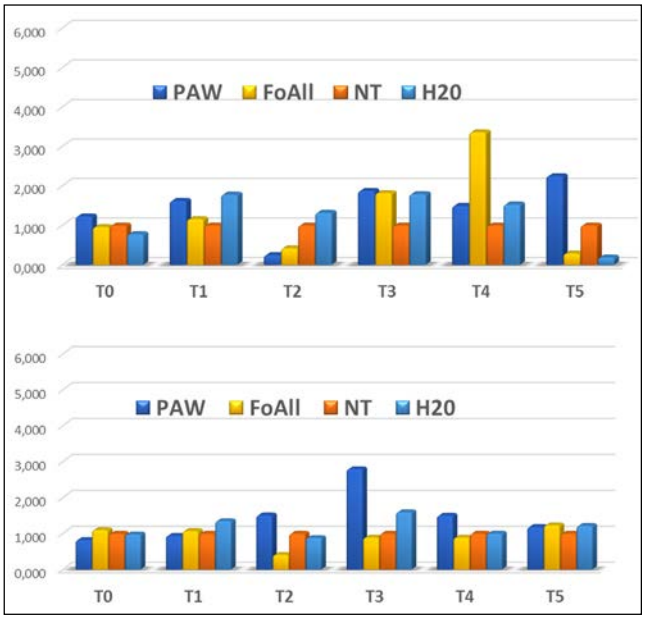


Figure 1. Gene expression changes in micropropagated healthy (top) and '*Ca.* P. asteris'-infected (bottom) periwinkle shoots after PAW treatment. Gene expression for deacetyl vindoline-O-acetyltransferase gene (*CrDAT*) as determined by qRT-PCR after PAW, SDW and FoAll treatments. The relative expression (ubiquitin was the internal control) to untreated shoots are shown at different time points after treatments.

Discussion

Periwinkle plants are of great medical value, producing about 150 alkaloids, including vinblastine, vindoline and catharanthine, used for their antitumoral effects. It is known that the synthesis of alkaloids can be influenced by pathogens, stress factors, or elicitors, therefore in this work the effect of PAW treatment and phytoplasma presence in periwinkle micropropagated shoots was investigated. PAW was confirmed a resistance inducer, enhancing some of the key enzymes in the phytoalexins pathway in both, healthy and phytoplasma infected shoots. In particular the analyses highlighted an overexpression of *CrDAT* gene, that plays an important role in the upstream regulation of the production of vindoline (Mujib *et al.*, 2012). It was shown to be more active in infected shoots treated with PAW at T3 and this finding suggests further studies aimed to the possible improvement of alkaloid biosynthesis stimulated by PAW in presence of phytoplasma infection.

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Control

Reaction of some Persian lime accessions on different rootstocks to witches' broom disease of lime

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Abstract

Persian lime (*Citrus latifolia* Tanaka) is the most tolerant citrus species against witches' broom disease of lime (WBDL), so far, the effects of different rootstocks on this feature have not been evaluated. To perform this evaluation an investigation was performed in randomized complete block design as factorial arrangement with two factors consisting in rootstock (Volkamer lemon, Bakraei, Sour orange and Mexican lime) and scion (Persian lime, IAC, Deperse lime and Tahitian lime) with three replications during 2013-2016. The reaction of T-budding grafted and non-grafted seedlings to WBDL was assessed via graft inoculation with Mexican lime scion infected with WBDL phytoplasma. PCR assay was used to detect WBDL phytoplasma in the inoculated plants and each PCR test was repeated three times. Detection of WBDL phytoplasma was late (116 days post-inoculation) in scions on Volkamer lemon rootstock and early (40 days post-inoculation) in the scions on Mexican lime rootstock. WBDL symptoms were not observed in the grafted plants during the course of the study but it was beholden in all seedlings except Volkamer lemon. Generally, Volkamer lemon rootstock is recommended as the best rootstock for Persian lime accessions in the WBDL-affected areas.

Keywords: graft inoculation, phytoplasma, Tahitian lime, Volkamer lemon, WBDL symptoms

Introduction

Witches' broom disease of lime (WBDL) associated with '*Candidatus* Phytoplasma aurantifolia' was reported from Oman in the late 1970s and then from United Arab Emirates in 1989. The disease almost completely destroyed Mexican lime trees in the two countries. In Iran, WBDL was first reported in Qasr-e-Qand area of Sistan and Baluchestan province in 1997 and then from other parts of this province and in most of Mexican lime orchards in Hormozgan, Kerman and Fars provinces. The disease in these areas has destroyed thousands of Mexican lime trees. Removal of sources of infection and use of resistant cultivars is the most economical and practical way to prevent and control of the disease (Salehi *et al*., 2017). At present, the planting of Persian lime trees is growing in southern regions of Iran and it is usually budded on Mexican lime and Bakraei rootstocks and it is provided to gardeners. However, both the above rootstocks are the most susceptible citrus species against WBDL (Hassanzadeh Khankahdani, 2016) and given that the Persian lime trees are grown in the WBDL-infected regions, despite its relative tolerance, the susceptibility of these two rootstocks (Mexican lime and Bakraei) may affect the growth of Persian lime trees. On the other hand, Mexican lime rootstock, that is the most usable citrus rootstock in south of Iran, is among the intermediate rootstocks in terms of alkalinity tolerance in soil. Therefore, it seems necessary the finding of a more suitable rootstock for Persian lime by investigating the scion reaction of this species to WBDL on different rootstocks.

Materials and Methods

To investigate the reaction to WBDL phytoplasma of some Persian lime genotypes scions on different rootstocks, an experiment was carried out as factorial arrangement in RCBD (randomized complete block design) with three replications including four rootstocks and four Persian lime genotype scions at the Agricultural and Natural Resources Research Center of Hormozgan during 2013-2016. The rootstocks were produced by seed and after transplantation and maintenance in the

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waiting nursery, budding was performed at 20 cm height from the soil surface in each rootstock and scion combination using T-budding method.

Results

Analysis of variance demonstrated that rootstock type had significant effect ($p<0.01$) on the number of day to detect WBDL phytoplasma. It was not observed significant difference between scions as well as interaction of scion and rootstock (data not shown). Presence of WBDL phytoplasma in the Persian lime scions on Mexican lime and Volkamer lemon rootstocks was detected 40 and 116 days after inoculation, respectively. The number of days until detection of phytoplasma in the scions on Bakraei and Sour orange rootstocks was 50 and 68 days, respectively (Figure 1).

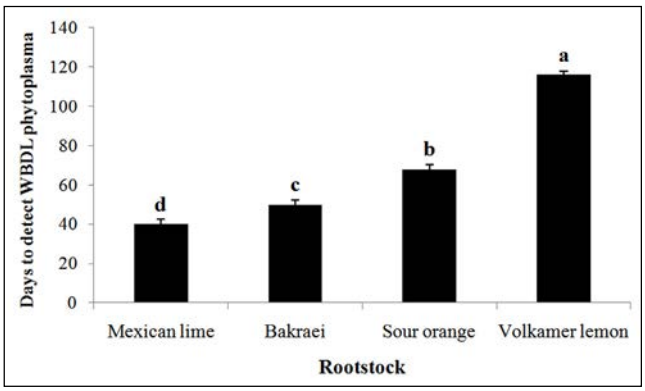


Figure 1. Individual effect of rootstock on the number of days between graft inoculation and detection of WBDL phytoplasma in the scions.

Based on the results of analysis of variance comparing non-grafted seedlings and grafted plants in viewpoint of the number of day to detect WBDL phytoplasma, it was observed a highly significant difference ($p<0.01$) among the inoculated plants (data not shown).

Mean comparison indicated that WBDL phytoplasma was detected in the inoculated Mexican lime seedlings after 27 days, which it had significant difference with all seedlings and the grafted plants except Persian lime on Mexican lime rootstock (37 days). Bakraei seedlings were in the next ranking, so that WBDL phytoplasma was detected in them after 40 days, although it had no significant difference with the grafted plants on Bakraei and Mexican lime rootstocks. WBDL phytoplasma was detected in Sour orange seedlings after 57 days that it had no significant difference with the grafted plants on Bakraei rootstock (50 days) as well as with IAC, Deperse lime and Persian lime on Sour orange rootstock (67 days). WBDL phytoplasma was detected in Volkamer lemon seedlings after 93 days that it had a significant difference with other seedlings and grafted plants. In this regard, WBDL phytoplasma was detected in the scions in Volkamer lemon rootstock later (113-117 days) than all seedlings and grafted plants (Figure 2).

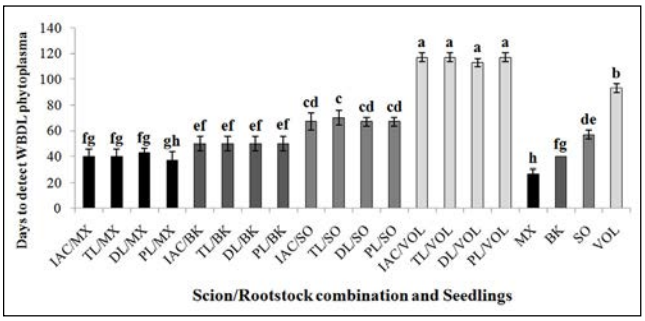


Figure 2. Mean comparison of the number of days between graft inoculation and detection of WBDL phytoplasma in the seedlings and the grafted plants. [Columns having same letters, are not significant different according to PLSD ($p<0.05$)]. TL, DL and PL: Tahitian lime, Deperse lime and Persian lime scions, respectively; MX, BK, SO and VOL: Mexican lime, Bakraei, Sour orange and Volkamer lemon rootstocks, respectively.

Discussion

The presence of WBDL phytoplasma in the Persian lime scions on Volkamer lemon as well as in Volkamer lemon seedlings was detected later than in other seedlings and in the grafted combinations using PCR. In contrast, the presence of WBDL phytoplasma was detected sooner than in the other seedlings and in the grafted combinations in Mexican lime and Bakraei seedlings as well as in the grafted combinations on these two rootstocks. It seems that the delay in detecting the presence of WBDL phytoplasma in Volkamer lemon rootstock implies that phytoplasma concentration is low in this rootstock. The later detection of WBDL phytoplasma in Volkamer lemon and Persian lime scions on the evaluated rootstocks may be due to their tolerance to WBDL in comparison to Bakraei and Mexican lime. In this study, for the first time, different genotypes of Persian lime were compared in terms of response to WBDL phytoplasma and their tolerance was proven, especially when they were budded on Volkamer lemon rootstock. In the study of the interaction between rootstock and scion, planting Persian lime trees including IAC, Tahitian lime, Persian lime and Deperse lime on Volkamer lemon rootstock are introduced as the best grafting combinations for the areas infected with WBDL.

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Control

Effect of temperature on symptom expression of witches' broom disease in a susceptible genotype of Persian lime

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Abstract

Previous studies showed that environmental factors like temperature affect symptom development and phytoplasma concentration within the plant tissues. In this study, under warm (35-37°C and 25-27°C day/night) and cool condition the reaction of a susceptible genotype of Persian lime to '*Candidatus* Phytoplasma aurantifolia' was investigated by graft inoculation. Under warm condition it took 9 weeks for the symptom expression in Persian lime shoots, but no symptoms were observed under cool conditions. Also, the mean copy numbers of '*Ca.* P. aurantifolia' per 100 ng of plant total DNA was higher than that under cool conditions and showed significant differences ($p<0.01$). These results indicate that temperature plays an important role in '*Ca.* P. aurantifolia' concentration and symptom development in citrus species.

Keywords: symptom development, grafting, quantitative PCR

Introduction

Lime witches' broom disease associated with '*Candidatus* Phytoplasma aurantifolia' is one of the most serious threats to lime production in Oman, United Arab Emirates and Iranian southern provinces (Noorizadeh *et al.*, 2021). Environmental conditions like temperature are one of the main factors that can either negatively or positively influence growth, population, and disease development from bacteria (Hoffman *et al.*, 2013). For example, it was reported that temperature sensitivity makes a clear distinction between '*Candidatus* Liberibacter asiaticus' and '*Ca.* L. americanus'. '*Ca.* L. americanus' is sensitive to heat and does not infect the plants maintained at 27–32°C, while '*Ca.* L. asiaticus' tolerates broader temperature range, from 22–24°C to 24–35°C. Recent study showed that under field conditions, the concentration of '*Ca.* P. aurantifolia' in infected Mexican lime trees was highest in warm season (Amiri Mazraie *et al.*, 2019). In addition, it has been reported that the severity of witches' broom disease of lime may be related to areas under warm climates (Queiroz *et al.*, 2016). In the present study, the results of experiments to investigate the interaction of host (Persian lime) and various temperatures on the concentration of '*Ca.* P. aurantifolia', witches' broom symptom expression and root development under controlled greenhouse conditions are reported.

Materials and Methods

The experiment was conducted in an insect-free greenhouse at Citrus and Subtropical Fruits Research Centre, Ramsar. Two-year-old Mexican limes were used as a rootstock. Healthy shoots of Persian lime genotypes (five replications) were grafted on Mexican lime rootstock. Then all the rootstocks were inoculated with two bark pieces infected with LWB-DL1 strain (GenBank accession number MK907878 for 16S rRNA gene sequence) from infected greenhouse-grown Mexican lime plants. Also, healthy citrus species buds (three replications) were grafted in lime rootstocks as negative controls. To examine symptom development, all the rootstocks were pruned by cutting the stem above the grafted site, kept under natural light conditions, transferred to warm and cool units (warm: 35–37°C in the day and 25–27°C in the night, cool: 24–26°C in the day and 18–20°C in the night), and inspected weakly for the symptom's appearance. For quantification of '*Ca.* P. aurantifolia' in infected plants, total DNA was extracted using the CTAB method at 60 weeks after inoculation and standards were prepared by amplifying 16S rRNA gene with the primer pair LWB^F/ LWB^R in PCR (Amiri Mazraie *et al.*, 2019). Absolute quantification of the phytoplasma was achieved by comparison with a dilution series from 1.7×10^2 to 1.7×10^8 copies of the PCR amplicons

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which were performed in duplicates on the same plate as the samples to be quantified.

Results

The disease symptoms only appeared under the warm condition (25–37°C). Symptoms started to appear within 9–10 weeks after inoculation (wai). Plants achieved the maximum symptom expression at 24 wai showing small leaves with shortened internodes (Figure 1). No symptoms were observed under the cool conditions.



Figure 1. Witches' brooms symptom expression in Persian lime scions under warm conditions. Left: infected; center: control; right: infected close-up.

At 60 weeks after inoculation, all the plants became symptomatic under warm conditions (Table 1). However, in cool conditions, the lowest percentage (60%) of phytoplasma positive plants was observed for Persian lime scions. Also, the average number of phytoplasma genomes in PCR-positive plants in warm condition was higher (144,000) than under cool conditions (14,000) (Table 2).

Table 1. Effect of temperature on witches' broom symptom development in Persian lime.

Citrus genotype	Temperature conditions	Replication				
		1	2	3	4	5
		Time (wai) to onset witches' broom symptom				
Persian lime	35–37°C day 25–27°C night	9	10	9	9	9
	24–26°C day 18–20°C night	-	-	-	-	-

Table 2. Quantitative PCR analysis of phytoplasma-infected plants 60 weeks after inoculation.

Citrus species	PCR-positive		Genomes copy numbers	
	Warm[1]	Cool[2]	Warm	Cool
Persian lime	100%	60%	144,000±33,683.294	14,000±18,075.821

[1]Temperatures 35–37°C day and 25–27°C night
[2]Temperatures 24–26°C day and 18–20°C night

Discussion

There are several reports that demonstrate the influence of the temperature on symptom expression of phloem-limited plant bacterial pathogens. It has been reported that in broad bean inoculated with "flavescence dorée" phytoplasma-infective *Euscelidius variegatus*, symptoms appeared sooner (1 week) at 25°C in comparison with 20°C under greenhouse conditions (Salar *et al*., 2013). Similarity, it has been reported that the severity of lime withes' broom infection is much higher in regions with high temperatures (Queiroz *et al*,. 2016). In this study, under the warm temperature the symptom development was accelerated and the phytoplasma concentration resulted increased. It can be hypothesized that higher temperature might have positive influence on the multiplication rate of the phytoplasma which grew to higher titters faster in the host plants (Salar *et al*., 2013).

Acknowledgements

Authors would like to acknowledge the Citrus and Subtropical Fruits Research Centre, for the financial support to this study.

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Control

Optimal management of witches' broom disease of lime in presence of extreme environmental factors using stochastic dynamic modelling

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Abstract

One of the major diseases affecting lime trees in Oman is witches' broom (WBDL). The occurrence, prevalence and severity of the disease are influenced by climate conditions. This study derives a stochastic dynamic model to determine the optimal management of witches' broom disease of lime while considering extreme environmental variables. The factors considered in the model include disease incidence, severity, spread, and weather conditions. The results indicate that increasing investment in disease management is the best way to reduce the risk of WBDL and the damage it causes to citrus fruits. However, scenario analysis reveals that the current disease management measures implemented by the government are insufficient to maximize the net benefit of citrus cultivation. Furthermore, adverse climate conditions have a significant negative impact on the economic and social value of citrus cultivation. These findings suggest that the government could introduce more effective educational programs to ensure that all citrus growers understand the damages due to WBDL and make socially and privately optimal decisions, thus reducing external costs.

Keywords: citrus industry, plant disease, optimal management, modelling

Introduction

Global food security is among the persistent issues for humanity in which efficient agricultural production can play a critical role. In particular, food and water security have become a serious threat to arid and semi-arid countries like Oman due to the unavailability of adequate fertile land and quality water supply. Oman is the largest grower of lime among Arab nations, especially in the coastal area of Al Batinah (Al-Yahyai *et al*., 2012). This important crop has been infected by a devastating disease named witches' broom which reduces its total yield and economic value. This disease is associated with the bacterium, '*Candidatus* Phytoplasma aurantifolia' that infects lime trees' phloem tissues. The disease can be transmitted by grafting, insect vectors, seeds, and dodder (Al-Yahyai *et al*., 2022).

The major symptom of WBDL is the turning of infected lime tree leaves into small light green or yellow colors with dense branching as shown in Figure 1. Additionally, the disease's severity is often aggravated by extreme environmental factors such as drought, temperature changes, inconsistent rainfall patterns and eventually leads to a decrease in the productivity of trees (Al-Yahyai *et al*., 2012). Even though the treatment of the disease is not yet fully identified, it can be controlled traditionally by chemical methods, biological methods such as rouging infected trees and using disease-free planting material (Grafton-Cardwell *et al*., 2013). The government has put in place projects such as citrus disease indexing and seedling production; and subsidized the treatment of the infected trees. However, the desired outcome can be achieved only if informed disease management strategies are employed. Hence, the objective of the study is to develop a stochastic dynamic bioeconomic model to obtain the optimal control strategy for WBDL insect vector and optimal replanting age of citrus trees after the first infection.



Figure 1. Lime tree exhibiting symptoms of WBDL (from Al-Sadi *et al*., 2017).

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Moreover, the study investigates different scenarios of disease occurrence given different climatic conditions that affect the incidence and spread of WBDL. Thus, the study seeks to determine the suitable areas in Oman for citrus cultivation. This study is the first attempt to simultaneously integrate the economic, environmental, and biological factors of the tree.

Materials and Methods

The study used primary data collected on citrus growing orchards in six different cities (Sohar, Liwa, Shinas, Salalah, Thumrait and Sharqiayah) in Oman while considering their climatic conditions. The choice of the study area is based on previous literature by Al-Ghaithi *et al.* (2017) who studied the expression of WBDL based on geographical locations to indicate highly susceptible areas. Variables such as cost of periodic tree and field management per season per feddan (=4.200 m^2), pruning, pesticide control, fertilization and the cost of labor used during periodic field management per day were considered.

Since the farmers have no control over the climate conditions in these regions, they need to make three nested decisions as to the optimal level and timing of disease control strategy [which month(s) in a growing period] as well as the optimal season of replanting the citrus trees. Although the control of the disease is usually carried out in the growing season, the optimal level and timing of the control was never studied and remain unknown. A dynamic optimization model, which considers an infinite time horizon and discrete time steps was used to simulate the farmer's decision-making process. It contains six different cases including the scenario of a healthy tree which serves as the baseline, infected tree that receives no treatment, infected and untreated tree with climate conditions consideration, infected and treated tree with climate conditions consideration, infected tree that is managed through seedling or sapling replacement, and lastly infected tree that is treated using new technologies. To model the farmer's decision-making process, a dynamic optimization model with an infinite time horizon and discrete time steps is utilized. The dynamic model was derived using a discrete-time Markov decision model. For the infected fruit, the problem was managed using discrete time dynamic bellman equation based on models derived by Al-Abri *et al.* (2023), Al-Abri (2022), and Al-Abri and Grogan (2019).

Results

There is a positive relationship between the spread of the disease and the increase in the number of insect vectors. The actions of the farmer to control the disease determine the insect vector density and the intensity of disease found in a field each month and season. The number of insect vectors found in a field in a particular month depends on the number of insect vectors from the previous month, insect vector growth over time which is impacted by climate conditions and any measures taken to control them. The growth of the insect vector population is expressed by a logistic function based on Trumper and Holt (1998) (Grogan, 2014). It has been demonstrated that climate conditions negatively affect fruit yield and stimulate the growth of insect vectors and associated diseases.

Results indicate that investing in disease management to reduce the risk of WBDL and resulting citrus fruit damage is crucial. However, the analysis of different scenarios reveals that the current disease management efforts by the government are insufficient to maximize the net benefits of citrus cultivation. Furthermore, adverse climate conditions significantly impact the economic and social value of citrus cultivation. As a result, the government could introduce more effective educational programs to ensure that all citrus growers understand the damages caused by WBDL and make socially and privately optimal decisions, thus reducing external costs. Both the government and citrus growers must recognize the importance of a well-managed and effective disease control program, as well as the program's positive impact on increasing citrus production and the negative effects of extreme environmental factors.

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Control

Enhancing the survival of sugarcane infected white leaf disease by using bacterial indoleacetic acid producers

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Abstract

Effects of bacterial indole acetic acid (IAA) producers (BIPs) on plant growth and recovery of sugarcane seedlings infected with phytoplasmas associated with white leaf disease (SWLD) were examined. Seven bacterial isolates showed β-indole acetic acid production in culture medium supplemented with tryptophan. Interestingly, two of them (BC17 and BTII2) produced the highest β-indol acetic acid after 4 days of culture. Treatment with two bacterial isolates of SWLD infected sugarcane plants also showed differences in height of sugarcane seedlings, while a gradual symptoms reduction decreased the plant mortality compared to non-treated plants as control. BIPs were shown to be efficient biofertilizer inoculants that promoted plant growth and also reduced the damage of phytoplasma-associated diseases at the sugarcane seedling stage.

Keywords: IAA-producing bacteria, biocontrol, plant growth, disease, phytoplasma-associated disease

Introduction

White leaf disease associated with 16SrXI phytoplasmas, is the most economically important disease in the sugarcane industry throughout Asian countries (Wongkaew *et al*., 2012). Although it is easy to recognize the infected plants with typical symptom of white strips along the entire leaf, the identification of the reservoir of phytoplasma at the beginning of infection is crucial for the management of sugarcane white leaf disease (SWLD). To date, management strategies of SWLD include various approaches by using resistant/tolerant sugarcane varieties against SWLD; implementing proper insect control measures; avoiding practices that promote insect breeding and spread of phytoplasmas; treating cuttings with hot water/antibiotics (Marcone, 2002). However, they are not suitable for sugarcane cultivation in Vietnam due to cost and complication in handlings. Vietnamese farmers usually have a habit of burning out all the plant debris and weed after harvesting to eliminate pathogens remaining in the field (Hoat *et al*., 2012). Bacterial indole acetic acid (IAA) producers (BIPs) have been characterized not only for plant growth but also for control of plant pathogens (Reetha *et al*., 2014; Janahiraman *et al*., 2016). In this work the effect of BIPs on growth and mortality rate of sugarcane plants infected with SWLD phytoplasmas was evaluated.

Materials and Methods

Among seven BIPs producing high quantities of IAA, two bacterial isolates (BCI7 and BTII2) were used after molecular identification in plant growth-promoting experiments in sugarcane cuttings of phytoplasma symptomatic KK3 variety originated from Khonkaen, Thailand with and without BIPs treatments. Each treatment was repeated three times with 10 sugarcane cuttings for each replicate. The suspension of BIPs was collected and sprayed on sugarcane cuttings after 14 days of growth at a concentration of 10^8 CFU ml^{-1} and this was continued once a week. Symptoms, survival rate, height of phytoplasma-infected sugarcane seedlings with and without treatment of BIPs were observed, measured and recorded every month for three months. Experimental data were collected and expressed as mean ± standard deviation (SD). Statistical analyses were conducted using one-way ANOVA. The p values were provided (**p<0.01; *p<0.05).

The detection of SWLD phytoplasma was done by nested PCR assay as described (Duduk *et al*., 2013). The results of nested PCR products were recorded under 254 nm UV light (UVP, USA) after running the amplification products in 1% agarose gel stained with gel red (TBR, Vietnam).

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Results

The isolation and characterization of BIPs were carried out on rice root samples collected in several provinces in the Mekong Delta, Vietnam. There are at least seven isolates of endophytic bacteria that were able to produce IAA and two of them exhibited the highest IAA production capacity. Biochemical and molecular analyses indicated that they are *Delftia lacustris* (BCI7) and *Rahnella aquatilis* (BTII2) (Thoa *et al*., 2022). The role of these bacterial isolates in promoting the growth of two rice varieties including high-yielding rice variety (Jasmine85) and local rice variety ("nanh chon") was demonstrated (Thoa *et al*., 2022).

The presence of phytoplasma 16SrXI in sugarcane seedlings was confirmed by using loop-mediated isothermal amplification (LAMP) as described previously (Quoc *et al*., 2021). Those plants infected with phytoplasma normally develop as stunted due to the disturbance of physiological activities. The results indicated that there was a significant difference in height at the second month when the controls (untreated plants) showed symptoms of white leaf disease and were shorter than the treated plants. Moreover, symptom rate of sugarcane seedlings treated with *D. lacustris* (BCI7) and *R. aquatilis* (BTII2) was significantly reduced in comparison with that in untreated plants resulting in the enhancement of survival rate of SCWL infected seedlings (Thoa *et al*., 2022). This result was clearly observed at the third month of BIP's treatment (Figure 1).

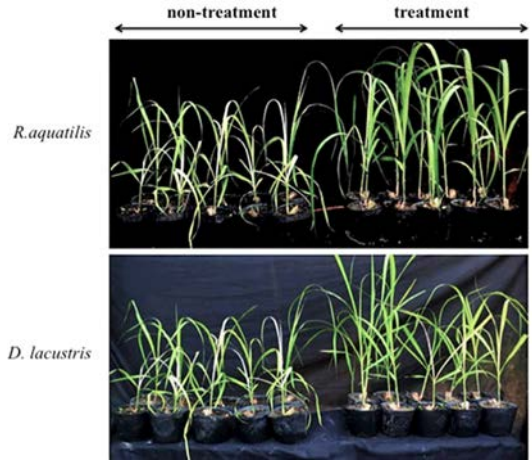


Figure 1. Symptoms of white leaf disease infected sugarcane seedlings after treatment with IAA-producing bacteria for three months.

The presence of phytoplasmas was verified by nested PCR method with a product of 1.2 kb as described (Hoat *et al*., 2012). The results depicted that the phytoplasma infection rate of the treated plants seems to be lower than that of the control plant even though the difference between treated and non-treated plants was not statically significant (Thoa *et al*., 2022).

Discussion

To minimize the damages due to SWLD, the sugarcane management along with the management of SWLD in the field was developed in sugarcane growing countries. Since it is difficult to eliminate phytoplasmas from the infected tissues, many thermotherapy methods including hot water, hot air, and aerated steam are used with moderate success. In addition, the usage of tissue culture approach is very useful for the farmers to raise disease-free plants. This approach along with the development of resistant sugarcane cultivars against SCWL phytoplasma has been followed routinely in India and Mauritius. However, their success is still limited. In Vietnam, various SWLD management approaches such as increasing the usage of healthy cuttings, pruning SCWL infected plants, increasing irrigation and biofertilizers in the sugarcane fields showed significant effects on the increase in yield and quality of sugarcane, reducing the incidence of SCWL disease at the early stages of development, although not completely eliminating the phytoplasmas. Additionally, the usage of phytoplasma resistant sugarcane varieties do not match with the requirement of yield by farmers. In this study, BIPs were used to provide IAA in the plant's auxin pool for enhancing the growth and survival rates of infected plants. Although BIPs cannot eliminate phytoplasmas from plants, they have the potential to reduce the amount of phytoplasmas in sugarcane seedlings helping to delay the appearance of white leaves and increasing the plant viability.

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Control

Production of sugarcane grassy shoot disease free setts by using hot water treatment

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Abstract

The use of healthy and vigorous setts plays a pivotal role in the sugarcane development program. The sugarcane grassy shoot disease (SCGS), which falls under major category of diseases, is associated with phytoplasmas in India, and responsible for severe losses in quality and yield. The SCGS is spread through vegetative propagating materials and insect vectors. To mitigate the infection of SCGS from the propagating materials and production of SCGS free sugarcane nursery, a hot water treatment (HTW) at 50ºC for 2 hours was found best in three tier breeder seed's program for disease free nursery. This treatment allows a significant reduction in disease incidence. It is suggested to sugarcane growers, sugar mill personnel and various extension workers to utilize the HWT which not only eliminate the SCGS from infected stalks but also improves the germination of the setts for better yield potential.

Keywords: phytoplasmas, thermotherapy, quality loss, healthy propagation materials

Introduction

Among the several sugarcane diseases, the white leaf (SCWL) and grassy shoot (SCGS) associated with the presence of phytoplasmas results in severe reduction in yield and quality. These diseases are reported as having up to more than 50% incidence and 40% reduction in qualitative and quantitative parameters in Uttar Pradesh, India, in several commercial varieties (Tiwari *et al*., 2012; Iqbal *et al*., 2015; Rao *et al*., 2017). The transmission of the disease occurs in nature through insect vectors and propagating infected sugarcane setts. The disease causes severe loss to the millable canes, and the severity increases multifold in ratoon crops resulting into a complete reduction in cane yield (Tiwari *et al*., 2012).

The use of healthy and vigorous setts plays a pivotal role in the sugarcane development program and setts are the main components of the all the inputs required for good quality and yield of sugarcane crop. Sugarcane, a vegetatively propagated crop requires 6-10 tons propagation materials for one hectare area, and sugarcane setts are costlier inputs which accounts for nearly 25% of total production cost. So, planting infected sett materials, which may carry latent infection is largely responsible for the annual recurrence of these diseases. The SCGS/WLD infection results in serious reduction not only in sugarcane yield but also in juice quality. So, to overcome the losses of the SCGS/WLD diseases, healthy propagation materials for nursery is required. This can be maintained through either producing diseases free materials through *in vitro* techniques (Tiwari *et al*., 2011) or by using heat therapy. The heat therapy has provided an effective control of SCGS, and other sett transmitted diseases of sugarcane. The heat therapy (50°C for 20 minutes) was first introduced by Kobus in 1989 in Java to control the "sereh" disease. Later, it was attempted in 1932 by Wilbrink against "sereh" and chlorotic streak diseases. Viswanathan (2001) used aerated steam therapy (AST) of seed cane for a period of 1 hour at 50°C to eliminate grassy shoot disease in sugarcane.

In the present communication successful utilization of heat therapy for producing SCGS/SCWL free propagation materials for nursery of breeder of propagation materials was done and it can be opted as regular feature program to produce disease free nursery materials and distribution of breeder propagation materials through three tier seed production program.

Materials and Methods

To produce the SCGS/SCWL free nursery materials, hot water treatments was applied in two segments. The variety CoS 08272 with the symptoms of white leaf/grassy shoot was

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Table 1. Hot water treatment of symptomatic SCGS/SCWL sugarcane plants.

Treatment	Number of buds planted (setts with two buds each)	Germination percentage	Disease incidence (%)
Symptomatic (SCGS) setts at 50°C for 2 hrs (X)	50	38%	-
Symptomatic (SCGS) setts at 52°C for 1 hr (Y)	50	30%	-
Symptomatic (SCGS) setts untreated (Z)	50	25%	35%

Table 2. Effect of water treatment on SCGS/SCWL disease symptoms.

Treatments	2017-18		2018-19		2019-20	
	Germination percentage	Disease incidence (%)	Germination percentage	Disease incidence (%)	Germination percentage	Disease incidence (%)
Symptomatic (SCGS) setts at 50° C for 2 hrs (T1)	40%	2%	54%	-	56%	-
Non symptomatic setts at 50°C for 2 hrs (T2)	50%	-	58%	-	60%	-
Symptomatic setts untreated (T3)	26%	33%	20%	50%	16%	48%

treated at two temperatures *i.e.* 50°C for two hours (treatment X) and 52°C for 1 hour (treatment Y) and for control symptomatic setts without treatment (treatment Z).

Equal number of bud populations (50 buds for each) were maintained initially in all the treatments and after planting germination percentage and diseases incidence were calculated. In the second segment, symptomatic (T1), non-symptomatic (T2) materials, were treated at 50°C for 2 hours and planted along with control *i.e.* non-symptomatic materials (T3), planted without hot water treatments and germination and symptoms were recorded.

Results and Discussion

From the study it is observed that HWT treatment of setts at 50°C for 2 hours (treatment X) was effective showing higher germination compared to setts treated at 52°C for 1 hour (treatment Y) which might be due to susceptibility of infected buds to temperature, however SCGS was absent in both treatments (Table 1). As treatment X showed better results, it was carried forward for the second segment of experiments.

SCGS affected sugarcane materials were treated at 50°C for 2 hours and the first year germination was 40% with disease incidence of 2%, however when similar materials were used in the next year, the germination percentage increased (54%) and the incidence of the disease was nearly absent and this trend continued for third year of the experiment. At the same time, non-symptomatic plants exposed to 50°C for 2 hours (T2) recorded higher germination in the first and in next two years. In the case of T3 (symptomatic untreated), the disease incidence was consistently increased with poor germination (Table 2).

The successful cultivation of sugarcane crop mainly depends upon the disease free good quality of propagation material. There is still an intense need to enhance the supply and quality of sugarcane setts since SCGS/SCWL are sett transmitted and affect the germination and ultimately led to poor quality production. The present study suggests to utilize the HWT at 50°C for 2 hours for producing SCGS/SCWL free setts for nurseries. Earlier, Steindil (1951) recommended the HWT for ratoon stunting disease but the treatment time was 2-3 hours. From India, Kirti and Singh (1958) did intensive studies on HWT and (moist hot air treatment) and recommended it for production of healthy setts for nurseries.

As it is established that heat therapy is an effective methodology for eliminating sugarcane pathogens like the agent of ratoon stunt disease, viruses and SCGS/SCWL infections from the vegetatively propagating materials (Viswanathan, 2001), treatments (HWT) of setts before planting to mitigate the infection of SCGS/SCWL is suggested. In addition to the control of the disease, fairly high yield was also obtained which is due to good germination and disease free development of the stalks. As use of HWT has become a part of regular feature programs, every year more than 0.015 Mt breeder propagation materials of different newly released cultivars are being distributed among growers for the foundation nurseries and this sett multiplication program has drastically reduced the incidence of the SCGS/SGWL diseases in sugarcane in Uttar Pradesh, India.

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Control

Control methods of alfalfa witches' broom phytoplasma disease

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Abstract

Alfalfa witches' broom (AWB) is the most important alfalfa disease in Iran. The infected plants appear severely stunted, erect and excessively branched and yellowish. Different methods of AWB disease control were studied in a randomized complete blocks design at three replications in Ardakan (Yazd province, Iran). The methods were dense planting of alfalfa, use of sorghum as windbreak and spraying with metasystox-R. The results indicated that sorghum and metasystox-R had no significant effect on disease control. Only dense planting of alfalfa in the field using twice the usual seed quantity significantly reduced the disease incidence by 16% compared the control that resulted at the 5% probability level. According to this findings, dense planting of alfalfa stands is recommended for the prevention of AWB disease in alfalfa farms.

Keywords: dense planting, disease incidence, *Medicago sativa*

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important and most cultivated forage crops in the world. The alfalfa is not only having nutritional value, but it also plays important roles in soil such as preventing erosion, increasing nitrogen fixation and maintaining and strengthening texture. Total losses caused by pest and disease damages to the alfalfa product in Iran was estimated up to 30%; the alfalfa witches' broom (AWB) disease is the most important one. It was reported for the first time in USA in 1925 (Haskell, 1926) and at present it is reported from many alfalfa growing areas of the world. In Iran, AWB disease is observed in all the major alfalfa growing areas in different cultivars of *M. sativa* especially in central and southern provinces of Iran (Esmailzadeh Hosseini *et al*., 2015). In 1997, epidemics of AWB disease were observed in alfalfa fields in Chahgeer (Abarkouh, Yazd province). The damage was so severe that the alfalfa production was reduced to less than one-third, alfalfa fields were destroyed completely, and farmers switched to other crops. In other infected areas in the country, depending on the severity of the disease, alfalfa fields in second, third and fourth years after planting have been ploughed and re-planted again. Until now no cure methods were identified for AWB disease control thus based on a farm infection that usually starts at the beginning of borders and thinning areas (Menzies, 1951), experiments on control methods for alfalfa witches' broom disease were performed in Iran.

Materials and Methods

Different methods of AWB disease control were studied in a randomized complete blocks design at three replications in Ardakan (Yazd province, Iran). The methods were dense planting of alfalfa, use of sorghum as windbreak and spraying with metasystox-R in randomized complete blocks with three replications.

The factors and their levels were density (25 kg/ha, the amount of seed according to the custom of the place and the use of the seed twice the custom of the place); windbreak (planting three rows of sorghum around the plot as a windbreak) and spraying with metasystox-R at a ratio of one per thousand, three times in March and two times in early spring. Three rows of sorghum were planted around the corresponding experimental plots to create a windbreak, which were harvested alternately.

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The infection of the plants was determined based on disease specific symptoms and PCR tests. The disease incidence was calculated by counting number of plants with symptoms out of total number of plants observed in two 1 m^2 in each plot by 100. The averages of the measured traits were compared by Duncan's test and the best treatment that had the low amount of disease incidence was determined. Furthermore, total nucleic acids were extracted from symptomatic alfalfa subjected to direct and nested PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), R16mF2/R16mR2 and R16F2n/R16R2 (Gundersen and Lee, 1996) primer pairs for 35 cycles and virtual RFLP analyses on sequenced amplicons that together with phylogenetic studies and DNA homology analyses of partial 16S ribosomal sequences were used to identify the detected phytoplasmas.

Results and Discussion

The infected plants appeared severely stunted, erect and excessively branched and yellowish (Figure 1). The results of molecular identification of the phytoplasmas showed that in this area the AWB phytoplasma disease was associated with a '*Candidatus* Phytoplasma australasia' strain (16SrII-D). Dense planting of alfalfa in the field using twice the usual seed quantity (50 kg/ha) significantly reduced the disease incidence by 16% compared to the custom of the place at 5% probability level. Use of sorghum as windbreak (Figure 2) and spraying with metasystox-R had no effect on disease control.

The spraying in March and early spring did not have any effect in reducing the disease incidence and this seems to be ineffective due to the special weather conditions and the occurrence of drought, that help the presence of active vectors of the alfalfa phytoplasma strain (*Orosius albicinctus*) throughout the year, especially in winter. Alfalfa cultivars in Iran have no resistance to AWB disease and the use of good agricultural practices and intensive cultivation resulted effective in reducing the spread of the disease to new crops. Using the results of this research, in areas where AWB diseases are widespread or likely to be present, in addition to management aspects such as destroying highly infected farms and transporting alfalfa dry, the farms should be planted with higher density. Considering that the condition of the soil, number and type of irrigation water have an effect on the amount of seed used, the best alfalfa planting density for reducing disease incidence is different in each region and should be tested locally.



Figure 1. Symptoms of alfalfa witches' broom, dwarfing and yellowing in Yazd, Iran.



Figure 2. Forage sorghum was used as a windbreak around alfalfa fields to prevent AWB disease.

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Quarantine

'*Candidatus* Phytoplasma aurantifolia' and '*Candidatus* Phytoplasma australasia': epidemiology meets quarantine

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Abstract

The use of '*Candidatus* Phytoplasma' as provisional taxon is helping both epidemiological studies and quarantine rules settling. A focused search in database evidences discrepancies and problems for strains enclosed in '*Ca.* P. aurantifolia' and '*Ca.* P. australasia' that share very often the same geographic distribution in Asian and Australian countries. An improved differentiation based on multigene approach could be useful to a more clear distiction of these phytoplasma strains.

Keywords: phytoplasmas, plant disease, taxon, phylogeny, 16S ribosomal gene, sequencing

Introduction

'*Candidatus* Phytoplasma aurantifolia' (Zreick *et al*., 1995) was the first phytoplasma taxon described associated with a severe lime witches' broom disease reported in Oman and other Arabian countries having destroyed a large number of citrus crops in the Arabian peninsula (Figure 1).



Figure 1. Lime witches' broom in a lime in a field in Oman in 2005.

After almost three decades the phytoplasma identification is still based on '*Candidatus* Phytoplasma' taxa defined on the basis of 16S ribosomal gene sequences (IRPCM, 2004). In spite the progresses in phytoplasma cultivation in artificial media (Contaldo *et al*., 2016) no pure culture is available to allow the establishment of taxonomically valid categories for phytoplasmas as it is for mycoplasmas and walled bacteria. The guidelines for the definition of '*Ca.* Phytoplasma' species were recently updated (Bertaccini *et al*., 2022) and could be useful also for quarantine purposes aimed to reduce the spread of the most dangerous and aggressive phytoplasma associated diseases. This provisional differentiation system is therefore very relevant for using and circulating healthy plants worldwide. The system is however not covering the whole phytoplasma associated diseases, *i.e.* "flavescence dorée" phytoplasma is not molecularly differentiable from described '*Ca.* Phytoplasma' and it is maintained under the name of the disease. The cultivation in artificial media of pure phytoplasma cultures is necessary not only to fulfil the Koch postulates for these bacteria but also to drop this provisional taxonomy. A large number of phytoplasma sequences of the 16S ribosomal gene were deposited under GenBank validating the presence of diverse '*Ca.* Phytoplasma' species worldwide, however some incorrect assignment is present and also some difficult situation for taxa differentiation can be evidenced. One of these cases is the designation of '*Ca.* P. aurantifolia' and '*Ca.* P. australasia' (White *et al*., 1998). Both phytoplasmas are quite widespread and often they share the same geographic areas such as Arabian peninsula, India and Australia, infecting agricultural relevant crops such as citrus, alfalfa, sesame and others. The database assignment of selected phytoplasma strains to these taxa was partly verified.

Materials and Methods

A search on the NCBI GenBank database was performed using the BLAST tool for '*Ca.* P. aurantifolia' (taxid: 180978) and '*Ca.* P. australasia' (taxid: 2754999). The 16S ribosomal gene 98.65% identity threshold to design new '*Ca.* Phytoplasma' species was used (Bertaccini *et al*., 2022). The

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search was done on 5,000 sequences with identities between 90 and 100% and coverage between 60 and 100% using filters above 98.65%, between 97.50 and 98.65% and below 97.50% for comparison to '*Ca.* P. aurantifolia' (GenBank accession number U15442) and '*Ca.* P. australasia' (GenBank accession number Y10097). A phylogenetic tree was built using selected strains among those retrieved in Mega 7 (Kumar *et al.*, 2016).

Results and Discussion

The results of blast search (Table 1) show a large number of deposited sequences (3,846 total) attributed to these two taxa, however using only the taxid at total of 682 sequence was identified. Among these sequences the attribution to '*Ca.* P aurantifolia' for 244 strains was based on the previous threshold of 97.5%, but further 44 strains were attributed to this taxon with a lower threshold. Based on the updated 98.65% threshold about 200 phytoplasmas in both taxa resulted wrongly named.

Table 1. Identity thresholds of phytoplasma strains in the NCBI.

Identity thresholds	'*Ca.* P. aurantifolia' (search with no name)	'*Ca.* P. australasia' (search with no name)
100-90%	485 (3,529)	197 (1,317)
100- 98.65%	199	192
98.65-97.50%	244	3
Below 97.50%	44	2

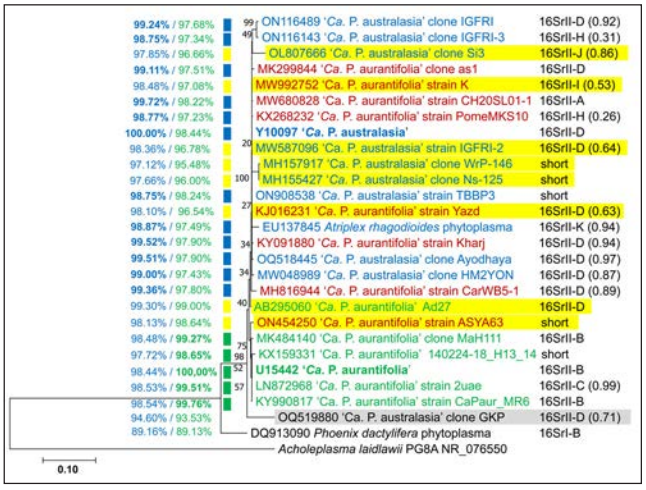


Figure 2. Neighbour-Joining phylogenetic tree of selected '*Ca.* P. aurantifolia' and '*Ca.* P. australasia' strains. Red color indicates wrong naming, yellow background uncertainity in taxon attribution, grey background questionable attribution. Blue: '*Ca.* P. australasia', green: '*Ca.* P. aurantifolia'. On the left identity percentages.

Strains selected among the four categories in Table 1 (five per group) were used to build a phylogenetic tree (Figure 2) and for verification of taxon identity thresholds. The following strains showed thresholds below 98.65% to both '*Ca.* Phytoplasma' species: GenBank accession numbers MW587096 and OL807666 (alfalfa and sesame, India); MH155427; MH157917 (*Phoenix dactilifera*, Saudi Arabia); OQ519880 (tomato, India); ON454250 (lettuce, Turkey); KJ016231 (sunflower, Iran); MW992752 (banana, Indonesia). The strains deposited in GenBank as '*Ca.* P. aurantifolia' under accession numbers MK299844 (Iran, *Artemisia sieberi*), MH816944 (India, carrot), KY091880 (Saudi Arabia, *P. dactilifera*), MW680828 (Taiwan, matted sea-lavender) and KX268232 (India, pomegranate) should be named '*Ca.* P. australasia' according to identity threshold. Finally the strain with GenBank accession number AB295060 (citrus, Oman) could be assigned to both taxa (thresholds 99.00% and 99.30%, respectively). The whole length 16S rDNA sequences (1,500 nt) is not available for these strains, but it could help to resolve taxa attribution, moreover also the available sequence signatures were not helpful. The 16SrII group (enclosing both taxa) is divided in at least 23 ribosomal subgroups (*sensu* Lee *et al.*, 1988). Using this differentiation system in the selected strains 16SrII-B and -C resulted referable to '*Ca.* P. aurantifolia' while subgroups 16SrII-A and -D, -H and -K to '*Ca.* P. australasia' (Figure 2). This verification should be performed for all the described ribosomal subgroups, however, also in the low number of strains examined it was not always able to help in the phytoplasma identity attribution. More biological (phenotypical) and molecular (multigene, genomes) features seem therefore necessary for a reliable differentiation to implement quarantine rules and to elucidate the epidemiological features that are also very relevant for the appropiate management of the diseases associated to both phytoplasmas.

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Quarantine

Multilocus RFLP characterization of '*Candidatus* Phytoplasma pyri' strains in pear from Chile and Italy

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Abstract

'*Candidatus* Phytoplasma pyri' associated with pear decline in Italy and Chile severely affects orchards. To its molecular characterization, results of multilocus RFLP analyses on seven phytoplasma genes (16Sr, *aceF*, *secY*, *leu*, *secA*, *tuf*, and *lsu36p*) resulted a promising tool. To complete this work and better understand the discrimination ability of the used genes the amplified DNAs obtained from Chilean and Italian strains will further be cloned and sequenced.

Keywords: pear decline, nested-PCR, phytoplasma genes, 16SrX-C

Introduction

Pear decline associated with the presence of '*Candidatus* Phytoplasma pyri' and transmitted by pear psylla, grafting and budding severely affects cultivations in both Europe and American continents (Fiore *et al*., 2018). Trees lose vigor over several seasons, leaves turn red early due to starch accumulation, and frequently the fall leaf colour occurs early (Figure 1). The disease was reported in Italy and north America since the sixties and more recently it was also reported in Chile (Facundo *et al*., 2017). To characterize the phytoplasma populations in the two countries, RFLP analyses on seven phytoplasma genes were performed.

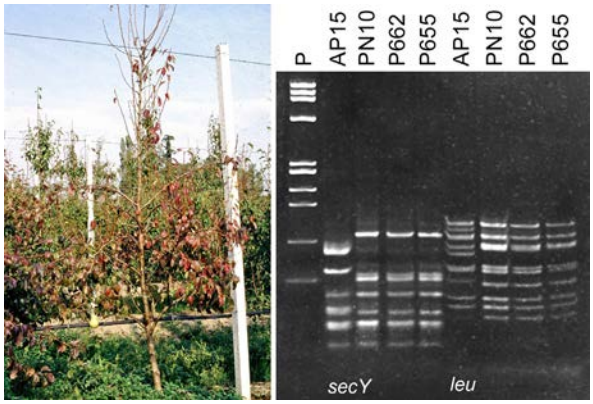


Figure 1. Symptoms associated to '*Ca.* P. pyri' in infected pear tree showing premature red colour and poor production (left). RFLP: on polyacrylamide 6.7% of *secY* and *leu* genes amplicons digested with *Tru1*I, sample acronyms are as in Table 1; P, marker ÖX174 *Hae*III digested (right).

Materials and Methods

Pear decline symptomatic samples were collected from Italian and Chilean orchards. A total of 10 samples from Italy were collected in Bologna area (North Italy) in 2019, while from Chile the 10 samples tested were collected in San Fernando, Graneros and Peumo (O'Higgins region) in April 2022. Total nucleic acids were extracted from 1 g of leaf midrib, phloem, and root tissues (Prince *et al*., 1993). PCR mixtures were prepared using master Mix (DreamTaq DNA Polymerase), and 20 ng of nucleic acid template. A '*Ca.* P. mali' strain maintained in periwinkle (AP15) (Bertaccini, 2023) and sterile distilled H_2O were used as positive and negative control, respectively. In nested PCR 1 µl of undiluted or 1: 30 of diluted amplicon solution were used as template. For phytoplasma detection and identification PCR with primer pair R16F2n/R2 (Gundersen and Lee, 1996), followed by nested reaction with the group specific primer pair R16(X)F1/R1 (Lee *et al*., 1995) and RFLP analysis with *Rsa*I, *Ssp*I (Fermentas, Vilnius, Lithuania) restriction enzymes, were used. The positive samples were further amplified on *aceF*, *secY*, *leu*, *secA*, *tuf*, and *lsu36p* genes using nested PCR according to published protocols (Danet *et al*., 2011; Lee *et al*., 2010; Abeysinghe *et al*., 2016; Hodgetts *et al*., 2008; Makarova *et al*., 2012; Cui *et al*., 2021). RFLP analysis was carried out on positive samples with *Tsp509*I, *Tru1*I, *Mbo*II and *Taq*I (Fermentas, Vilnius, Lithuania) according with the different amplicons (Table 1). The fragments obtained were separated in 6.7% polyacrylamide gel, stained with ethidium bromide, visualized under an UV transilluminator at 312 nm then photographed with Kodak DC 290 zoom digital camera.

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Table 1. Results of RFLP analyses on multigene amplicons of the '*Ca*. P. pyri' strains from Italy and Chile.

Pear sample	Genes					
	*leu – Tru*1I	*aceF – Tsp509*I	*secY – Tru*1I	*secA – Mbo*II	*lsu36p – Taq*I	*tuf – Tru*1I
PN10 (Italy)	+ A	+ A	+ A	+ A	+ B	+ A
PN11 (Italy)	-	-	+ B	-	+ C	+ C
PN13 (Italy)	+ A	+ A	+ B	+ A	-	+ C
PN14 (Italy)	+ nd	+ A	-	+ A	-	-
P655 (Chile)	+ A	+ A	+ A	+ B	+ B	+ A
P662 (Chile)	+ A	+ A	+ A	+ B	+ A	+ A
P663B (Chile)	+ A	+ A	+ A	+ B	+ B	+ A
'*Ca*. P. mali' AP15	+ B	+ B	+ C	+ B	+ A	+ B

nd: undigested; for each restriction enzyme, same letter indicates identical restriction profile

Results and Discussion

The PCR results on 16S ribosomal gene provided amplification for 7 samples from Italy and three from Chile. All phytoplasmas were enclosed in subgroup 16SrX-C after RFLP analyses with *Rsa*I and *Ssp*I on 16SrX specific amplicons (data not shown). These 10 pear samples were further analysed with PCR on other six phytoplasma genes, and only in 7 samples the amplification was obtained (Table 1). The strains PN10, P655, P662 and P663B were amplified on all the genes; PN13 on 5 genes; PN11 and PN14 on 3 genes. With *leu*, *aceF* and *secA* genes only PN11 strain was not amplified; on *secY* and *tuf* genes only PN14 was not amplified; the strains PN13 and PN14 were not amplified on *lsu36p* gene. The RFLP analyses showed identity in the restriction profiles with the enzymes used for the *leu* and *aceF* amplified genes that however distinguished these strains from the 16SrX-A ('*Ca*. P. mali') strain used as reference. The *secY* and *tuf* gene profiles also were distinguished from the one of 16SrX-A, while the *secA* and *lsu36p* amplicons of '*Ca*. P. mali' showed profiles identical to some of those obtained in pear samples. The *secA* gene distinguished the Chilean pear phytoplasmas from the Italian ones, however failed to distinguish the Chile phytoplasmas from the AP15 strain. The profiles of *lsu36p* and *tuf* amplicons resulted mixed in the samples from the two countries. These amplicons are being cloned and sequenced to better understand the discrimination ability of these DNA fragments.

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Quarantine

Screening of European stone fruit yellows, apple proliferation and pear decline diseases in fruit nurseries with focus on latent infection

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Abstract

The occurrence of phytoplasmas was monitored in nurseries by PCR testing to determine the level of infection with these regulated non-quarantine pests in propagation materials of pome and stone fruits in the Czech Republic (central Europe) in 2022. Fourteen fruit nurseries were visited in ten regions of the Czech Republic. Out of a total of 640 samples (various cultivars of apple, pear, plum, apricot and peach) collected, only 7 apricots were detected as infected by '*Candidatus* Phytoplasma prunorum' which represents 1.09% infection rate out of all tested trees. The obtained results show that the current occurrence of phytoplasmas in the propagation material of fruit trees in the Czech Republic is very low and the situation is promising, reflecting the successful application of phytosanitary measures.

Keywords: phytoplasmas, *Psylloidea*, pome fruit, non-quarantine, pathogens, nursery

Introduction

Phytosanitary regulated '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum' are associated with apple proliferation (AP), pear decline (PD) and European stone fruit yellow (ESFY) diseases. These pathogens are reliably detected in symptomatic trees. However, they can also occur in the form of latent infections, where a reliable detection can be problematic. The economic competitiveness of the Czech nurseries, and consequently of the Czech fruit production sector, depends also on the limiting the spread and harmfulness of these phytoplasma diseases. Therefore, it was planned to monitor the occurrence of phytoplasmas in nurseries by PCR testing to determine the level of presence with these regulated infectious bacteria in propagation materials of pome and stone fruits.

Materials and Methods

A total of 14 fruit nurseries were visited in ten regions of the Czech Republic with higher fruit tree nursery production (Figure 1). The shoots of pome and stone fruit species without visible symptoms of phytoplasma disease were collected from each nursery in September and October 2022. In total, 640 samples were collected from various apple, pear, plum, apricot and peach cultivars (Table 1 and Figure 1) for phytoplasma detection. The phloem from mature shoot was used as a plant material for the detection of phytoplasmas. The total DNA was extracted from the phloem scrapings using the Exgene Plant SV mini commercial kit (GeneAll). The presence of phytoplasmas was determined by quantitative PCR or nested PCR using P1/P7 followed by R16F2n/R2 primers (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Gundersen and Lee, 1996). RFLP analysis by *Rsa*I, *Mse*I, and *Ssp*I was used to identify the phytoplasmas detected in nested PCR at the ribosomal subgroup level.

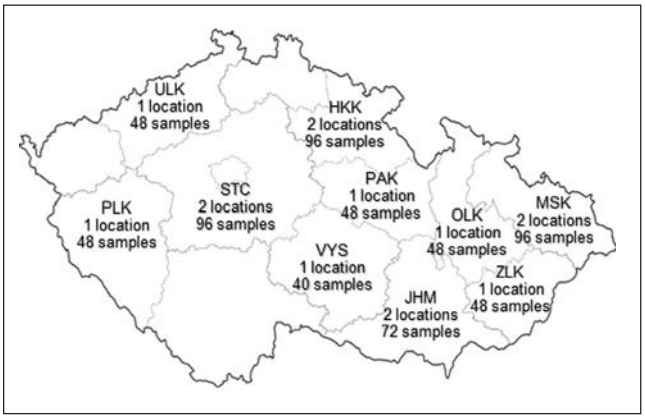


Figure 1. Fruit nurseries surveyed in ten regions of the Czech Republic during the screening for ESFY, AP and PD phytoplasma presence.

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Results

Towards the end of the vegetation period 2022, the fruit nurseries were screened for the presence of phytoplasmas. In total, 640 samples of 5 fruit species from 98 cultivars were analysed; the number of cultivars of particular fruit species and their respective percentage shares is summarized in Figure 2. Apples accounted for the largest proportion of samples collected for testing, which is in accordance with the representation of individual fruit species in the nursery sector in the Czech Republic. The results are summarized in Table 1.

Table 1. Results of phytoplasma detection in Czech Republic nurseries.

Fruit crop	Number of samples	Number of infected samples	Positive samples %	Phytoplasma
Apple	236	0	-	No
Pear	184	0	-	No
Apricot	100	7	7.0%	'*Ca.* P. prunorum'
Plum	104	0	-	No
Peach	16	0	-	No

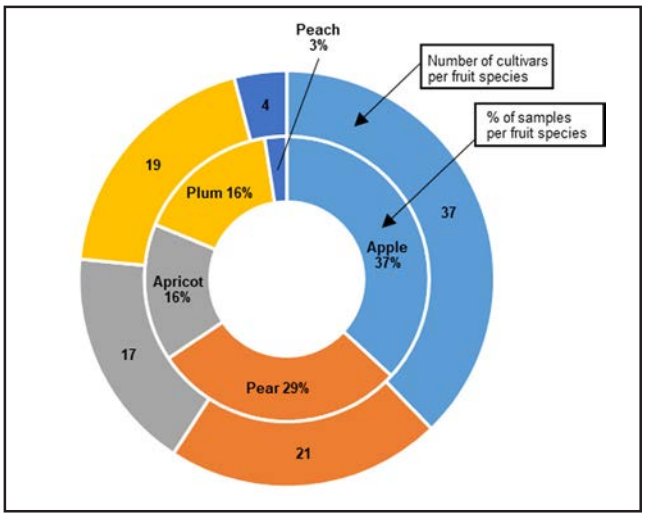


Figure 2. Number of cultivars of the diverse fruit species and their respective percentage share during the screening.

Among the tested fruits trees, phytoplasma presence was not detected in apple, pear, plum and peach samples. Only apricots were positive for '*Ca.* P. prunorum'. This ESFY phytoplasma was detected in 7 out of 100 accessions of apricot, which represents 1.09% infection rate out of all tested trees. Infected plants were immediately destroyed in collaboration with nurserymen.

Discussion

The results obtained show that the current occurrence of phytoplasmas in the Czech nurseries is relatively low. Education by phytosanitary authorities and research organizations aimed at describing the symptoms of phytoplasma associated diseases and subsequent measures taken by nurserymen to prevent their spread, including measures against phytoplasma vectors, have contributed to this good health status in the Czech fruit propagation sector.

Nevertheless, it is necessary to pay constant attention to the presence and spreading of phytoplasmas in plantings and propagating materials also in the context of the current climate changing and global warming. Symptoms of ESFY vary among stone fruit species, cultivars and rootstocks (Cieslinska, 2011; EPPO, 2021). They may also be influenced by environmental factors. There are many tolerant host plant species that do not show symptoms despite being infected with '*Ca.* P. prunorum' (Sullivan, 2014). However, in the case of increased disease spread, there is a risk of significant economic damage, especially for susceptible cultivars of stone and pome fruits grown in commercial orchards. Particular attention should be paid to imports of propagating material from abroad. The screening results show that for the reliable detection of ESFY, it is useful to combine the results of visual assessment with those of molecular detection especially in the case of young plant material in fruit nurseries. Apricots are one of the stone fruit species more susceptible to ESFY infection. The disease can cause considerable economic losses in late developmental stages due to the mortality of apricot trees.

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Quarantine

Multigene characterization of phytoplasmas enclosed in 16SrIX group infecting different host plant species

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Abstract

The presence of phytoplasmas enclosed in 16SrIX-C subgroup is spread in several countries of at least three continents and the molecular differentiation of phytoplasmas in this group is relevant for epidemiological and quarantine reasons. A differentiation of phytoplasmas detected in diverse host plant species in different geographic areas in Europe and America by multigene analyses was carried out on cultivated (daisy and periwinkle) and wild species (common dandelion). The 16SrIX-C phytoplasma RFLP and sequencing results of *rp*, *secA*, *secY*, *leu*, *imp* and *tuf* genes resulted in the differentiation of the studied strains mainly according with both host species and geographic distribution.

Keywords: *Leucanthemum vulgare*, *Taraxacum officinale*, phyllody, witches' broom, virescence, sequencing

Introduction

The presence of phytoplasmas enclosed in 16SrIX-C subgroup is spread in several countries of at least three continents and molecular differentiation is therefore relevant for their epidemiology and for quarantine purposes.

Phytoplasmas in some subgroups of 16SrIX group are enclosed in the '*Candidatus* Phytoplasma phoenicium' (Verdin *et al*., 2003) and are of quarantine relevance in several countries, therefore appropriate tools for their differentiation are relevant. Samples of *Catharanthus roseus*, *Leucanthemum vulgare* and *Taraxacum officinale* with symptoms of phyllody, virescence, stunting or witches' broom (Figure 1) collected in Italy and Colombia were analyzed to verify the presence of 16SrIX phytoplasmas and to molecularly characterize them using a multigene approach.

Materials and Methods

DNA was extracted from symptomatic plant samples collected in north Italy, in Sicily (NAXOS) and in Colombia (CO) and from the PEY strain maintained in micropropagated collection (Bertaccini, 2023) (Table 1) by a CTAB method (Angelini *et al*., 2001). Nested PCR amplification was performed on 16S rRNA gene out using 1 µl of the 1: 30 diluted DNA with P1/P7 primers followed by R16F2n/R16R2 (Bertaccini *et al*., 2019). The amplicons were subjected to



Figure 1. From top left: daisy with phyllody (a), witches' broom and leaf reddening in common dandelion (b) and flower virescence in periwinkles from Italy (c) and Colombia (d, courtesy B. Duduk).

RFLP analysis with *Tru*I (Fermentas, Vilnius, Lithuania). The same enzyme was used for RFLP analyses on the amplicons obtained for the *rp*, *secA*, *secY*, *leu*, *imp* and *tuf* genes that were amplified using published protocols (Martini *et al*., 2007; Lee *et al*., 2012; Hodgetts *et al*., 2008; Abeysinghe *et al*., 2016; Contaldo *et al*., 2011; Quaglino *et al*., 2015), respectively. The PCR and RFLP products were analyzed by

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Table 1. Results of RFLP and sequencing on multigene amplicons of the 16SrIX-C phytoplasmas analyzed.

Samples (acronym)	Origin	Gene amplification, RFLP profile and sequence identity % to '*Ca*. P. phoenicium'					
		*rp Tru*1I	*secY Tru*1I	*secA Tru*1I	*Leu Tru*1I	*tuf Tru*1I	*Inmp*
Leucanthemum vulgare (LV1)	E-R*, Italy	+ A 89.47	+ A nd	+ A nd	+ A nd	+ A 84.06	-
Leucanthemum vulgare (LV2)	E-R*, Italy	+ A 88.72	+ A nd	+ A nd	+ A nd	+ A nd	-
Taraxacum officinale (TO5R)	Veneto, Italy	+ A nd	+ B nd	-	+ A nd	+ A nd	-
Taraxacum officinale (TO8R)	Veneto, Italy	+ A 89.37	+ A nd	-	+ A nd	+ A nd	-
Taraxacum officinale (TO11R)	Veneto, Italy	+ A nd	+ B nd	-	+ A nd	+ A nd	-
Catharanthus roseus (PR)	E-R*, Italy	+ A nd	+ A nd	+ A nd	+ A nd	+ B nd	-
Catharanthus roseus (P1)	E-R*, Italy	+ A nd	+ A nd	+ A nd	+ A nd	+ B nd	+
Catharanthus roseus (P2)	E-R, Italy	+ A nd	+ A nd	+ A nd	+ A nd	+ B nd	+
Catharanthus roseus (PEY)	Basilicata, Italy	+ A 89.58	+ A nd	-	+ A nd	+ B nd	-
Catharanthus roseus (CO)	Colombia	+ B 88.05	+ C 81.97	+ B nd	+ B nd	+ C 90.03	-
Catharanthus roseus (NAXOS)	Sicily, Italy	+ A 89.31	+ A nd	+ C nd	+ C nd	+ D nd	+

*E-R: Emilia-Romagna; nd: not determined

electrophoresis on 1% agarose gel and in 6.7% polyacrylamide gel, respectively and stained with ethidium bromide. The results were documented with KODAK EDAS 290 digital camera with a bench-top UV transilluminator at 312 nm. RFLP selected gene amplicons for differential profiles were directly sequenced in both senses using the same primers of amplification, and the obtained sequences were assembled, aligned, and compared with nucleotide sequences available at NCBI database using BLAST (www.blast.ncbi.nlm.gov) function.

Results and Discussion

The PCR assays with the primers amplifying the 16S ribosomal gene of phytoplasmas produced the expected size amplicons from all symptomatic samples and control strains. RFLP analysis showed patterns identical to each other's and to the strains in subgroup 16SrIX-C. The 16S rRNA gene sequences showed a hight identity percentage to '*Ca*. P. phoenicium'. In particular for LV1 (1,124 nt) it was 99.03% (6 SNPs and 3 GAPs); for TO5R (1,273 nt) 99.21% (10 SNPs); for TO8R (1,270 nt) 99.29% (9 SNPs); for NAXOS (1,773 nt) 98.08% (23 SNPs and 10 GAPs) and for CO (890 nt) 98.65% (9 SNPs). These percentages did not allow a differentiation from '*Ca*. P. phoenicium' according with the current identity threshold (Bertaccini *et al*., 2022). However the identity percentages on *rp* gene and the collective restriction profiles on the other genes tested allow a better differentiation (Table 1). Moreover, *inmp* gene was only amplified from samples P1, P2 and NAXOS confirming the reported high variability for this gene (Quaglino *et al*., 2015) and the *secA* gene was not amplified in *T. officinale* and PEY samples. Considering this variability together with the geographic distribution and host species a new '*Candidatus* Phytoplasma' species could be proposed for phytoplasmas in the 16SrIX-C subgroup.

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Mixed infection

Mixed phytoplasma infection in *Cressa cretica* showing witches' broom symptoms in Iran

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Abstract

A *Cressa cretica* witches' broom disease was observed in Ardakan (Yazd province, Iran) during 2018-2021. Total DNAs extracted from eighteen symptomatic and four asymptomatic plants were subjected to nested PCR assays using primers amplifying the phytoplasma 16S ribosomal DNA. Expected length of PCR amplification products was detected in all symptomatic plant samples but not in those asymptomatic. RFLP analysis of the R16F2n/R16R2 amplicons showed mixed infection of phytoplasmas enclosed in 16SrXXIX and 16SrI groups.

Keywords: phytoplasma diseases, 16SrI, 16SrXXIX, molecular detection, RFLP analyses

Introduction

Phytoplasmas are wall-less plant-pathogenic bacteria, associated with a wide range of plant diseases worldwide. They are transmitted mainly by leafhoppers and psyllids. Currently 49 '*Candidatus* Phytoplasma' species were described based on their 16S rRNA gene sequences (Bertaccini *et al*., 2022). Recent reports suggest an increased susceptibility to pathogens in high salinity tolerant plant species (Besri, 1993). Several of these plants are common in some areas of Iran since they grow in a wide range of saline soils and are often used in traditional medicine and can be also used as ornamental, animal fodder, biofuel, and edible oil production, and therefore have a significant economic importance. One of these plant species is *Cressa cretica* living on the edge of deserts and dry lands of desert and semi-desert areas. This perennial plant is herbaceous, with small leaves, without petiole, and belongs to family Convolvulaceae (Priyashree *et al*., 2010). Symptoms of witches' broom (Figure 1) were observed with 4% incidence in *C. cretica* plants in Ardakan (Yazd province, Iran), therefore the possible phytoplasma association with the disease was investigated.

Materials and Methods

During 2018-2021 surveys in Chah-Afzal (Ardakan, Yazd province) samples from symptomatic (CrcWB) and asymptomatic *C. cretica* plants were collected. Total DNAs extracted from eighteen symptomatic and four asymptomatic plants were subjected to nested PCR assays using P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), R16mF2/R16mR2 and R16F2n/R16R2 primer pairs for 35 cycles under described conditions (Gundersen and Lee, 1996). RFLP analysis was done on the amplicons obtained from nested PCR with R16F2n/R16R2 primers using *Mse*I, *Hha*I, *Alu*I, *Hae*III, *Rsa*I, and *Taq*I restriction enzymes to identify the ribosomal subgroups of the detected phytoplasmas. The verification of the amplicon presence and RFLP profiles was achieved by electrophoresis in agarose gels at 1% and 3%, respectively, followed by ethidium bromide staining and UV light visualization and photographing.



Figure 1. Small leaves, shortened internodes, proliferation of axillary buds, bushy growing and witches' broom in *Cressa cretica* (left) in Ardakan (Yazd province, Iran) compared to a asymptomatic plant (right).

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Results and Discussion

Symptomatic *C. cretica* plants showed small leaves, shortened internodes, proliferation of axillary buds, bushy growing and witches' broom (Figure 1). DNA fragments of about 1.8, 1.4 and 1.25 kbp respectively were obtained from CrcWB plants, but not from those symptomless. RFLP analysis on R16F2n/R16R2 amplicons showed the presence of mixed phytoplasma infection in the majority of the profiles with some of the above listed enzymes. In particular, *Mse*I and *Rsa*I profiles confirmed the presence of phytoplasmas of 16SrXXIX and 16SrI groups (Figure 2). The *C. cretica* witches' broom samples resulted therefore infected with mixed phytoplasma population in 16SrI and 16SrXXIX groups.

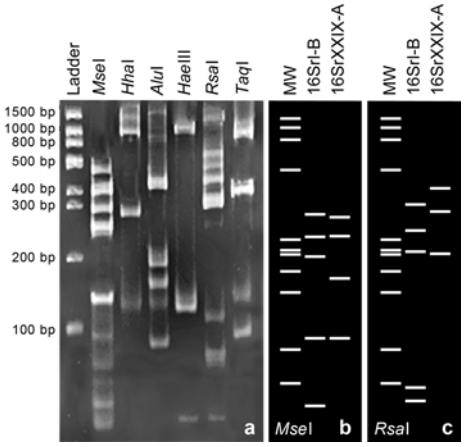


Figure 2. Restriction fragment length polymorphism (RFLP) analysis of nested PCR products amplified with primers R16F2n/R16R2 (1,248 bp) from *C. cretica* witches' broom phytoplasma. Ladder: 100 bp DNA (a) and virtual RFLP results using *Mse*I and *Rsa*I of '*Candidatus* Phytoplasma asteris' and '*Ca*. P. omanense' R16F2n/R2 sequences in the *i*PhyClassifier (Zhao *et al.*, 2009) MW, molecular marker (b and c).

So far, phytoplasmas enclosed in twelve ribosomal groups 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXIV, 16SXXIX and 16SrXXX were reported infecting different plant species in Iran. The 16SrI, aster yellows, '*Ca*. P. asteris' is one of the most widespread in Iran and also worldwide infecting a number of plants species within large distribution areas. Furthermore, phytoplasmas in the 16SrXXIX group, '*Ca*. P. omanense' were detected in *C. cretica* and were previously detected in *Cassia italica*, and *Convolvulus arvensis* in Oman, in Lebanon (Al-Saady *et al*., 2008) and in Iran (Esmailzadeh Hosseini *et al*., 2016), respectively. Moreover, they were reported in Lebanon in grapevine, bindweed and in a potential insect vector (Foissac *et al*., 2019). '*Ca*. P. omanense' was also identified in *Prunus persica* and *Diospyros kaki* in Iran (Esmailzadeh Hosseini *et al*., 2017; 2019) therefore *C. cretica* may have an important role in the epidemiology of this phytoplasma in the middle East countries. '*Ca*. P. omanense' in Iran seems to be more widespread than initially described especially considering its distribution in arid areas in middle East, north Africa and several southern Mediterranean areas. Collectively, *C. cretica* may have an important role in the epidemiology and spreading of this phytoplasma.

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Mixed infection

Mixed infection of phytoplasmas and potyvirus in *Phlox drummondii* in India

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Abstract

Phytoplasma symptoms of virescence and leaf yellowing were observed in *Phlox drummondii* in Indian Agricultural Research Institute with an incidence of about 20%. PCR assays were performed on the genomic DNA to amplify 16S rDNA region using universal primer pairs P1/P7 followed by nested primer pairs R16F2n/R16Rn. Further to examine if there was virus infection, the total RNA was used for cDNA production using RT-PCR, then amplified with primer pair targeting conserved coat protein (CP) gene of potyvirus. The specific amplicons of 1.2 kb and 860 bp of 16S rRNA and *cp* genes were obtained, respectively. Sequence comparison, phylogenetic and virtual RFLP analysis of 16S rRNA gene sequences confirmed the presence of '*Candidatus* Phytoplasma asteris' (16SrI-B subgroup). Further, sequence comparison and phylogenetic analysis of *cp* gene confirmed the presence of bean common mosaic virus.

Keywords: '*Candidatus* Phytoplasma asteris', potyvirus, virescence, ornamental crop

Introduction

In addition to fungal diseases and adverse environmental conditions, phlox plants are susceptible to viral (mosaic virus, curly top virus) and phytoplasma diseases belonging to group 16SrIX (Madhupriya *et al*., 2013) and 16SrI (Valiunas *et al*., 2001) that were reported. from India and Lithuania, respectively. Symptoms of virescence and leaf yellowing were observed in phlox plants in the Division of Plant Pathology, Indian Agricultural Research Institute, India. To verify their association with the presence of phytoplasmas and viruses molecular assays were performed.

Materials and Methods

During a survey, three symptomatic (PDVY-1, PDVY-2, and PDVY-3) and two asymptomatic (PDVY-4 and PDVY-5) leaf samples were collected from flower beds of *Phlox drummondii* in gardens of Plant Pathology Division, IARI during November 2019 and used for DNA and RNA extraction. The DNA was extracted from midribs by DNeasy plant DNA Extraction kit (Qiagen). A concentration of 100 ng/µl was used for amplification of the phytoplasma 16S rRNA gene using specific universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by nested primers R16F2n/R16R2 (Gundersen and Lee, 1996). The PCR cycling parameters for P1/P7 were 94°C for 4 minutes, followed by 30 cycles of 94°C for 45 seconds; 55°C for 1 minute; 72°C for 2 minutes followed by final extension of 72°C for 10 minutes. Two µl of the PCR product were diluted 1: 20 and used as template in nested PCR with the same cycling conditions except for annealing at 56°C for 1 minute. DNA from *Catharanthus roseus* (16SrVI-D; GenBank accession number KP866410) was used as positive control. RNA was extracted from the same samples using QIAGEN RNeasy Plant Mini Kit as per manufacturer description and used for RT-PCR with specific primers to virus coat protein as described by Flores-Estévez *et al*. (2003). PCR amplicons were visualized on a 1% TAE agarose gel and purified using the Wizard^{R} SV Gel and PCR Clean-up System (Promega, Madison, USA) and cloned in competent *Escherichia coli* (DH5-α). Cloned products of about 1,250 and 800 bp were sequenced bi-directionally at Agri genome, Kerala, India. The sequences were assembled using DNA Baser version 4 and aligned with Clustal W in BioEdit7 (Thompson *et al*., 1994). The consensus sequences were submitted to GenBank and used in BLAST analysis. The 16Sr DNA sequences generated and reference strain sequences retrieved from GenBank were used to construct phylogeny through MEGA 7.0 employing the neighbor-joining method (Kumar *et al*., 2016). The phytoplasma sequences were subjected to *in-silico* RFLP with

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*i*PhyClassifier (Zhao *et al*., 2009). *Acholeplasma laidlawii* (GenBank accession number AB680603) and a potato virus Y isolate (GenBank accession number FJ214726) were used as outgroups to root the trees in phytoplasma and BCMV phylogeny analyses respectively.

Results

Virescence and leaf yellowing (Figure 1) were observed in *P. drummondii* plants with about 20% incidence in different flower beds. The phytoplasma presence was confirmed by amplification of about 1.25 kb product only from all the symptomatic samples.



Figure 1. Symptoms of leaf yellowing (a) and virescence (b) in phlox plants.

Pairwise sequence comparison of the partial 16S rRNA gene sequences showed 100% identity among themselves and sequences of strains PDVY-2 and PDVY-3 were submitted to GenBank (accession numbers MT757134-5). They both showed 100% sequence identity to '*Candidatus* Phytoplasma asteris' and cluster with strains in 16SrI group. The virtual RFLP analysis classified the PDVY phytoplasma strains within the 16SrI phytoplasma subgroup B with a similarity coefficient of 1.00 to the representative strain of 16SrI-B subgroup (GenBank accession number M30790) (data not shown). The coat protein gene sequences of virus isolates showed 100% sequence identity among themselves, were submitted to GenBank under accession numbers MT741491-2 and showed 100% sequence identity with Bean common mosaic virus strain NL-7 from India (GenBank accession number JN692257). Phylogenetic analysis showed that these isolates cluster with other BCMV isolates (Figure 2).

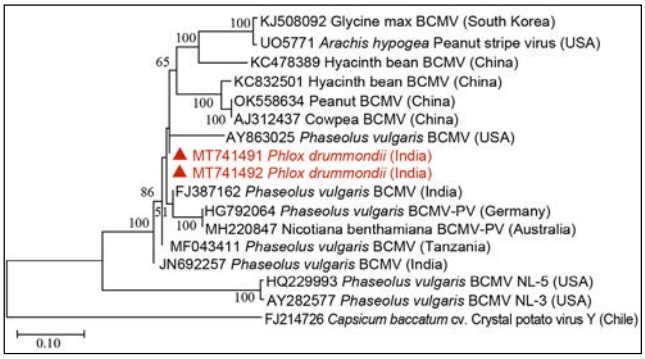


Figure 2. Phylogenetic tree based on coat protein sequences constructed by neighbor-joining method showing the relationship of the BCMV in *P. drummondii* with the other BCMV isolates. Potato virus Y strain from Chile was used as outgroup.

Discussion

In the present study, mixed infection of phytoplasmas (16SrI-B subgroup) and BCMV was reported associated with phlox virescence and yellowing in India. Previously, individual association of '*Ca.* P. asteris' and begomovirus infection in phlox were also reported from two different regions (Kumar *et al*., 2010; Rao *et al*., 2018). However, no report of mixed infection of phytoplasma and virus is yet reported in phlox anywhere in the world. Hence, this is the first detection of mixed infection of a potyvirus and a phytoplasma ('*Ca.* P. asteris') in virescence and leaf yellowing disease of phlox. Further, work on epidemiology and management of this phlox disease complex will help to prevent the further spread of both pathogens.

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Mixed infection

Detection and identification of '*Candidatus* Phytoplasma rubi' and viruses in *Rubus idaeus* using total RNA

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Abstract

In a survey for viruses in a production field of raspberry in central Bohemia, several plants had symptoms reminiscent of phytoplasma and virus. The samples were analysed using PCR and Sanger sequencing. '*Candidatus* Phytoplasma rubi' was identified in seven raspberry bushes (five plants with symptoms resembling phytoplasma infection, one plant with diffuse mosaic and one asymptomatic plant). A total of 31 plants tested positive for viruses (mainly raspberry leaf mottle virus and raspberry bushy dwarf virus).

Keywords: phytoplasma, PCR, Sanger sequencing, virus, raspberry

Introduction

Raspberry (*Rubus idaeus* L.) is cultivated for its excellent fruit mainly used fresh or in the food industry. Fruits and leaves are also used in pharmacology, herbal medicine and cosmetics. However, the plants are susceptible to virus and phytoplasma infections, causing significant economic losses in fruit yield and quality (Martin *et al*., 2013; Linck and Reineke, 2019).

During a survey for viral diseases of raspberry at a production field, numerous bushes showing symptoms typical of phytoplasma disease were observed. Further molecular tests confirmed presence of phytoplasmas and viruses in both symptomatic and asymptomatic shrubs.

Materials and Methods

The sampling of the raspberry production field in Central Bohemia took place on 18 May and 28 July 2021. In May, leaf samples were collected from 16 plants, only one of which showed symptoms of chlorosis and necrosis. In July, samples were collected from a total of 35 plants among which three plants were asymptomatic, five showed symptoms resembling those of phytoplasma infection, and the other 27 showed leaf mosaic and growth abnormalities. About 100 mg of leaf lamina tissue was used to extract total RNA using the Ribospin Plant kit (GeneAll, Korea), transcribed into cDNA, and tested by PCR for the presence of the most common raspberry viruses (Raspberry leaf blotch virus, RLBV; Black raspberry necrosis virus, BRNV; Raspberry bushy dwarf virus, RBDV; Raspberry leaf mottle virus, RLMV; Raspberry vein chlorosis virus, RVCV; Rubus yellow net virus, RYNV) as described by Jones *et al*. (2002), McGavin *et al*. (2011, 2012), and Tzanetakis *et al*. (2007). For phytoplasma detection cDNA was used as template in PCR with P1/P7 and P1A/P7A primer pairs. PCR products were diluted with sterile distilled water (1: 29) prior amplification by nested PCR using P1A/P7A and R16F2m/R1m, R16(V)F1/R1 primer pairs, respectively (Bertaccini *et al*., 2019). Amplified DNA fragments were sequenced in both directions. The sequences were compared with sequences available in the GenBank using the BLAST service.

Results

In May 2021, five symptomless plants tested positive for RLMV, two plants for RBDV and RLMV, one plant with chlorosis and necrosis for RBDV, and no virus was detected in the other eight plants. One plant (A939, Table 1) was positive in nested PCR using generic phytoplasma primers P1A/P7A and R16F2m/R1m. Sanger sequencing revealed the 99.7% nt identity (1008 nt identical/1011 nt) with both '*Candidatus* Phytoplasma rubi' strains GBFC_EY_02 and RS (GenBank accession numbers MH801133 and CP114006).

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Table 1. Identification of viruses and phytoplasmas in raspberry plants.

Sample	Symptoms[1]	Virus[2]	Phytoplasma
A939	none	none	´*Ca.* P. rubi´
A993	Ph, Y	RBDV	´*Ca.* P. rubi´
A999	DM	RYNV	´*Ca.* P. rubi´
B1	FM, Ph	none	´*Ca.* P. rubi´
B9	M, Ph	RBDV, RYNV	´*Ca.* P. rubi´
B10	FM, Ph	RYNV	´*Ca.* P. rubi´
B11	DM	RYNV	´*Ca.* P. rubi´

[1]DM: diffuse mosaic; FM: fruit malformation; M: mosaic; Ph: flower phyllody; Y: yellowing; [2]RBDV: raspberry bushy dwarf virus; RYNV: Rubus yellow net virus.



Figure 1. Symptoms of flower phyllody and enlargement of calyx petals in raspberry cv. Enrosadira infected with '*Ca.* P. rubi'.

In July, '*Ca.* P. rubi' was identified in all five plants with symptoms of flower virescence and phyllody, abnormal enlargement of calyx petals (Figure 1), deformation of fruits, premature leaf yellowing/reddening as well as in one plant with diffuse mosaic. The co-infections with viruses in these plants is reported in Table 1.

Additionally, the viruses were detected in one asymptomatic (BRNV) and 18 symptomatic plants (6 RLMV, 5 RBDV, 1 BRNV, 1 RYNV, 2 RBDV and RYNV, 2 BRNV and RLMV, 1 BRNV, RBDV and RYNV). None of the tested viruses was detected in 11 plants (two asymptomatic, nine symptomatic). In summary, 31 plants were positive for viruses (mainly RLMV and RBDV) and 7 were infected with '*Ca.* P. rubi' (5 co-infections with viruses) out of the 51 shrubs tested.

Discussion

For phytoplasmas detection, usually DNA extracted from symptomatic parts, leaf midribs or phloem tissues is used in many studies. In the presented work, however, it was successfully employed RNA from leaf lamina tissue. ´*Ca.* P. rubi´ was detected not only in plants with symptoms previously described as rubus stunt (Linck and Reineke, 2019), but also in plants with diffuse mosaic and in plants completely lacking symptoms (at the time of sample collection). In both cases, it may be a latent infection where the symptoms typical of phytoplasma infection have not yet developed. According to Linck and Reineke (2019), latent infection of '*Ca.* P. rubi' can last up to one year in raspberry plants. However, it is important to note that the sequences obtained from cDNA amplicons had lower quality than those obtained by DNA amplification (Fránová *et al.*, submitted). Sanger sequences obtained with forward primers [P1A, R16F2m, R16(V)F1] were mostly unreadable and '*Ca.* P. rubi' was identified from the reverse primer (P7A, R16R1m, R16R1) sequences. It was also possible to detect viruses (BRNV, RBDV, RLMV, RYNV) in both phytoplasma-infected and phytoplasma-free plants that can also impair plant health and raspberry production. The detection of viruses and phytoplasmas in symptomless plants in the present study confirms the need to use sensitive molecular methods to detect the presence of these pathogens and thus prevent their uncontrolled spread.

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Mixed infection

Multigene characterization of '*Candidatus* Phytoplasma palmae' strains infecting citrus species in Cuba

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Abstract

Surveys for the presence of '*Candidatus* Liberibacter asiaticus' in citrus showing "huanglongbing" symptoms in Cuba allowed the detection of citrus plants coinfected with '*Candidatus* Phytoplasma palmae'. The molecular characterization of these citrus infecting phytoplasmas confirmed that they are indistinguishable from phytoplasma strains previously detected in coconut during the lethal yellowing outbreak in the island in 2000. This finding suggests the possible loss of specificity in some '*Ca.* P. palmae' strains confirming that their presence could represent a further threat to the Cuban citriculture.

Keywords: phytoplasmas, lethal yellowing disease, "huanglongbing", molecular characterization, epidemiology

Introduction

Coconut lethal yellowing is the most important disease affecting this crop worldwide. Diverse ribosomal groups of phytoplasmas have been detected infecting several citrus species in plants showing "huanglongbing" (HLB)-like symptoms (Martinello-Sanches *et al.*, 2016). Coconut lethal yellowing in Cuba was firstly reported associated with 16SrIV-A phytoplasmas (Llauger *et al.*, 2002) and recently different ribosomal groups were detected in symptomatic coconut plants (Paredes-Tomás *et al.*, 2019). On the other hand, a mixed infection between '*Candidatus* Liberibacter asiaticus', and various phytoplasmas in citrus was also reported in Cuba. The mixed infection was mainly detected in the Matanzas province where the predominant phytoplasmas in citrus were identified as member of the 16SrIV group (Bertaccini *et al.*, 2019; Luis-Pantoja *et al.*, 2021). This study was undertaken to molecularly compare the strains of '*Candidatus* Phytoplasma palmae' (Bertaccini *et al.*, 2022) detected in citrus and in coconuts to clarify the possible epidemiological relationships between them.

Materials and Methods

Detection of '*Ca.* L. asiaticus' by nested PCR with primers rP1/fD1 and OI1/OI2c (Jagoueix *et al.*, 1996) was carried out on 58 symptomatic citrus samples collected from Jaguey Grande, Matanzas province (Table 1). DNA extraction was performed from 1 g of leaf midribs, using CTAB and/or phenol-chloroform methods. '*Ca.* P. palmae' positive controls from coconut infected plants from Cuba, Mexico and Jamaica were used (Luis Pantoja *et al.*, 2021). Nested PCR amplification was done on 16S rDNA gene with universal primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by 16SrIV group specific primers LY16Sf/LY16Sr (Harrison *et al.*, 2002). Moreover, the positive samples were amplified on the *groel* gene with primers GroELF1/R1 followed by GroELF2/R2 (Myrie *et al.*, 2011) in nested PCR. RFLP analyses were then performed with *Alu*I on LY16Sf/LY16Sr and *Hinf*I on GroELF2/R2 amplicons.

The PCR and RFLP products were analysed by electrophoresis on 1% agarose gel and in 6.7% polyacrylamide gel, respectively, stained with ethidium bromide and visualized under UV. Selected amplicons were directly sequenced using the same primers used for their amplification and compared with nucleotide sequences in NCBI database using BLAST (www.blast.ncbi.nlm.gov).

Results

All the citrus samples analysed resulted positive to '*Ca.* L. asiaticus' (Table 1); 19 out of 58 samples yielded PCR amplification with LY16Sf/LY16Sr primers and four of these samples were amplified on the *groEl* gene.

Table 1. Results of the surveys in citrus samples from Cuba.

Sampled species	'*Ca.* P. palmae'	'*Ca.* L. asiaticus'
Sweet orange	9/21	21/21
Grapefruit	6/19	19/19
Persian lime	0/9	9/9
Eureka lemon	4/9	9/9
Total	19/58	58/58

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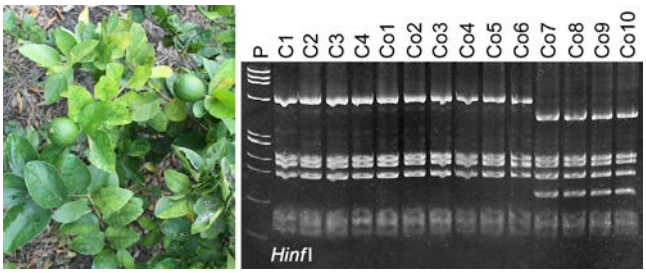


Figure 1. Symptoms of "huanglongbing" in citrus (left). RFLP profiles of LYgroELF2/R2 amplicons digested with *Hinf*I of samples C1-C4, citrus-Cuba; Co1 to Co3, coconuts-Cuba; Co4 to Co6, coconuts-Jamaica and Co7 to Co10, coconuts-Mexico. P: marker ÖX174 *Hae*III digested (right).

Two RFLP profiles were identified on the 16S ribosomal gene sequence in citrus trees using *Alu*I restriction enzyme: profile 1 was present in 11 citrus samples and profile 2 was identified in 8 citrus and in coconuts control samples from Cuba, Jamaica and Mexico (data now shown). The four positive samples on the *groEl* gene were obtained from phytoplasmas from citrus samples showing profile 1. Their *Hinf*I RFLP profiles resulted identical to those of '*Ca*. P. palmae' strains from old Cuban infected coconut plantations and from Jamaican coconut strains and were differentiable from those detected in the Mexican strains (Figure 1). The sequence from profile 1 (GenBank accession number OQ600016) resulted 99.92% identical to that of '*Ca*. P. palmae' (GenBank accession number U18747) confirming the phytoplasma identification.

Discussion

The copresence of '*Ca*. L. asiaticus' and '*Ca*. P. palmae' in the symptomatic citrus samples highlight a complex epidemiological situation in these Cuban citrus orchards. The RFLP characterization of the detected '*Ca*. P. palmae' strains based on the *groEl* gene sequence shows that they are identical to those detected in coconut with lethal yellowing 20 years ago in Cuba and more recently in Jamaica (Luis Pantoja *et al*., 2021). Considering that the Cuban coconut plantations were almost destroyed by the previous epidemics (Llauger *et al*., 2002) and coconut replanting is just starting, one hypothesis may be that the old phytoplasma strains were able to thrive in alternate host plants and were possibly transmitted from coconut to citrus trees by insect vectors (Paredes Tomas *et al*., 2022). The strain detected based on the 16Sr gene and having profile 2 yielded no amplification with primers targeting the *groEl* gene. Lethal yellowing is the most important disease affecting coconut production worldwide. In America it is associated with the presence of different 16S ribosomal subgroups in the '*Ca*. P. palmae' taxon. The identification of phytoplasma strains in citrus trees that can be differentiated based on the 16S ribosomal gene sequence and amplified with primers specific for '*Ca*. P. palmae' (Harrison *et al*., 2002) confirms that different phytoplasmas are present in citrus orchards infected with HLB in Cuba (Luis Pantoja *et al*., 2021). This finding suggests that the presence of '*Ca*. P. palmae' is a threat not only to palms but also to other crops grown within the same agricultural environment. In Cuba this situation may become a further threat to citriculture aggravating, and very likely increasing, the severity of HLB disease. Further work is necessary to verify the economic impact of this mixed bacterial infections in citrus in Cuba.

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Mixed infection

Duplex PCR assay for simultaneous detection of citrus witches' broom and "huanglongbing" pathogens

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Abstract

Witches' broom (WB) and "huanglongbing" (HLB) associated to phloem limited bacteria '*Candidatus* Phytoplasma aurantifolia' and '*Ca.* Liberibacter asiaticus', respectively are the most serious and destructive citrus diseases in the south of Iran. Although it is possible to detect each of these pathogens by PCR separately, this is expensive and time-consuming. In this study a duplex PCR detection system using P4/P7 and fp400/rp400 primer pair sets for simultaneous detection of both pathogens was developed. Two 400 and 500 bp amplicons were simultaneously amplified and no bands were detected from healthy plants. The consistent results of the developed duplex PCR were compared with simplex PCR for detection of each pathogen and confirmed with sequencing analysis. This rapid, sensitive, specific and robust duplex PCR assay should be useful for detection of WB and HLB especially for quarantine purposes, disease surveys, certification and plant improvement programs for obtaining pathogen-free plant materials..

Keywords: '*Candidatus* Phytoplasma aurantifolia', '*Candidatus* Liberibacter asiaticus', polymerase chain reaction

Introduction

Iran has been ranked between 8th and 10th in global citrus production in different years. Witches' broom (WB) and "huanglongbing" (HLB) diseases associated with '*Candidatus* Phytoplasma aurantifolia' and '*Ca.* Liberibacter asiaticus', respectively are the most destructive diseases of citrus trees in some of the southwest Asian countries including Iran. A number of citrus cultivars, such as Mexican lime, orange, grapefruit and mandarins have been reported as common hosts for both pathogens (Azadvar *et al.*, 2023). Recently, these two pathogens have also been reported in association with newly emerging citrus decline disease (CDD) in Iran (Alizadeh *et al.*, 2017; Passera *et al.*, 2018).

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Materials and Methods

Total DNA was extracted from leaf midrib tissues of WB, HLB and CDD symptomatic citrus trees by a CTAB method (Zhang *et al.*, 1998) and subjected to PCR tests using OI1/OI2c (Jagoueix *et al.*, 1994) and P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) primer pairs for '*Ca.* Liberibacter' and '*Ca.* Phytoplasma' detection, respectively. Baranwal *et al.* (2005) developed a duplex PCR technique for simultaneous detection of HLB bacterium and Citrus yellow mosaic virus in citrus trees. Therefore a literature search was conducted to find the appropriate primers for detection of '*Ca.* Liberibacter' and '*Ca.* P. aurantifolia' and the candidate primers for duplex PCR tests using BioEdit (Hall 1999), BLAST (http://www.ncbi.nlm.nih.gov/blast) and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) softwares based on the primer properties including Tm, % CG, annealing temperature, size of the replication fragment, primer dimer. The efficiency of the selected primer pairs including rp(II)F1/rp(II)R1, FU5/rU3, SecYF2/SecYR1, rpF1C/rp(I)R1A, P4/P7, P3/P7, R16F2n/R16R2, and OI1/OI2c, A2/J5, fOI2/r23S1, fP535/rP535, fP400/rP400 was evaluated for detecting both bacteria, respectively in single and duplex PCR tests. PCR reactions were optimized using different primers, changing the reaction components of the master mix and PCR conditions including the PCR buffer, polymerase enzyme, $MgCl_2$ concentration, PCR temperature profile. The PCR products were electrophoresed in agarose gel and photographed. The resulting amplicons were extracted from gel using a DNA gel extraction kit (Fermentas) and directly sequenced (Bioneer, South Korea)

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on both forward and reverse directions. The sequences were analyzed by BLAST search.

Results

Results showed that the duplex PCR assay using primers fP400/rP400 for detecting '*Ca.* L. asiaticus' (Ding *et al.*, 2008) and P4/P7 for detecting '*Ca.* P. aurantifolia' (Schneider *et al.*, 1995) was able to simultaneously detect HLB and WB associated pathogens in one reaction. In each PCR reaction, two bands with approximate sizes of 400 bp and 500 bp were simultaneously amplified in the samples infected by WB and HLB, while no bands were formed in the healthy samples of lime and grapefruit (Figure 1).

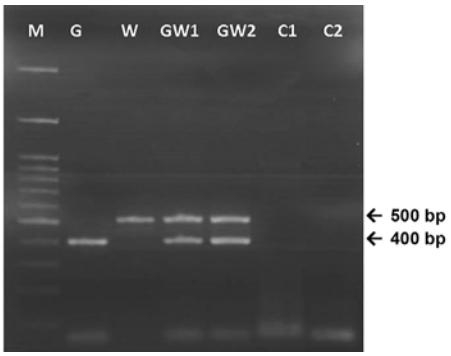


Figure 1. Agarose gel of duplex PCR products using fp400/rp400 and P4/P7 primer pairs for WB and HLB bacteria detection. G: '*Ca.* L. asiaticus' infected tree, W: '*Ca.* P. aurantifolia' infected tree, GW1 and GW2: '*Ca.* L. asiaticus' and '*Ca.* P. aurantifolia' mixed infected trees, C1: healthy acid lime tree, C2: healthy grapefruit tree, M: 100 bp DNA Ladder.

PCR master mix was containing 1X PCR buffer, 1 µl of each primer (10 pm), 3 µl $MgCl_2$ (25 mM), 3 µl dNTPs (10 mM), 1 µl *Taq* DNA Polymerase (5 U/µl), and 3 µl DNA (50 ng/µl) in 50 µl reaction mixture. PCRs were performed in a thermal cycler (Cleaver Sciences, GTX, South Korea) with 5 minutes at 94°C for denaturation, and the following 40 cycles with 40 seconds of denaturation at 94°C, 1 minute annealing at 52°C and 1.5 minutes primer extension at 72°C and final extension of 10 minutes at 72°C.

BLAST analyses of the sequences indicated that the 400 bp amplified with the primer pair fp400/rp400 was identical to the nucleotide sequence of the NusG and 50S ribosomal protein L11 (*rplK*) genes of the gxpsy strain of '*Ca.* L. asiaticus' (GenBank accession number CP004005). The nucleotide sequences of 500 nt DNA fragment amplified with the primer pair P4/P7 was identical with the nucleotide sequence of the tRNA-Ile and a part of the 23S ribosomal RNA genes of '*Ca.* P. aurantifolia' (GenBank accession number U15442) agent of witches' broom disease of lime.

Discussion

Witches' broom and "huanglongbing" are the most destructive citrus diseases in southern Iran, important for both national and international quarantine. Every year, millions of plant samples from citrus nurseries and quarantine stations must be checked for these diseases by CR, which is very time-consuming and costly. The developed duplex PCR technique significantly reduces the time consumed and costs for '*Ca.* L. asiaticus' and '*Ca.* P. aurantifolia' detection, while the accuracy and sensitivity is the same.

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Mixed infection

The biology and epidemiology of '*Candidatus* Phytoplasma asteris' and '*Candidatus* Liberibacter solanacearum' and their contribution to risk management in carrots

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Abstract

Between 2020 and 2021, a total of 18 carrot fields were surveyed in east and west Flanders to monitor '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter solanacearum' presence. While '*Ca.* P. asteris' was detected in 3 out of 18 sampled carrot fields, '*Ca.* L. solanacearum' was found in none. Vector monitoring on '*Ca.* P. asteris' positive sites resulted in the identification of 502 insects, mostly Auchenorrhyncha belonging to the genus *Empoasca*, *Macrosteles*, *Eupteryx*, *Fieberiella*, *Euscelis*, *Zyginidia*, *Javesella* and *Aphrophora*, as well as insects belonging to the *Psyllidae* family. '*Ca.* P. asteris' presence was demonstrated in specimen from the five first mentioned genera. Subsequently, transmission assays were set up for *Empoasca pteridis*, *Eupteryx aurata*, *Macrosteles sexnotatus*, and *Euscelis incisus*. Setting up a *M. sexnotatus* culture failed, and for the other three, '*Ca.* P. asteris' acquisition was only demonstrated for *E. pteridis* and *E. incisus*, however, without demonstrating successful transmission to healthy carrot plants.

Keywords: aster yellows phytoplasma, insect vectors, leaf- and planthoppers, psyllids

Introduction

In Belgium, a large survey in 2015-2016 demonstrated a major infection of aster yellows phytoplasma ('*Candidatus* Phytoplasma asteris'; AY) in carrot. The phloem inhabiting bacterium revealed to be widely spread and was identified in the vast majority of carrots showing yellowing and reddening symptoms. More recently, '*Ca.* Liberibacter solanacearum' haplotype D was detected for the first time in two Belgian commercial carrot fields (De Jonghe *et al.*, 2019). Phytoplasma diseases in carrot are known to be transmitted by a number of plant-sucking plant- and leafhoppers (Auchenorrhyncha), whereas '*Ca.* L. solanacearum' transmission is associated with psyllid presence. A subsequent survey attempted to identify potential insect vectors that play a role in the phytoplasma spread in carrot fields since yet many gaps still remain. In addition, the unexpected presence of '*Ca.* L. solanacearum' also requires more insight in the presence and role of psyllids in the carrot fields. The objective of this study was to further investigate the role of different insects (plant-, leafhopper, psyllid and other species) in the spread of phytoplasmas and '*Ca.* L. solanacearum' in commercial carrot fields and the surrounding (weeds and trees) borders, by means of field surveys and transmission assays.

Materials and Methods

Between 2020 and 2021, a total of 18 carrot fields located in east and west Flanders (Belgium) were surveyed for phytoplasma and '*Ca.* L. solanacearum' presence. Depending on the symptom severity, 3 to 10 carrot plants were sampled and tested at every location. On confirmed positive sites an extensive vector survey was performed by means of sweep netting, suction trapping, tree beating and the use of sticky plates in the carrot field as well as the surrounding borders. Trapped insects were identified morphometrically and molecularly. While live trapped insects from the sweep netting and suction trap were used to establish cultures and transmission assays, those from sticky plates were used to test '*Ca.* L. solanacearum' and/or AY presence. Transmission experiments were set up with *Empoasca pteridis* and *Eupteryx aurata* according to Galetto *et al.* (2011) and with *Euscelis incisus* following the method of Jakovljevic *et al.* (2015).

Results

Laboratory tests confirmed the presence of '*Ca.* P. solanacearum' in symptomatic carrot plants sampled at 3 locations. Insect monitoring was only carried out at 2 AY positive sites because of early harvesting. During these two

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years survey a total of 502 insects were identified among which a number of psyllids (*Psyllidae* family) and specimens of the genera *Eupteryx (aurata, atropunctata* and *calcarata)*, *Empoasca (decipiens, pteridis), Zyginidia, Macrosteles (sexnotatus), Aphrophora (salicina), Euscelis (incisus), Fieberiella (florii)* and *Javesella (pelucida)*. Part of the collected insects were tested for phytoplasma presence. Out of those, 2/15 *Empoasca*, 3/33 *Macrosteles*, 2/89 *Eupteryx* and 1/3 *Fieberiella* specimens tested positive in nested PCR (EPPO, 2018) and Sanger sequencing (Table 1). Due to the large number of *Eupteryx* individuals trapped, 29 pools of three specimens and two individual specimens were tested.

Table 1. Total number of trapped and AY positive insects caught on AY positive sites.

Genus/Family	Number of trapped insects	Number of AY positive insects
Empoasca	115	2/15
Macrosteles	63	3/33
Eupteryx	147	2-4/89*
Fieberiella	3	1/3
Euscelis	6	0/1
Zyginidia	98	0/8
Javesella	1	0/1
Aphrophora	7	0/7
Psyllidae	62	0/12
Unknown	9	/

*1 individual and 1 pooled sample

Trapped living insects were used to set up colonies and subsequent transmission assays. Only colonies of *E. pteridis*, *E. aurata* and *E. incisus* survived. Colonies of all other species collapsed probably due to unfavourable conditions. Despite the availability of well written procedures, transmission assay optimization was still required with great insect losses as a consequence. Eventually, 465, 100 and 3 individuals of *E. pteridis*, *E. aurata* and *E. incisus*, respectively, were used in transmission assays with AY. At the end of the transmission experiments only 105 *E. pteridis* individuals and 1 individual each for *E. aurata* and *E. incisus* survived. Insects were collected and molecularly tested for AY presence. A total of 12/105 (11.43%) *E. pteridis*, 0/1 *E. aurata* and 1/1 (100%) *E. incisus* tested positive in nested PCR and Sanger sequencing. In contrast, none of the test plants expressed symptoms after 4 weeks or tested positive for AY presence in molecular analyses.

Discussion

The results of a two-year field survey showed the presence of '*Ca.* P. asteris' in carrot fields in East and West Flanders. While '*Ca.* P. asteris' was detected in 3 out of 18 sampled carrot fields, '*Ca.* L. solanacearum' was not found at all. The number of infections was much lower in comparison with the 2015-2016 survey (VECTRACROP data). This lower incidence might be a consequence of climatic conditions (heavy rains during the summer) in which the majority of field samplings took place, as poor weather conditions negatively impact vectors abundance, which is essential for AY and '*Ca.* L. solanacearum' spread. The results of the field survey might therefore give a distorted picture on the real epidemiological relevance of the two pathogens in Flanders.

The vector survey at AY positive sites further confirmed previous findings: Auchenorrhyncha play a key role in the spread of phytoplasmas. The vector survey identified species from the *Empoasca*, *Macrosteles*, *Eupteryx*, *Fieberiella* and *Euscelis* genuses as AY carriers. *Macrosteles* is a confirmed vector, and *Empoasca* and *Eupteryx* in particular are also of high concern because of their suspected involvement and high abundance in both carrot fields and the surrounding borders. These findings can however not be taken as proof for their vector capacity. For that reason transmission assays were set up with *E. pteridis* and *E. aurata*, and also with *E. incisus*. Even though bacteria acquisition was demonstrated for both *E. pteridis* (11.43%) and *E. incisus* (100%, 1 individual), none of the test plants expressed characteristic symptoms or tested positive in nested PCR. Low inoculation densities might be an important cause for these results, with 5 and 1 as maximum number of AY positive insects per test plant for *E. pteridis* and *E. incisus* respectively. Unfortunately, this number was hard to control as the infection status of inoculated insects could only be assessed at the end of the experiment.

Previous studies identified species from the *Empoasca* genus as inefficient transmitters. In the study of Galetto *et al.* (2011) only an estimated 2.5% of *Empoasca decipiens* transmitted chrysanthemum yellows phytoplasma to *Chrysanthemum carinatum* cv Tricolor. Inefficient vectors such as *Empoasca* however often appear in high populations. Their epidemiological relevance for phytoplasma transmission should therefore not be underestimated. There are clear indications that species belonging to the *Empoasca*, *Eupteryx*, *Macrosteles*, *Fieberiella* and *Euscelis* genuses are potential AY vectors. Generalisation based on vector field studies for transmission capability is however tenuous. Further studies need to be performed to elucidate the obtained results.

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Mixed infection

Phytoplasma, proteobacterium and fungus in single and mixed infections of sugar beet in central Europe

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Abstract

The "basses richesses" syndrome (SBR) and rubbery taproot disease (RTD) associated with *'Candidatus* Arsenophonus phytopathogenicus' and *'Ca.* Phytoplasma solani', respectively are hampering the sugar beet production in Europe. A series of experiments shed light on the presence of these bacteria in sugar beet in central Europe - Pannonian plain (Serbia and Slovakia), with epidemic occurrences of RTD, and Germany. Only *'Ca.* P. solani' was found in sugar beet in the Pannonian plain, while both pathogens were present in Germany. *Reptalus quinquecostatus* was identified as the insect vector responsible for the of the epidemic strain of *'Ca.* P. solani' and thus for the epidemic occurrence of RTD. The RTD affected sugar beet is susceptible to charcoal root rot caused by *Macrophomina phaseolina* which exacerbates the losses associated to the phytoplasma presence. These results suggest that more attention should be given to phytoplasma infection in sugar beet in Europe.

Keywords: "stolbur", SBR, RTD, *'Candidatus* Arsenophonus phytopathogenicus', *'Candidatus* Phytoplasma solani'

Introduction

Sugar beet production in Europe is hindered by the syndrome of "basses richesses" (SBR) and the rubbery taproot disease (RTD) associated with two bacterial pathogens, *'Candidatus* Arsenophonus phytopathogenicus' and *'Ca.* Phytoplasma solani' ("stolbur" phytoplasma), respectively (Sémétey *et al.*, 2007; Curcic *et al.*, 2021a). They share common symptoms such as yellowing and proliferation of leaves. However, there are some specific symptoms, discoloration of vascular tissue and deformation of young leaves in SBR and necrosis of older leaves, wilted and rubbery taproot and plant decline in RTD. '*Ca.* A. phytopathogenicus' (γ-proteobacterium) is an intracellular symbiont, widely distributed among arthropods, while phytoplasmas are associated with a multitude of plant diseases. To further elucidate the prevalence of association of infections of sugar beet with these two phloem-limited bacteria in Central Europe, a series of surveys and experiments were conducted over a four-year period (2019-2022).

Materials and Methods

Phytoplasmas and γ-proteobacteria presence was assessed in sugar beet in the Pannonian plain (Serbia and Slovakia) where epidemic occurrence of RTD was reported and Germany where both pathogens are known to be present. The investigation about RTD was conducted in 2019 in four localities with long history of this disease presence in Northern Serbia. The survey also included several localities in the Pannonian plain, particularly in Slovakia. The differentiation between epidemic and non-epidemic strains of the '*Ca.* P. solani' was obtained on variable epidemiologically informative non-ribosomal genes.

Epidemiological research conducted in 2020 and 2021 included potential reservoir plants and insect vectors. Potential reservoir plants (weeds) were collected in and around RTD affected field. Transmission experiments included single plant and semi-field trials. Because RTD-affected sugar beets are susceptible to rot, the possible relationship with fungal root rot was also evaluated.

Results

In the Pannonian Plain, only phytoplasmas were detected in sugar beet, while '*Ca.* A. phytopathogenicus' was not found in any of the five countries studied.

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The results show an epidemic occurrence of RTD in Serbia (Figure 1) and Slovakia, while the disease was sporadic in the other studied countries of the Pannonian plain. A strain, distinguished by non-ribosomal genes, was identified as responsible for the epidemic occurrence in the two countries which are located in opposite parts of the Pannonian plain. Transmission experiments confirmed the role of *Reptalus quinquecostatus sensu* Holzinger *et al.* (2003) and *Hyalesthes obsoletus* in the transmission of *'Ca.* P. solani' to sugar beet and inducing RTD. *R. quinquecostatus* transmitted only the strain responsible for RTD epidemics (Figure 2), whereas *H. obsoletus* transmitted strains which are present with sporadic occurrence. In Germany, both phytoplasma and '*Ca.* A. phytopathogenicus' were detected, with phytoplasma dominating in the eastern part of the country.

RTD-affected sugar beets are susceptible to rot, and this is usually due to the charcoal root rot caused by *Macrophomina phaseolina* that was found to occur exclusively in *'Ca.* P. solani' infected sugar beets, exacerbating the losses associated with the presence of the "stolbur" phytoplasma alone.



Figure 1. Field of sugar beet affected with RTD (*'Ca.* P. solani') in Serbia.



Figure 2. Transmission of *'Ca.* P. solani' to sugar beet with *R. quinquecostatus*. Healthy sugar beet (left) and infected (right).

Discussion

While phytoplasmas play a minor role in France, they dominate over '*Ca.* A. phytopathogenicus' in part of the studied Central Europe (Pannonian plain and east Germany) (Sémétey *et al.*, 2007a; Curcic *et al.*, 2021b). Two insect vectors were found to be involved in the transmission of *'Ca.* P. solani' to sugar beet in the case of RTD. The strain responsible for the epidemics and the transmission experiments pointed to the cixiid *R. quinquecostatus* as the vector responsible for the transmission of the epidemic strain of *'Ca.* P. solani' and thus for the epidemic occurrence of RTD (Kosovac *et al.*, 2023). While charcoal root rot has been regarded as the most significant root pathogen of sugar beet in Serbia (Budakov *et al.*, 2015), elsewhere it is considered a minor pathogen of weakened plants. Based on these results, *M. phaseolina* appears to exacerbate the sugar beet yield loss associated with the presence of *'Ca.* P. solani, which may explain why there are different reports on the significance of *M. phaseolina* as a fungal pathogen of sugar beet in Serbia compared to other regions where it is considered a less serious threat. The results presented here suggest that more attention should be focused on phytoplasma infection in sugar beet in Europe.

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Mixed infection

'*Candidatus* Phytoplasma solani' and '*Candidatus* Arsenophonus phytopathogenicus' in sugar beet in Germany and Switzerland

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Abstract

'*Candidatus* Phytoplasma solani' ("stolbur" phytoplasma), associated with rubbery tap root disease (RTD), and '*Ca.* Arsenophonus phytopathogenicus', associated with "syndrome basses richesses" (SBR), were surveyed in sugar beet in eastern and southern Germany and Switzerland. '*Ca.* P. solani' was more detected in eastern than in southern Germany. None of the samples from Switzerland tested positive. '*Ca.* A. phytopathogenicus' was more frequently detected in southern Germany and Switzerland than eastern Germany. The results confirmed the observation that RTD is prevalent in the eastern part of Germany and SBR in the southern part of the country.

Keywords: rubbery tap root disease, syndrome basses richesses, bacteria, distribution

Introduction

The "stolbur" phytoplasma, '*Candidatus* Phytoplasma solani', and the γ-proteobacterium '*Ca.* Arsenophonus phytopathogenicus' can cause severe economic losses and are of increasing importance for sugar beet (Sémétey *et al.*, 2007). '*Ca.* P. solani' has been associated with rubbery tap root disease (RTD) in sugar beet (Curcic *et al.*, 2021a). RTD has been first reported in the 1960s and has in the past years increased in importance, having reached epidemic proportions in Serbia (Curcic *et al.*, 2021a). RTD has been recorded further in Austria, Croatia, Hungary and Germany (Curcic *et al.*, 2021b). The syndrome basses richesses (SBR; low sugar content disease) has been associated with '*Ca.* A. phytopathogenicus' presence.

The disease was first observed in sugar beet in eastern France in 1991 and has since spread to Germany, Switzerland and Hungary (Gatineau *et al.*, 2001; Bressan *et al.*, 2009; Schröder *et al.*, 2012; Schaerer *et al.*, 2019). Symptoms of both diseases include yellowing of leaves and abnormal leaf re-growth (Figure 1). RTD is further characterized by a rubbery tap root without vascular bundle discoloration. SBR affected plants have a firm tap root with brownish vascular discolouration (Figure 2). The planthopper species *Hyalesthes obsoletus* and *Pentastiridius leporinus* (Hemiptera, Cixiidae) have been reported as main vectors of '*Ca.* P. solani' and '*Ca.* A. phytopathogenicus', respectively (Jovic *et al.*, 2019; Kosovac *et al.*, 2023).



Figure 1. Common RTD and SBR symptoms. Yellowing of whole leaves and abnormal leaf re-growth in sugar beet.



Figure 2. Rubbery tap root (RTD; left) and brownish vascular discolouration (SBR; right) symptoms in sugar beet.

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Table 1. Number of sampling and positive sites for '*Ca.* P. solani' and the γ-proteobacterium '*Ca.* A. phytopathogenicus' in Germany and Switzerland.

Region/Country	Number of sampling sites	'*Ca.* P. solani' Number of positive sites (%)	'*Ca.* A. phytopathogenicus' Number of positive sites (%)
Eastern Germany	63	22 (35)	16 (25)
Southern Germany	33	1 (3)	21 (64)
Switzerland	43	0 (0)	20 (47)

Materials and Methods

A survey was carried out in 2021 to determine the presence and distribution '*Ca.* P. solani' and '*Ca.* A. phytopathogenicus' in eastern and southern Germany and Switzerland in symptomatic sugar beet collected from more than 130 sites.

Results

The detection of '*Ca.* P. solani' presence decreased from 35% in eastern Germany to 3% in southern Germany. None of the samples from Switzerland tested positive (Table 1). '*Ca.* A. phytopathogenicus' was detected at all sites. The highest percentage of positive plants was recorded in southern Germany (64%) followed by Switzerland (47%). eastern Germany had the lowest number of plants infected with '*Ca.* A. phytopathogenicus' (25%). Mixed infection where higher in eastern Germany (16%) compared with southern Germany (3%).

Discussion

The survey confirmed the dominance of '*Ca.* P. solani' in eastern Germany. Conversely, '*Ca.* A. phytopathogenicus' was more abundant in southern Germany and Switzerland than eastern Germany. A more detailed survey of insect vector presence and abundance is needed to get a better understanding of their importance as vectors associated with RTD and SBR and their incidence in the respective regions.

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Worldwide

First evidence of 16SrII-D phytoplasmas association with *Luffa cylindrica* phyllody from India

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Abstract

The phytoplasma detected in smooth loofah gourd species (*Luffa cylindrica*) showing phyllody in Ayodhya, Uttar Pradesh, India was characterized by molecular analyses. Phylogeny and restriction fragment length polymorphism analysis of the 16S rRNA gene sequences of nested PCR products identified this phytoplasma as member of the 16SrII-D subgroup, '*Candidatus* Phytoplasma australasia'. This is the first molecular characterization of a 16SrII-D phytoplasma infecting smoot loofah gourd in world.

Keywords: cucumber phyllody, "turai", phytoplasma, '*Candidatus* Phytoplasma australasia'

Introduction

Phytoplasmas are cell-wall less bacteria with very small genome size and are amongst the smallest self-replicating living organisms (Rao, 2021). They infect numerous vegetable crops, including cucurbitaceous, causing significant losses and are transmitted by sap-feeding insects (Kumari *et al*., 2019; Rao, 2021). Smooth loofah gourd (*Luffa cylindrica*, common name "turai") is a commonly grown and consumed cucumber vegetable in different parts of India. In 2020, smooth loofah gourd plants showing phyllody symptoms were observed in agricultural fields of ND University of Agriculture & Technology campus, Ayodhya, Uttar Pradesh, India. The present work reports molecular characterization of phytoplasmas associated with *L. cylindrica* phyllody.

Materials and Methods

Leaves of loofah gourd were collected from symptomatic and asymptomatic plants from fields located at ND University of Agriculture & Technology, Ayodhya in 2020 and used as sources for molecular studies of the detected phytoplasmas. Leaf midribs were subjected to DNA extraction by Plant DNA extraction kit (Qiagen, Germany). Healthy loofah gourd plants grown from seeds were employed as negative controls. For PCR assay, 100 ng of DNA extract was used for amplification of the 16S rRNA gene and spacer region with universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995). The amplification products were amplified in nested PCR assay with the primer pair R16F2n/R2 (Gundersen and Lee, 1996). The amplification was carried out using a thermal cycler (Eppendorf, Germany) and the cycling protocol described by Mitra *et al*. (2022). The 16S rDNA virtual RFLP patterns of smooth loofah gourd (SLG) phytoplasma, Ayodhya strains were analyzed and compared to that of other phytoplasmas using *i*PhyClassifier (Zhao *et al*., 2009). Cloning and sequencing the 1.25 kb R16F2n/R2 primed PCR products of the SLG phytoplasma were done and used for further analyses. The sequences were compared with 16S rDNA sequences of phytoplasmas in GenBank using Blast from the NCBI (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences of SLG phytoplasma strains were aligned using Clustal W. A phylogenetic tree was constructed with the neighbor-joining method of MEGA 11.0 software (Tamura *et al*., 2021). *Acholeplasma laidlawii* was used as outgroup to root the tree.

Results

Severe symptoms of smooth loofah gourd phyllody including reduced size of leaves and shortening of internodes were observed in Ayodhya with an infection rate of 20% (Figure 1).

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Figure 1. Phyllody and shoot proliferation in loofah (*Luffa cylindrica*) at Ayodhya, Uttar Pradesh.

Amplicons of about 1.8 kb were obtained from symptomatic *L. cylindrica* plants (5 out of 5 samples) with P1/P7 primer pair (data not shown). R16F2n/R2 PCR products were obtained from all the five samples and no amplification was observed in asymptomatic loofah gourd plants (negative control) (data not shown). The R16F2n/R2 nested amplified products tree generated by the analysis of nearly full length 16S rDNA sequences of different 16Sr phytoplasma groups, revealed that the SLG phytoplasmas clustered with phytoplasmas belonging group 16SrII supported by high bootstrap values (Figure 2). Virtual RFLP analysis results derived from *in silico* digestions of the R16F2n/R2 sequence indicated that the two strains produced virtual RFLP profiles identical to each other and to the representative of 16SrII-D subgroup. This confirms that SLG phyllody phytoplasma is enclosed in the 16SrII-D subgroup. Identity percentage of 100% with '*Candidatus* Phytoplasma australasia' (GenBank accession number Y10097) provided the identification of the detected strains.

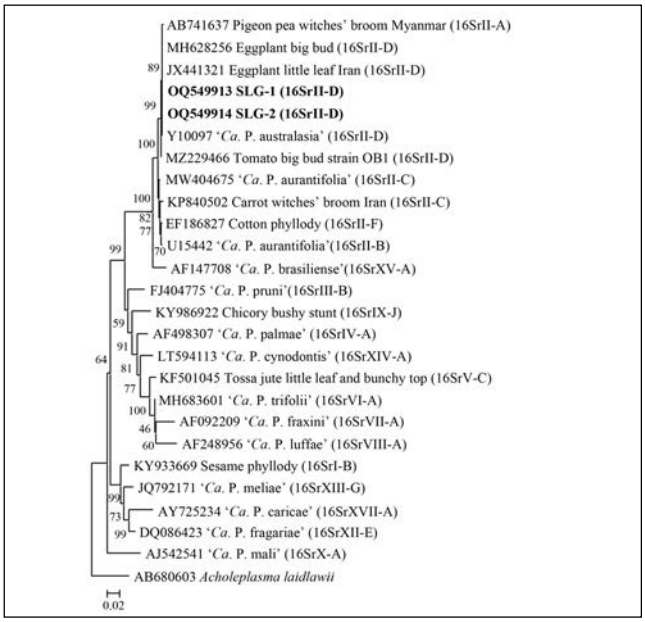


Figure 2. Phylogenetic tree constructed using the neighbour-joining method showing the phylogenetic relationship among the smooth loofah gourd phytoplasma, Ayodhya strains (SLG-1, -2) (in bold) with reference phytoplasma strains.

Discussion

Phytoplasma diseases are an emerging threat to vegetables including cucurbitaceous crops in India and abroad and induce serious yield losses in case of early infection (Kumari *et al*., 2019; Rao, 2021). Phytoplasmas affecting *L. cylindrica* were reported in Taiwan, Brazil and India (Lee *et al*., 1993; Montano *et al*., 2006; Kumar *et al*., 2010). The presence of phytoplasma strains in different 16SrII subgroups in different plant species and of their efficient insect vectors in India suggest a high level of genetic diversity and host susceptibility among the 16SrII phytoplasmas infecting distinct plant host species in India. This is the first molecular detection in SLG of phytoplasmas of the 16SrII-D subgroup in India. The results of present work suggest that loofah gourd fields may be the sources of 16SrII phytoplasmas to other important crops in Uttar Pradesh province with efficient vectors, which needs further studies.

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Worldwide

First report of a phytoplasma associated with little leaf and witches' broom of *Tecoma stans* in India

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Abstract

Little leaf and witches' broom symptoms were observed on *Tecoma stans* plants at the AND University campus, Ayodhya, India, during 2021-2022. A 1,250 bp product was amplified from DNA extracted from symptomatic *T. stans* plant samples subjected to nested PCR assays with P1/P7 and R16F2n/ R16R2 primers. Sequence analysis of the 16Sr RNA gene product through phylogeny and virtual RFLP analyses of the *T. stans* phytoplasma strain allowed its identification as '*Candidatus* Phytoplasma australasia' (16SrII-D). This is a first record of phytoplasma presence in *T. stans*.

Keywords: trumpet flower, yellow bells, 16S rRNA gene, 16SrII-D phytoplasma subgroup

Introduction

In India, numerous plant species are grown as ornamentals for their landscape value. *Tecoma stans* (L.) Juss. ex Kunth is an ornamental shrub, belonging to the family Bignoniaceae and commonly known as yellow bells, yellow elder, trumpet flower. Leaves, bark and roots of *T. stans* also contain biologically active chemicals, used in traditional medicine. It is usually planted as a decorative landscape plant in warmer climates of Australia, south and south-east Asia, South Africa, eastern Africa and some oceanic islands because of its beautiful yellow flowers and pinnate foliage (Srivastava *et al*., 2021).

Materials and Methods

In December 2021, phytoplasma suspected symptoms of little leaf and witches' broom (Figure 1), were observed in *T. stans* at the AND University campus of Acharya Narendra Deva University of Agriculture and Technology, Ayodhya in Uttar Pradesh, India. Leaves of two asymptomatic and two symptomatic plants were collected. DNA was extracted from leaf midrib by using the Plant DNA Kit (Qiagen) following the manufacturer's instruction and subjected to a nested PCR assay using primers P1/P7 and R16F2n/R16R2 (Deng and Hiruki 1991; Schneider *et al*., 1995; Gundersen and Lee, 1996). The amplified products were diluted 1: 30 with nuclease free water and 2 µl were used as template in nested PCR assays. DNA extracted from a sesame phyllody phytoplasma maintained in *Catharanthus roseus* in a greenhouse (16SrII, GenBank accession number KC920747) was used as positive control. PCR reactions were carried out in a thermal cycler (Mastercycler, Eppendorf, Germany) under a reported cycling protocol (Mitra *et al*., 2022).

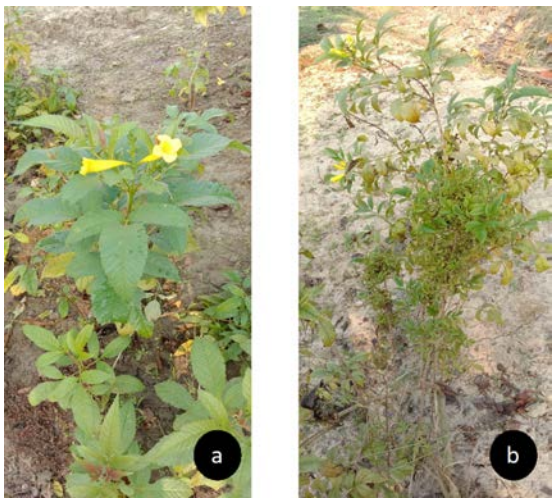


Figure 1. Asymptomatic *Tecoma stans* plant (a); symptoms of little leaf and witches' broom in *T. stans* at Ayodhya, Uttar Pradesh, India (b).

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The PCR products were purified using WizardR SV Gel, were ligated into pGEM®T vector (Promega, Madison, USA) and cloned in competent cells of *Escherichia coli* (DH5-α). The cloned products were outsourced for sequencing using M13Fwd/M13Rev primers in both directions at AgriGenome, Kakkanad, Kerala, India. The sequences obtained were compared with those of phytoplasmas strains in GenBank using the BLASTn (https://www.ncbi.nlm.nih.gov/) and the sequences of the *T. stans* little leaf and witches' broom strains (TLL&WB-1 and -2) were submitted to GenBank. Sequence assembly and phylogeny were carried out by neighbour joining method using the software MEGA 11 (Tamura *et al*., 2021) with 1,000 bootstrap replications. Sequences of the *Acholeplasma laidlawii* (GenBank accession number NR074448) was used as outgroup to root the trees. 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 (Zhao *et al*., 2009).

Results

The expected PCR-amplified products of the phytoplasma 16S rRNA gene (about 1.25 kb) were obtained from the two symptomatic plant samples but not from the asymptomatic plants. BLAST analysis revealed that the partial 16S rRNA gene sequences (GenBank accession numbers OQ119726 and OQ119727) of the TLL&WB phytoplasma strains showed maximum sequence identities (99.84 to 100%) with that of '*Candidatus* Phytoplasma australasia' strains (GenBank accession numbers MN733142, KY082674, JF340076 and MK181635). Phylogenetic analysis and an *i*PhyClassifier virtual RFLP analysis based on the 16S rRNA gene sequences, demonstrated that the TLL&WB phytoplasma strains were members of the 16SrII-D subgroup (Figure 2).

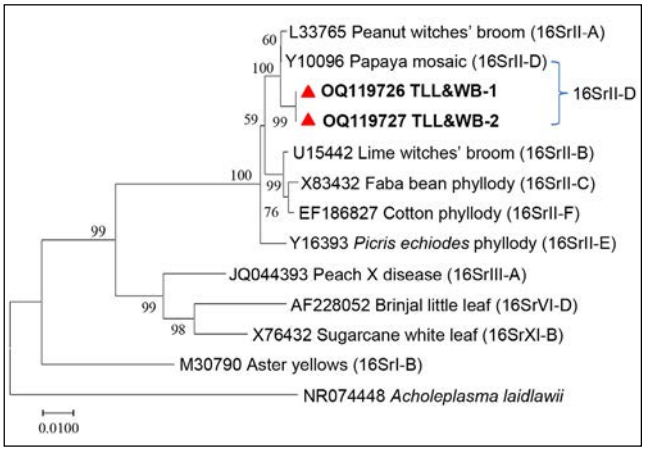


Figure 2. Phylogenetic tree based on the nucleotide sequences of the 16S rRNA gene sequence of phytoplasmas, illustrating the relationships between *T. stans* little leaf and witches' broom phytoplasmas (red triangles) and other phytoplasma strains.

Discussion

The economic importance of ornamental plant species has been progressing significantly all over the world with an international demand expanding continuously. The phytoplasmas are an important group of plant pathogens which drastically damage growth and marketing potentials and values of ornamental plants and affect their commercial value worldwide (Bellardi *et al*., 2018). So far, eleven ribosomal groups (16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXII, 16SrXIII, and 16SrXIV) of phytoplasmas were identified in 45 ornamental species in India (Rao, 2021). The present findings expand the current knowledge regarding the identification of a new host of '*Ca*. P. australasia' (16SrII-D subgroup) in *T. stans*.

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Worldwide

Identification of 16SrII group phytoplasma strain associated with witches' broom of spinach and hemp in India

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Abstract

Witches' broom and little leaf symptoms were observed in spinach and hemp plants in Ayodhya, Uttar Pradesh, India. Phylogenetic study and *in silico* RFLP analysis of 16S rDNA sequences confirmed the presence of '*Candidatus* Phytoplasma australasia' (16SrII-D) in both species. Its report in spinach is new while its finding in hemp suggests its role as potential natural reservoir of the phytoplasma.

Keywords: *Spinacia oleracea*, *Cannabis sativa*, 16SrII group, alternate host, PCR, RFLP, weed

Introduction

Phytoplasmas are a group of phloem-limited cell wall-less bacteria enclosed in class *Mollicutes* that infect diverse range of plant species throughout the world (Bertaccini *et al*., 2014). Spinach (*Spinacia oleracea* L.) in an annual leafy vegetable that belongs to the family Chenopodiaceae and is native to south-west Asia widely distributed and cultivated throughout the world. During December 2022, spinach plant expressing symptoms of witches' broom, indicative of phytoplasma infection was observed at Ayodhya, Uttar Pradesh, India. Hemp (*Cannabis sativa* L.) plants grown in nearby fields also show phytoplasma suspected symptoms. In the present study, molecular identification of phytoplasmas associated with the severe symptoms of witches' broom in spinach and hemp growing nearby spinach field were attempted.

Materials and Methods

Spinach and hemp plants exhibiting severe witches' broom symptoms were sampled from Ayodhya, Uttar Pradesh, India. Leaf midribs from three symptomatic samples each of spinach and hemp along with two non-symptomatic samples were used for DNA extraction (Ahrens and Seemüller, 1992). PCR and nested PCR assays with the universal primer pair for phytoplasma P1/P7 and R16F2n/R16R2, respectively, were used as described (Deng and Hiruki 1991; Schneider *et al*., 1995; Gundersen and Lee, 1996). Electrophoresis of the nested PCR products was performed in a 1% (w/v) agarose gel, stained with ethidium bromide and observed under UV transilluminator. Three nested PCR amplicons were purified (Wizard SV gel kit, Promega) and sequenced (Eurofins, India). The sequences were assembled using BioEdit software (Hall, 1999) and aligned using CLUSTAL W. Aligned and edited sequences were deposited in NCBI GenBank. The aligned sequences and selected phytoplasma strain sequences retrieved from GenBank were utilised for phylogenetic tree construction via the Neighbour-Joining method with 1,000 replicates for each bootstrap using MEGA 11 software (Tamura *et al*., 2021). *Acholeplasma laidlawii* served as the out-group for rooting the tree. Aligned sequences were used for *in silico* RFLP analysis and 16Sr subgroup identity using the *i*PhyClassifier tool (Zhao *et al*., 2009).

Results

During a survey in ND University of Agriculture and Technology campus at Ayodhya, witches' broom and little leaf symptoms were observed on spinach and hemp plants growing nearby spinach fields (Figure 1). P1/P7 primers followed by R16F2n/R16R2 primers during nested PCR yielded amplicons of 1.25 kb in all the symptomatic samples (data not shown). For identification of the phytoplasma strain, 1.25 kb amplicons were sequenced and submitted to GenBank with the accession numbers OQ518444 for spinach witches' broom (SpWB) and OQ518445 for *Cannabis* witches' broom (CaWB). In BLASTn search comparison, both the SpWB and CaWB strains showed 99.5% sequence identity

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with the 16S rRNA gene sequence of '*Candidatus* Phytoplasma australasia'. Phylogenetic analysis further confirmed the BLASTn results (Figure 2). Both SpWB and CaWB strains showed identical restriction profiles to the reference phytoplasma strain of 16SrII-D subgroup.



Figure 1. Infected plants showing witches' broom symptoms: spinach witches' broom (a) and *Cannabis* witches' broom (b).

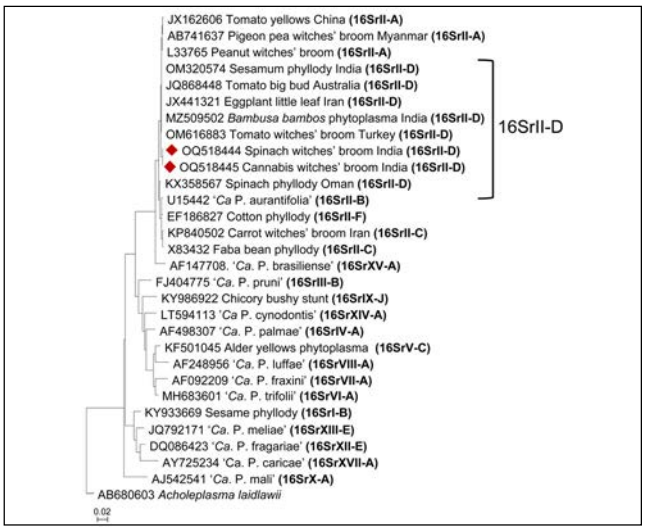


Figure 2. Phylogenetic tree showing the relationship among SpWB and CaWB phytoplasmas and selected phytoplasma strains. *Acholeplasma laidlawii* was used as outgroup. The tree was constructed using neighbour-joining method and bootstrap values from 1,000 replicates.

Discussion

Spinach is a highly nutritious crop and SpWB phytoplasma infection is therefore a significant threat to spinach and detection of the same phytoplasma group in adjacent growing *Cannabis* plants alerts its epidemiological significance. Only phytoplasmas in 16SrI group were reported in spinach from India (Kumari *et al*., 2021); on the contrary *Cannabis* species showing witches' broom, little leaf symptoms have been reported as associated with several phytoplasmas including 16SrV group in China (Zhao *et al*., 2007), 16SrI and 16SrXIV in India (Mall *et al*., 2011; Chaube *et al*., 2014) and 16SrXII in Iran (Sichani *et al*., 2011). These findings suggest that *Cannabis* plants can act as natural reservoir of 16SrII-D phytoplasmas during the off-season and phytoplasmas can be effectively transmitted by insect vectors during the following crop season (Tran-Nguyen *et al*., 2000). This is the first report of spinach as new 16SrII-D phytoplasma host and of *C. sativa* as alternate host and potential reservoir of the same phytoplasma.

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Worldwide

Occurrence of a '*Candidatus* Phytoplasma aurantifolia' strain associated with *Euonymus japonicus* fasciation

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Abstract

Euonymus japonicus is grown in parks and gardens in Iran. During 2017-2021, stem fasciation symptoms were observed up to 8.4% in the Yazd area. Nested polymerase chain reaction using P1/P7, followed by R16mF2/R16mR2 and R16F2n/R16R2 primer pairs was carried out on DNAs extracted from twenty-eight symptomatic and six symptomless plants. After 35 cycles, DNA fragments of about 1.8, 1.4 and 1.25 kbp respectively were obtained from symptomatic *E. japonicus* samples but not from the symptomless ones. R16F2n/R16R2 trimmed sequences for six samples showed 100% sequence identity to each other showing 99.2% identity with '*Candidatus* Phytoplasma australasia', ribosomal group 16SrII. This is the first report a 16SrII phytoplasma strain associated with fasciation of *E. japonicus*. Since this species is grown on the margins of parks and gardens it may have an important role in the epidemiology and spread of this phytoplasma as reservoir.

Keywords: 16SrII phytoplasma group, flat limb, RFLP analyses, Iran

Introduction

Phytoplasmas are cell wall-less prokaryotes associated to plant diseases worldwide and transmitted mainly by leafhoppers and psyllids, causing devastating losses in more than one thousand plant species (Bertaccini *et al*., 2014).

Currently, they are divided into ribosomal groups based upon RFLP analysis of 16S rRNA gene sequences (Lee *et al*., 1998), and 49 '*Candidatus* Phytoplasma' species (Bertaccini *et al*., 2022). Ornamental plants were reported as hosts of different phytoplasmas worldwide. *Euonymus japonicus*, family Celastraceae, is a popular ornamental plant grown in parks and gardens in Iran as green and ornamental wall, pruned into desired shapes and sizes. The present work reports detection and identification of phytoplasmas in *E. japonicus* showing fasciation (Figure 1) in Yazd, Iran.

Materials and Methods

During 2017-2021, total DNAs were extracted from twenty-eight symptomatic and six symptomless plants using the method described by Zhang *et al*. (1998). Collected samples were subjected to direct and nested PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), R16mF2/R16mR2 and R16F2n/R16R2 (Gundersen and Lee, 1996) primer pairs for 35 cycles under published conditions. Samples with sterile H_2O were used as negative controls. Virtual RFLP, phylogenetic and DNA homology analyses of partial 16S ribosomal sequences were used to identify the detected phytoplasmas.



Figure 1. The flat limb (fasciation) disease symptoms in *Euonymus japonicus* in Yazd, Iran.

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Results and Discussion

The prevalent disease symptom observed in Yazd, Iran in *E. japonicus* in the surveyed areas was the stem fasciation (flat limb) and it was observed in up to 8.4% of the plants. The results of nested PCR with P1/P7, followed by R16mF2/R16mR2 and R16F2n/R16R2 on DNAs extracted from twenty-eight symptomatic and six symptomless plants produced fragments of approximately 1,800, 1,400 and 1,250 bp from all the symptomatic *E. japonicus* plant samples in both direct and nested PCR, respectively. No amplification was obtained from DNAs of asymptomatic samples and negative controls. The R16F2n/R16R2 trimmed sequences from six of the positive samples resulted 100% identical to each other, therefore one sequence named EuF, was deposited in GenBank under accession number OQ448205. BLAST analysis showed its 99.92% identity with '*Ca.* P. australasia' (GenBank accession number Y10097) and phylogenetic analysis using neighbor-joining method in MEGA 7 (Kumar *et al.*, 2016) showed its clustering with phytoplasmas enclosed in the 16SrII group (Figure 2).

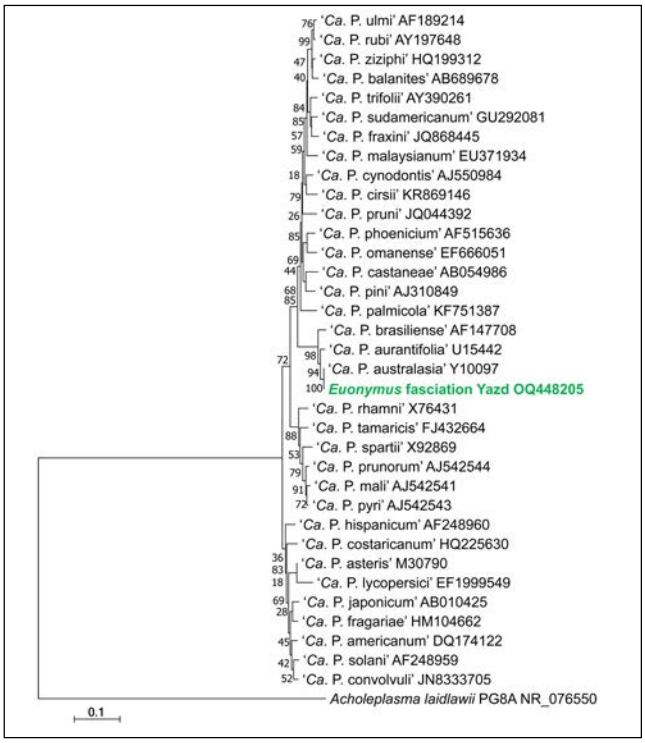


Figure 2. Phylogenetic tree showing *E. japonicus* phytoplasma (in green) position. *Acholeplasma laidlawii* is used as outroot.

This is the first report of a 16SrII phytoplasma strain associated with Euonymus fasciation disease in Iran. Phytoplasmas associated with fasciation symptoms were reported from different plant species in Iran. *Carthamus tinctorius* fasciation from Birjand, South Khorasan province (Mahmoudi *et al.*, 2019), *Celosia christata* fasciation from Ahvaz, Khuzestan province (Azimi *et al.*, 2018) and *Conocarpus erectus* little leaf, leaf roll and stem fasciation from Ahvaz and Dezful, Khuzestan province (Azimi *et al.*, 2017) were reported always associated with phytoplasmas in the 16SrII group. Moreover, in *C. erectus* stem fasciation from Bandar Abbas, Hormozgan province (Hemmati *et al.*, 2021) 16SrIX-A phytoplasmas were identified. *E. japonicus* in Iran is grown on the margins of parks and gardens and may have an important role in the epidemiology and spread of '*Ca.* P. australasia' phytoplasma as reservoir.

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Worldwide

Phytoplasma-associated diseases in stone fruits, pomegranate and grapevine in Jordan

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Abstract

Field surveys and molecular-based analyses revealed the presence of almond and pomegranate phytoplasma-associated diseases and confirmed the diffusion of grapevine yellows in Jordan. Seven '*Candidatus* Phytoplasma' species belonging to eight ribosomal subgroups were detected in symptomatic almond trees, four '*Ca.* Phytoplasma' species belonging to five ribosomal subgroups were identified in symptomatic pomegranate trees, and four '*Ca.* Phytoplasma' species belonging to four subgroups were identified in symptomatic wine and table grapevine cultivars. Preliminary results were obtained in additional host plants and putative insect vectors of these phytoplasmas.

Keywords: almond, molecular detection, reservoir plants, insect vectors

Introduction

In Jordan, phytoplasma-associated diseases as well as their epidemiology are still poorly studied. '*Candidatus* Phytoplasma solani' was associated with "bois noir" of grapevine and plum yellowing and witches' broom, '*Ca.* P. trifolii' with tomato big bud (Anfoka *et al.*, 2003), '*Ca.* P. asteris' with peach yellowing and reddening (Anfoka and Fattash, 2004), '*Ca.* P. aurantifolia' with potato reddening and tuber deformation, and '*Ca.* P. ulmi' with date palm stunting and yellowing (Hemmati *et al.*, 2021). In this study, a national survey on phytoplasma-associated diseases was conducted in Jordan from 2019 to 2021 on almond, pomegranate, and grapevine, three of the main fruit crops cultivated in the whole country as commercial and family farming. The aims of the study were: (i) survey the presence of phytoplasma-associated diseases in familiar and commercial orchards and vineyards throughout the country, (ii) detect and identify the phytoplasmas associated with these diseases, (iii) explore the presence of putative insect vectors and reservoir plants of the identified phytoplasmas..

Materials and Methods

Field surveys were conducted in 22 pomegranate orchards localized in three northern Jordan governorates, 23 almond orchards localized in two northern Jordan governorates, and 13 vineyards (11 with table grapevine and 2 with wine grapevine cultivars) localized in four northern and one southern Jordan governorates. Leaf samples were collected from symptomatic and symptomless pomegranate, almond, and grapevine plants. Moreover, leaf samples were collected from non-crop weed species showing suspicious symptoms, observed within and around the surveyed orchards and vineyards. In parallel, insects within examined orchards and vineyards were collected by entomological sweeping net and identified based on stereomicroscope observation of morphological features. Nested PCRs were carried out using 25 to 50 ng of total nucleic acids extracted from plant and insect samples with the primer pairs P1/P7 followed by R16F1/R16R0 (Quaglino *et al.*, 2009). F1/R0 PCR products were sequenced in both strands by a commercial service (Eurofins Genomics, Germany), assembled, trimmed to the F1/R0 annealing sites, aligned using the ClustalW Multiple Alignment program and analyzed by Sequence Identity Matrix in the software BioEdit. For attribution to '*Ca.* Phytoplasma' species, one 16S rDNA nucleotide sequence randomly selected among each group of identical sequences obtained in this study was compared with those of the 49 '*Ca.* Phytoplasma' species described in literature (Bertaccini *et al.*, 2022). '*Ca.* Phytoplasma' species attribution was confirmed searching the species-specific signature sequences within the analyzed F1/R0 nucleotide sequences. For ribosomal group/subgroup attribution, 16S rDNA sequences were analyzed by virtual RFLP using the online tool *i*PhyClassifier (Zhao *et al.*, 2009). *In silico* RFLP profiles related to new ribosomal subgroups were confirmed by enzymatic digestions.

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Results

During field surveys, phytoplasma-like symptoms, including leaf yellowing/reddening and rolling, little leaf, and witches' broom were observed in pomegranate; early flowering along with evergreen pattern, witches' broom, yellowing, dieback, slim leaf and leaf rolling, and stem fasciation were observed in almond trees; leaf reddening/yellowing and rolling symptoms, typical of grapevine yellows, were observed in wine and table grapevine cultivars. Nested PCR amplification detected phytoplasmas in 17%, 21%, and 19% of symptomatic pomegranate, almond, and grapevine samples, respectively. Nucleotide sequence analyses allowed attributing the detected phytoplasmas to '*Ca.* P. solani' (ribosomal subgroup 16SrXII-A), '*Ca.* P. aurantifolia' (16SrII-C), '*Ca.* P. asteris' (16SrI-B and -R), and '*Ca.* P. ulmi' (16SrV-A) in pomegranate; '*Ca.* P. asteris' (16SrI-B and -R), '*Ca.* P. aurantifolia' (16SrII-B and -C), '*Ca.* P. omanense' (16SrXXIX-A and -B), '*Ca.* P. phoenicium' (16SrIX-B), '*Ca.* P. pyri' (16SrX-C), '*Ca.* P. solani' (16SrXII-A), and '*Ca.* P. ulmi' (16SrV-A) in almond; '*Ca.* P. solani' (16SrXII-A), '*Ca.* P. omanense' (16SrXXIX-A and -B), '*Ca.* P. aurantifolia' (16SrII-C), and '*Ca.* P. asteris' (16SrI-R) in grapevine.

These phytoplasmas were found in plants showing specific symptoms and differentially distributed in the considered locations. In pomegranate orchards, four cicadellids (*Macrosteles sexnotatus*, *Cicadulina bipunctata*, *Psammotettix striatus*, *Zyginidia sohrab*) and two non-crop plants (*Plantago major*, *Capsicum annuum*) resulted hosting '*Ca.* P. asteris' (16SrI-B and -R), and one cicadellid (*Balclutha incisa*) was carrying '*Ca.* P. solani'. In almond orchards, '*Ca.* P. asteris' (16SrI-R) was identified in putative insect vectors such as *Empoasca* sp., *Reptalus* sp., and *Hyalesthes obsoletus*, '*Ca.* P. pyri' (16SrX-C) in *Cacopsylla bidens*, and '*Ca.* P. omanense' (16SrXXIX-B) in the non-crop plant *Amaranthus* sp. In vineyards, '*Ca.* P. solani' was identified in the putative insect vectors *Orosius cellulosus*, *Euscelidius mundus*, *Laodelphax striatellus*, and *Circulifer* sp., and in *Convolvulus arvensis*; '*Ca.* P. aurantifolia' in the insect *O. cellulosus* and in bindweed; '*Ca.* P. omanense' (16SrXXIX-A) in the insect *Psammotettix striatus*; '*Ca.* P. asteris' (16SrI-B and -R) in *Arboridia adanae*, *Cicadulina bipunctata*, *Circulifer* sp., *L. striatellus*, *Hyalesthes obsoletus*, and *P. striatus*.

Discussion

This study described a new pomegranate disease complex associated with multiple phytoplasmas, firstly reported the presence of putative insect vectors (*M. sexnotatus*, *B. incisa*, *O. cellulosus*) in Jordan, and the ribosomal subgroup 16SrXXIX-B (About Alloush *et al.*, 2023). Interestingly, '*Ca.* P. ulmi', *Ca.* P. pyri', and '*Ca.* P. omanense' in almond, and '*Ca.* P. ulmi' in pomegranate are reported for the first time in this study. The other phytoplasmas identified in almond and pomegranate were previously reported in the Middle East (Hemmati *et al.*, 2021). Moreover, obtained results suggest that grapevine yellows phytoplasma diversity and ecology in Jordan are more complex than previously known (Salem *et al.*, 2013), leading to a potential risk of disease outbreaks.

In conclusion, this study provided new insights on phytoplasma diffusion in important crops in Jordan, identifying putative insect vectors and non-crop plants. Further studies must focus on verify the capability of putative insect vectors, identified in the present work, to transmit the associated phytoplasmas by transmission trials; survey the insect population diversity and dynamics throughout the whole season; upscale the survey on almond, pomegranate and grapevine in the whole Country, focusing on the canopy and the roots of symptomatic trees and exploring differences among the available cultivars.

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Worldwide

Investigation on phytoplasma diseases of sweet pepper in the Bekaa valley of Lebanon

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Abstract

In July 2018, several sweet pepper plants showing symptoms of leaf chlorosis, yellowing and stunted leaves, typically associated with phytoplasma infection, were observed in different locations in the Bekaa Valley, Lebanon. Polymerase chain reaction (PCR) was used to amplify the 16S ribosomal gene of phytoplasmas with the universal primers P1/P7 and R16F2n/R16R2 primers in nested PCR assay. The amplifications from all the symptomatic plants yielded a product of 1.2 kb in nested PCR. BLAST and phylogenetic analysis of the amplified 16S rRNA gene sequences confirmed the presence of '*Candidatus* Phytoplasma solani' and '*Ca.* P. trifolii' phytoplasmas. This is the first report of the occurrence of '*Ca.* P. solani' in pepper in Lebanon.

Keywords: pepper, '*Candidatus* Phytoplasma trifolii', molecular analyses, 16S ribosomal gene, nested PCR

Introduction

In Lebanon, fruiting vegetables are of great economic importance. The main crops are tomatoes, eggplants, cucumbers, peppers, squash and zucchini, melons and watermelons that represent about 38% of vegetable crops. Pepper (*Capsicum annuum*) started to spread in several agricultural areas occupying an area of 800 ha, especially in the Bekaa region and the coastal plains for domestic and foreign markets. Like several other crops, pepper cultivation is exposed to many pathogens, causing serious economic losses (Choueiri *et al.*, 2004). Like many solanaceous species, pepper could be infected by phytoplasmas, that are wall-less phloem limited bacteria associated to many important plant diseases worldwide. The most typical phytoplasma symptoms in pepper are leaf chlorosis, proliferation, shortening of internodes, stunting and/or decline (Bertaccini, 2022). Phytoplasma diseases in *C. annuum* have been reported worldwide, in association to different phytoplasma ribosomal groups according to the geographic areas (Castro and Romero, 2002; Choueiri *et al.*, 2007). Previous surveys conducted in tomato and pepper cultivations in Lebanon showed the presence, in the majority of the samples tested, of phytoplasmas belonging to 16SrVI ribosomal group ('*Candidatus* Phytoplasma trifolii', PTL strain) (Choueiri *et al.*, 2007). Recently, Lebanese pepper growers frequently complain about disease epidemic yield losses, and the appearance of unusual symptoms. In July 2018, several symptoms assumed to be due to viral/phytoplasma diseases (leaf chlorosis, yellowing and stunted leaves) were observed in sweet pepper plants in different locations at Bekaa valley, Lebanon. Investigations were conducted to verify the identity of these pathogens and study their epidemiology after a decade from their first detection.

Materials and Methods

In July 2018, a total of 90 leaf samples were collected from symptomatic and asymptomatic pepper plants in six commercial fields from three locations in Bekaa valley, Lebanon. The nucleic acid of the collected plant samples was extracted from the midribs of the fresh leaves according to a CTAB protocol (Angelini *et al.*, 2001). The phytoplasma identification was carried out by nested-PCR analyses on 16S rDNA. DNA was amplified by PCR using the universal primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) which amplify a DNA fragment of 1.7 kb, then a 1: 30 dilution of the first step PCR product was used as target for nested PCR, with the primer pair R16F2n/R16R2 (Lee *et al.*, 1995). Aliquots (10 µl) of amplified nested PCR products were analyzed by electrophoresis in 1.2% agarose gel in 1 TAE buffer together with 1 kb plus DNA ladder (Thermo Fisher Scientific, Wilmington, DE, USA) and then visualized with a UV transilluminator. PCR amplicons from positive samples were directly sequenced on both strands (Macrogen, Korea) using the same primers employed for their amplification.

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Obtained sequences were assembled, aligned, and compared with nucleotide sequences available at NCBI database using BLAST (www.blast.ncbi.nlm.gov) function.

Results

The sweet pepper plants showed symptoms of leaf chlorosis, yellowing and stunted leaves, typically associated with phytoplasma presence (Figure 1). PCR analyses for the detection of phytoplasmas confirmed the expected correlation between the observed symptoms and the presence of phytoplasma infection (Figure 2). Out of 90 samples collected, 6 plants resulted positive after nested amplification of the 16Sr ribosomal gene, with an incidence of 6.6%.



Figure 1. Sweet pepper (*Capsicum annuum*) plants showing phytoplasma symptoms in Bekaa valley, Lebanon: leaf yellowing and stunting (a and c) and leaf chlorosis (b).

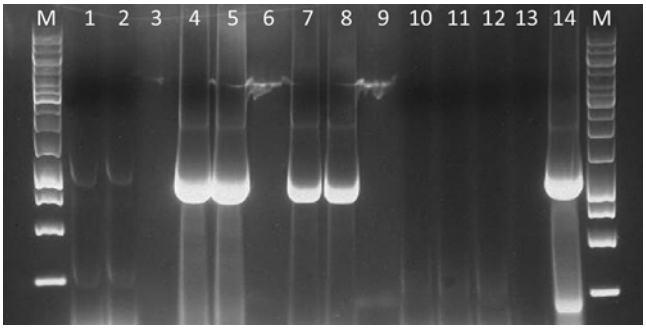


Figure 2. Agarose gel 1.2% showing R16F2n/R16R2 amplification products. M: 1 kb DNA ladder, 1- 12: amplicons obtained from pepper; 13: negative control; 14: positive control (PTL phytoplasma strain).

The 16Sr DNA sequences obtained from selected amplicons showed the presence of two phytoplasmas in the collected samples: a '*Ca.* P. trifolii' strain (WB-2, GenBank accession number OQ297264), with 100% identity with the Turkish strain GRS93 (GenBank accession number MN224665) and '*Ca.* P. solani' strain (WB-16, GenBank accession number OQ297265), having 99.76% identity with the Turkish strain Bigol D11 (GenBank accession number MT279680). The samples infected with the '*Ca.* P. solani' strain were further analysed on *stamp* gene. The sequences obtained from selected amplicons showed 100% identity with '*Ca.* P. solani' strain P7 from *Catharanthus roseus* from Lebanon (GenBank accession number FN813258).

Discussion

'Ca. P. solani' was reported in sweet pepper in different countries around the world. In Lebanon the presence of the '*Ca.* P. solani' in pepper plants was detected in one field located at Ammiq, only 5-6 km away from Tal Danoub, where '*Ca.* P. solani', the "bois noir" associated agent was reported in grapevine (Mortada *et al.*, 2013). This is the first report of the occurrence of '*Ca.* P. solani' in pepper in Lebanon. This finding highlights the necessity to conduct a detailed survey for phytoplasmas associated with stunted and chlorotic pepper plants in other regions of Lebanon.

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Worldwide

Molecular detection of '*Candidatus* Phytoplasma mali' associated with virescence in *Narcissus tazetta* in Turkey

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Abstract

In the winter 2019 virescence, flower sterility, and stunting symptoms were observed in daffodil plants in Mersin province of Turkey. To detect the presence of phytoplasmas ten plants showing symptoms and ten asymptomatic plants were assayed by PCR analysis. The nested-PCR analysis of extracted DNA with universal primer pairs amplifying 16S phytoplasma ribosomal gene were positive for 9 out of the 10 symptomatic daffodil samples. BLAST analysis of the 16S rDNA sequences, virtual restriction fragment length polymorphism (RFLP) and RFLP analyses confirmed the presence of '*Candidatus* Phytoplasma mali' in *Narcissus tazetta* plants. The detected strains of '*Ca.* P. mali' were sequenced and compared in multiple alignments using sequences in NCBI GenBank. The nucleotide identity among the 16S rDNA gene fragments ranged between 99.26 and 99.91% to '*Ca.* P. mali', ribosomal subgroup 16SrX-A. Although phytoplasmas in this subgroup have been reported frequently from woody plants, this is the first report of a '*Ca.* P. mali' strain infecting daffodil plants.

Keywords: daffodil, phyllody, PCR/RFLP, 16SrX-A subgroup

Introduction

Daffodil is an important cut flower as it has great demand for bulb trade. It has 80 species with 60 genera in the Amaryllidaceae family (Takos and Rook, 2013). Nine taxa belong to genus *Narcissus*, and the most common species cultivated in Turkey are *Narcissus tazetta* and *N. serotinus* (Davis, 1984). Many phytoplasma associated diseases in ornamental plants were identified and considered as virus diseases on the basis of symptoms and insect spread behaviour. The first report of phytoplasmas in *Narcissus* sp. was published by Bellardi *et al.* (1990) showing leaf chlorosis, phyllody and flower malformation and some multivesicular membrane bounded bodies in the sieve tubes. Recently, two more reports were published from Iran on phytoplasma detection in daffodil plants. Gholami *et al.* (2018) reported the presence of '*Candidatus* Phytoplasma solani' in daffodil plants showing phyllody and virescence symptoms. Molecular and phylogenetic analyses revealed that the phytoplasma strains detected in daffodil were enclosed in the 16SrXII-A subgroup. Mahmoudi *et al.* (2020) observed yellowing symptoms in the daffodil grown in the Southern Khorasan Province, Iran. Molecular and phylogenetic analyses revealed that the phytoplasma strains present were enclosed in 16SrII-B subgroup.

In the present study, symptomatic daffodil plants exhibiting phyllody, flower sterility, and stunting were examined for phytoplasma presence by nested PCR, RFLP, 16S rRNA gene sequence analyses and comparison.

Materials and Methods

Samples from 10 symptomatic and 10 asymptomatic daffodil plants were collected from field in Mersin province, Turkey in 2019. DNA extraction was performed as described by Doyle and Doyle (1990). The universal phytoplasma specific 16S rRNA gene primer pairs P1 and P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) were used in PCR assay. Nested PCR reaction was performed using primer R16F2n/R16R2 (Gundersen and Lee, 1996). Phytoplasma positive controls employed for the molecular analyses included DNA from phytoplasma strains maintained in periwinkle [*Catharanthus roseus* (L.) G. Don.] (Bertaccini *et al.*, 2000). Nested-PCR products of detected phytoplasmas of 1,200 bp, including control strains, were subjected to RFLP analysis with restriction endonucleases *Rsa*I and *Ssp*I. Restriction

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fragments were resolved through agarose gels. DNA amplicons were sequenced bi-directionally using R16F2n and R16R2 primers and were assembled using UGENE software (Unipro). The resulting consensus sequences were deposited in the NCBI database and aligned by MEGA 11 software. BLASTn was used to compare the phytoplasma 16S rRNA gene sequences to DNA sequences available in the NCBI database. Phylogenetic analysis was performed using 1,000 replicates for bootstrap analysis by neighbor joining algorithm. Virtual RFLP patterns were generated by *in silico* digestion of the 16S rRNA gene assembly with 17 restriction endonucleases including *Alu*I, *BamH*I, *Bfa*I, *BstU*I (*Tha*I), *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3A*I (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I (Lee *et al*., 1998) using pDRAW32.

Results

A fragment of approximately 1.2 kb was amplified from DNA of 9 out of 10 symptomatic daffodil plants showing virescence and dwarfing (Figure 1). The RFLP analysis of the amplicons obtained with R16F2n/R16R2 primers showed that all of the phytoplasma strains from daffodil plants were differentiable from '*Ca.* P. prunorum' (ESFY) and '*Ca.* P. pyri' (PD) by *Rsa*I and *Ssp*I restriction enzymes (Figure 1). The partial 16S rRNA sequence analysis confirmed the presence of phytoplasmas having high identity percentages to '*Ca.* P. mali' ranging from 99.26 to 99.91%.

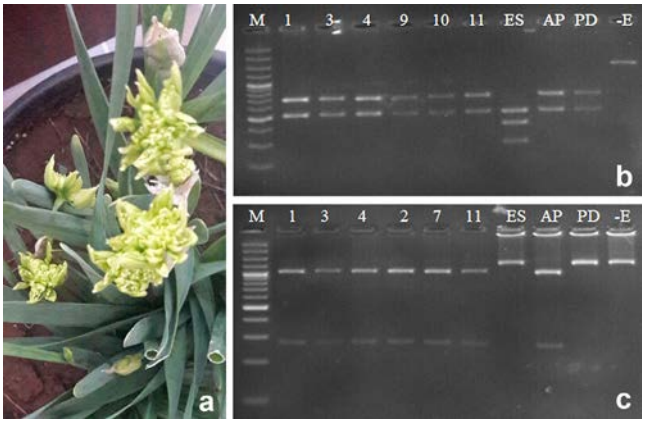


Figure 1. Daffodil plant infected by '*Ca.* P. mali' showing virescence and dwarfing (a). Agarose gels of RFLP analyses of 16S rRNA gene amplified in nested PCR with R16F2n/R2 from *N. tazetta* strain SMO321 (lanes 1, 2, 3, 4, 7, 9, 10, 11) and controls. Banding patterns resulting from *Ssp*I (b) and from *Rsa*I (c) restriction endonuclease digests. Phytoplasma strains used as reference: ES, AP and PD: '*Ca.* P. prunorum' (ESFY), '*Ca.* P. mali' (AP) and '*Ca.* P. pyri' (PD), respectively. -E: negative control uncut.

Discussion

The presence of phytoplasmas in daffodil plants has been reported for subgroups 16SrXII-A and 16SrII-B and associated to yellowing symptoms in Iran (Gholami *et al*., 2018; Mahmoudi *et al*., 2020). Here the presence a 'Ca. P. mali' strain (16SrX-A) is reported for the first time in daffodil plants, and it is one of the few reports of this phytoplasma in herbaceous host plant. The epidemiological relevance of the finding should be further evaluated together with the presence of insect vectors in the studied environment.

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Worldwide

Multilocus next-generation sequencing of leafhopper-associated phytoplasmas highlights gaps in knowledge for some phytoplasma lineages and genetic *loci*

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Abstract

Analysis of DNA sequence data for 6 housekeeping genes obtained from leafhopper-associated phytoplasmas using anchored hybrid enrichment next-generation sequencing yielded a well resolved phylogeny largely congruent with previous phylogenetic estimates, despite the substantial amounts of missing data. In general, phytoplasmas belonging to previously well studied groups (*e.g.*, 16SrI) were placed on the phylogeny with high confidence while the placements of strains belonging to poorly studied groups were less stable. This suggests that major gaps in knowledge of phytoplasma diversity remain, especially for groups largely distributed in the tropics.

Keywords: target-capture, Cicadellidae, vector, biorepository

Introduction

Shortcomings of the classification scheme, now in widespread use for phytoplasmas (Zhao *et al.*, 2009), include reliance on relatively small numbers of molecular (restriction site) characters from a single gene (16Sr), the inability of single *loci* to characterize distinct strains within certain groups unambiguously, and the paucity of whole genome sequences, with only half of the 16Sr groups represented so far (Cho *et al.*, 2020). Comparative genomic analyses have begun to identify additional candidate *loci* that provide greater discriminatory power useful for grouping phytoplasmas (*e.g.*, aster yellows phytoplasmas in group 16SrI) (Cho *et al.*, 2020), but some *loci* show highly variable evolutionary rates that may limit their use to certain lineages.

Recent investigations on leafhopper-associated phytoplasmas in natural areas worldwide led to discovery of several new strains and subgroups over the past 2 years (Wei *et al.*, 2021; Trivellone *et al.*, 2022). It was also shown that anchored hybrid enrichment next-generation sequencing (AHE) (Lemmon *et al.*, 2012), is more efficient and sensitive than traditional PCR and Sanger sequencing for obtaining phytoplasma data from DNA extracted from potential insect vectors. Here preliminary results on a new AHE probe kit that specifically targets 129 phytoplasma genes in host DNA samples are presented. This method can yield large amounts of data for multiple *loci*, allowing new phytoplasmas to be placed accurately within a phylogenetic context, even in the absence of 16S rDNA data.

Materials and Methods

Leafhopper specimens were selected from a large collection at the Illinois Natural History Survey, assembled over more than 20 years and covering all major biogeographic regions. DNA was extracted as described by Trivellone *et al.* (2022). Initial screening of 634 single specimens was carried out using qPCR of the 16S ribosomal gene following Angelini *et al.* (2007). All phytoplasma-positive samples and 29 samples that yielded phytoplasma sequence data using the probe kit of Cao *et al.* (2022) were analyzed using the AHE probe kit containing 58,000 DNA probes targeting 129 genes shared among 50 partial or full phytoplasma genomes available so far, to obtain additional DNA sequence data. The quality of the probe design was verified using available phytoplasma genomic resources following the procedures of Lemmon *et al.* (2012). A preliminary phylogenetic analysis of concatenated sequences of 6 housekeeping genes (*tuf*, *secA*, *secY*, *rplV*, *rpsC*, *groEl*) was constructed, with sequences aligned using Concatenator-0.2.1 and analyzed by maximum likelihood using raxmlGUI 2.0.

Results

A total of 54 leafhopper specimens (accounting for about 8.5% of the 634 leafhoppers analyzed) tested positive for the

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presence of bacterial 16Sr gene during preliminary qPCR screening. For 22 of the 83 samples (54 new + 29 previously analyzed), AHE assembly yielded phytoplasma 16Sr sequences belonging to six ribosomal groups (16SrI, 16SrIII, 16SrV, 16SrVI, 16SrXI, 16SrXIV) as identified using BLAST or *i*PhyClassifier. For 78 samples, AHE assembly yielded sequences of 19 additional putative phytoplasma genes. Phylogenetic analysis was carried out using a subset of 33 samples, 17 of them having data for all 6 housekeeping genes and the others with data for at least 2 genes (Figure 1). Two well-supported clades were detected: one comprising '*Ca.* P. asteris' (GCA_013372025.1) and 7 strains all blasting to 16SrI related strains; the other comprising '*Ca.* P. ziziphi' (GCF_003640545.1) and 2 phytoplasma strains blasting to 16SrV strains. One subclade comprising 2 strains is sister to the 16SrV subclade and includes strains that blasted to 16SrVI and 16SrV with different pairs of genes. Another well-supported subclade includes 1 strain and '*Ca.* P. phoenicium' (GCF_001189415.1) (Figure 1).

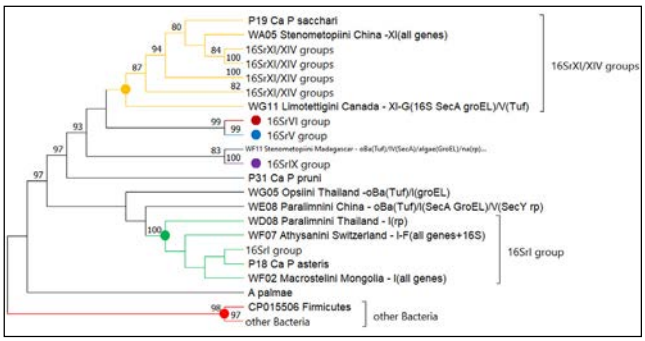


Figure 1. Phylogenetic tree inferred by Maximum Likelihood (ML) based on the Kimura 2-parameter model for 6 concatenated housekeeping genes (*tuf, secA, secY, rplV, rpsC, groEl*). Bootstrap values >80% (1,000 replicates) shown. Reference strains '*Ca.* P. asteris', '*Ca.* P. phoenicium', '*Ca.* P. pruni', '*Ca.* P. sacchari and 'Ca. P. ziziphi'. *Acholeplasma palmae* and two *Firmicutes* are outgroups.

Another large subclade with relatively low branch support (Figure 1) includes 12 phytoplasma strains and '*Ca.* P. sacchari' (GCF_009268105.1). Another unresolved subclade includes 4 strains sister to 16SrIX group and another two strains (E08 and G05) blasting to other bacteria using the *tuf* gene only. However, these strains could be unknown phytoplasmas that cannot be properly identified as such because they are not closely related to any phytoplasma strains that have sequence data available in GenBank. Another group of 3 strains that blasted to other bacteria (on *tuf* and *secA* genes) is more distantly related (Figure 1).

Discussion

Overall, these results suggest that the AHE approach using the new probe kit performed well at capturing data for 6 housekeeping genes proposed for effective distinction of some problematic '*Candidatus* Phytoplasma' species (Bertaccini *et al.*, 2022). Although data for the complete set of *loci* was not obtained for some samples, the phylogeny presented is largely congruent with previous phytoplasma phylogenies despite these missing data. Not surprisingly the samples belonging to previously well studied phytoplasma groups (*e.g.*, 16SrI and 16SrV) were placed with relative certainty on the phylogeny compared to samples belonging to poorly studied groups, including groups 16SrXI and XIV, which previous studies have also shown to be problematic. This may reflect the overall paucity of data in GenBank for many phytoplasma groups, especially those distributed mainly in tropical countries, where diversity is expected to be higher, but overall knowledge of phytoplasmas remains low. These results highlight gaps in knowledge of phytoplasma phyletic and genetic diversity. The studies of leafhopper-associated phytoplasmas from natural areas worldwide aim to improve knowledge of phytoplasma diversity overall by providing multilocus sequence data for many new or poorly known phytoplasma lineages. This should enable to a more fully evaluation of the variability of multiple genetic *loci* and their suitability for use in phytoplasma differentiation. For future analysis it is necessary to increase the number of samples and reference strains included in the concatenated dataset, allowing comparison with more comprehensive phylogenetic trees based on single genes (including 16Sr), and the inclusion of sequence data for other more variable genes captured using the AHE approach.

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Worldwide

Preventing phytoplasma emerging diseases: phylogenetic relatedness and landscape analyses to assess the risk of outbreaks

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Abstract

Ecological fitting is a natural process that may drive new phytoplasma-host associations. Environmental changes across habitat interfaces are one of the most important factors in providing new opportunities for a pathogen to encounter a new host. In this study the contribution of geography and phylogenetic relatedness in explaining the rate of phytoplasma detection in insect vectors was studied. Furthermore, the landscape characteristics of the areas inhabited by the phytoplasma positive insect specimens were evaluated.

Keywords: phylogenetic conservatism, Hemiptera, leafhopper vectors, phytoplasma diseases

Introduction

New associations between phytoplasmas and their hemipteran insect vectors may emerge through the process known as ecological fitting (Janzen, 1985). By evaluating three mechanisms (phenotypic plasticity, correlated trait evolution, and phylogenetic conservatism) driving the ecological fitting process, it is possible to assess the degree of which a host switch by phytoplasmas is likely to happen (Agosta and Klemens, 2010). Altered habitat interfaces in a mixed, fragmented landscape trigger new opportunity for a pathogen to encounter a new host (Brooks *et al*., 2019). As such, given an opportunity, pathogens and hosts with compatible traits may set the stage for new emerging diseases and outbreaks (Trivellone *et al*., 2022a). In this study the contribution of geography and phylogenetic relatedness in explaining the rate of phytoplasma positive detection was explored.

Materials and Methods

By applying a stratified random selection process, 191 insect vector (leafhopper) samples were chosen using two factors: phylogenetic relatedness (4 phylogenetic lineages of grass and non-grass insect specialists) and geographic origin (3 regions, New World-NW, Palearctic + Oriental region-PaOr, Afrotropical + Australian region-AtAu). DNA extraction and TaqMan quantitative PCR (qPCR) analysis of the 16Sr gene were carried out using the same protocol as in Trivellone *et al*. (2022b) to detect the presence of phytoplasma DNA. A two-way ANOVA and Bayesian regression were used to determine whether geographic origin and the phylogenetic group of the insect host influence phytoplasma infection rate in the sample. The analyses were carried out using the *brms* package (Berkner, 2017) in R (R Core Team, 2008).

Results and Discussion

Preliminary results from this experiment suggested that phylogenetic relatedness and geography are significant factors ($p<0.001$) driving the rate of positivity in leafhoppers collected in natural areas. In particular, the presence of phytoplasmas was found higher in the phylogenetic groups 1, including deltocephalini, chiasmini and paralimnini, and significantly higher in the Palearctic and Oriental regions. The influence of geographic origin and phylogenetic group on presence or absence of phytoplasmas is summarized in Figure 1. The landscape factors that may facilitate phytoplasmas to further spread across interfaces between habitats (including managed anthropogenic habitats) and to switch into susceptible hosts are presently under analyses.

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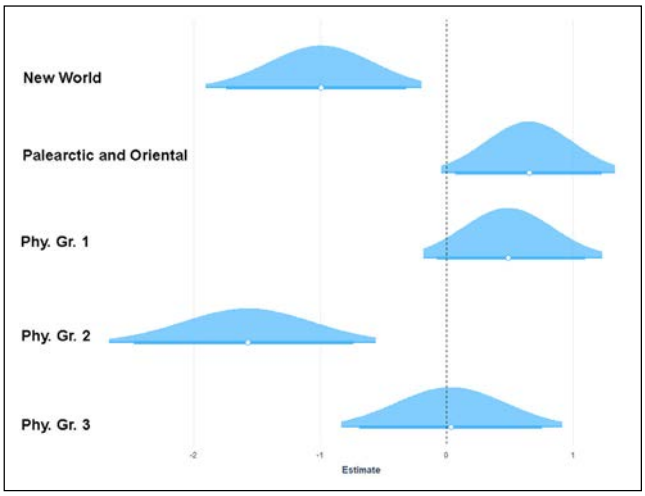


Figure 1. Density plots of the posterior distributions of the estimated parameters from the Bayesian regression model. Points show posterior means, and horizontal lines are 95% Credible Intervals.

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Worldwide

Main phytoplasmas infecting crops in South America

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Abstract

The diseases associated to phytoplasmas in South America are responsible of serious economic losses. The 16SrIII-J phytoplasmas have a wide host range and are transmitted by numerous leafhopper species and seems thus the most critical.

Keywords: '*Candidatus* Phytoplasma pruni', '*Ca.* P. fraxini', '*Ca.* P. hispanicum', '*Ca.* P. meliae', '*Ca.* P. pyri'

Introduction

During the last 20 years, the information about '*Candidatus* Phytoplasma' species infecting different crops in South America has increased, offering important indications about their presence in the region. Some of them seems to be native to South America, having been detected exclusively or prevalently in this geographical area. The main phytoplasmas infecting different crops of importance for South America are summarized here.

'*Ca.* P. pruni'

Among the phytoplasmas present in South America, the 16SrIII ribosomal group has been detected in many crops, herbaceous and woody plants, in different countries. The most widespread phytoplasmas belong to the 16SrIII-J subgroup and are infecting several plant species: chayote, cabbage, cauliflower, eggplant (Brazil); sugar beet (Argentina, Chile); lettuce, Swiss chard, potato, cactus pear, carrot, grapevine, cherry (Chile); tomato, garlic, summer squash, sunflower, cassava (Argentina); corn (Peru); China-tree (Bolivia); *Solanum quitoense* (Colombia). Also, five insect potential vectors and two vectors of these phytoplasmas were found in Chile and different weeds have been reported as reservoirs, explaining the high spread of this pathogen (Guzmán *et al.*, 2014; Quiroga *et al.*, 2018, 2019, 2022; Fernández *et al.*, 2019, 2022; Bedendo and Spotti, 2019; Gamarra *et al.*, 2022). The 16SrIII-B phytoplasmas were detected in China-tree (Argentina, Brazil, Paraguay), peach, plum, tomato (Argentina), sweet orange, cassava (Brazil) (Guzmán *et al.*, 2014; Bedendo and Spotti, 2019; Bongiorno *et al.*, 2020; Quiroga *et al.*, 2018); the 16SrIII-L, in cassava (Colombia and Brazil) and the 16SrIII-A, in cassava (Brazil). Other phytoplasma subgroups were incidentally reported such as 16SrIII-X in lettuce, *Conyza bonariensis* (Argentina), sweet orange (Brazil) (Guzmán *et al.*, 2014; Barbosa *et al.*, 2021; Fernández *et al.*, 2022); 16SrIII-W in *Heterothalamus alienus* (Argentina), and 16SrIII-V in passion fruit (Brazil) (Guzmán *et al.*, 2014). Not characterized phytoplasmas in the 16SrIII group were reported in cassava (Paraguay), broccoli (Brazil), tomato and dandelion (Peru), bell pepper and strawberry (Bolivia) (Arocha *et al.*, 2010; Bedendo and Spotti, 2019).

'*Ca.* P. fraxini'

'*Ca.* P. fraxini' (16SrVII-A) was reported to infect ash in Colombia, grapevine, *Ugni molinae* and *Paeonia lactiflora* in Chile. The 16SrVII-B subgroup was identified in Brazil in *Erigeron* sp. and *Catharanthus roseus*, and 16SrVII-C subgroup was described in alfalfa and strawberry from Argentina. The phytoplasma 16SrVII-D was detected in *Erigeron bonariensis* from Brazil (Gajardo *et al.*, 2009; Arismendi *et al.*, 2011; Fernández *et al.*, 2013; Guzmán *et al.*, 2014; Flôres *et al.*, 2015).

'*Ca.* P. hispanicum' - '*Ca.* P. meliae'

The 16SrXIII ribosomal group is only reported in the American continent, however the 16SrXIII-A ('*Ca.* P. hispanicum') was not reported as such in South America.



Figure 1. Severe phyllody associated to 16SrXIII-F phytoplasmas in infected strawberry fruits from Chile.

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The subgroup 16SrXIII-F (Figure 1), found in strawberries (Argentina and Chile), *Berberis microphylla*, mandarin, and sweet orange (Chile) seems to be the most widespread. Phytoplasmas 16SrXIII-C were detected in China-tree (Argentina, Bolivia, Paraguay), and 16SrXIII-E in papaya (Brazil). Another phytoplasma no further characterized, belonging to the 16SrXIII ribosomal group, was detected in broccoli from Brazil (Bedendo and Spotti, 2019). The phytoplasma 16SrXIII-G ('*Ca.* P. meliae') was only reported from Argentina, infecting China-tree and plum (Fernández *et al*., 2016; Bongiorno *et al*., 2020).

'*Ca.* P. pyri' (16SrX-C)

It is the main phytoplasma detected in pear (Chile, Uruguay) and peach (Argentina) trees in South America. Its epidemiological cycle in the three countries is still not defined because among the two known insect vectors, *Cacopsylla pyri* and *C. pyricola*, the first one has never been found in South America, while the latter has only been found sporadically in Argentina (Quiroga *et al*., 2018).

'*Ca.* P. asteris'

'*Ca.* P. asteris' (16SrI-B) was found in oil palm (Colombia), corn (Colombia, Peru), potato (Bolivia), sugarcane (Brazil), and papaya (Peru). Phytoplasmas in the 16SrI group were detected in several plant species in Colombia, Brazil and Peru (Jones *et al*., 2005; Perilla-Henao *et al*., 2012; Álvarez *et al*., 2014; Hodgetts *et al*., 2009; Gamarra *et al*., 2022).

Conclusions

In general, the information generated to date in South America about phytoplasmas is not exhaustive. It is necessary to expand the monitoring to find out the health status of the different crops, it is also urgent to start with epidemiological studies to optimize the control of the diseases associated with these pathogens. Certification programs must be implemented for production and commercialization of healthy plants, especially for the strategic crops in the region, taking also into account the impact that climate change may have on these diseases.

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Worldwide

First report of 16SrII-D phytoplasmas in *Verbesina encelioides* showing phyllody in Oman

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Abstract

Verbesina encelioides, Asteraceae, in the sunflower family, is commonly known as golden crownbeard. It was found in Oman developing phyllody symptoms, a phytoplasma-associated disease. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA, *secA*, *imp* and SAP11 genes of phytoplasmas using direct and nested techniques. The PCR amplifications from all infected plants was amplifying expected length fragments in both direct and the nested PCR from symptomatic plant samples. Sequence identity results on BLAST and phylogenetic analyses of these genes sequences confirm that the phytoplasma detected in *V. encelioides* is a member 16SrII-D subgroup and shares more than 99.5% of all four genes sequence identity to the other phytoplasmas enclosed in this ribosomal subgroup.

Keywords: plant disease, PCR, sequencing, RFLP, molecular characterization

Introduction

Verbesina encelioides, also known as golden crownbeard, is a medicinal plant widely used in traditional medicine. *V. encelioides* roots play an important role in the management of diabetes (Sindhu *et al*., 2010). Ramakrishnan *et al*. (2017) reported a low molecular weight protein from *V. encelioides* of 14 k Da which is responsible for antibacterial and antifungal activity. The flowers and plants of *V. encelioides* are used or visited by moths, butterflies, honeybees, native bees and other insects in search of nectar, food or shelter and protection. One-year old *V. encelioides* plants grown in a field at Al-Saleel National Park (N: 22 212 46.83 , E: 59 112 49.23), Al-Kamil, Sharqiyah, Oman, were observed with symptoms of phyllody which are classically reported as associated with phytoplasma diseases. The *V. encelioides* symptoms are similar to sesame phyllody symptoms reported previously in Oman as associated with 16SrII-D subgroup phytoplasmas (Al-Subhi *et al*., 2018).

Materials and Methods

Leaf samples from four symptomatic and two asymptomatic *V. encelioides* plants were sampled for nucleic acid DNA extraction using the Doyle and Doyle (1987) method with some modifications. Polymerase Chain Reaction (PCR) was used with specific primers for phytoplasma 16S rRNA gene using primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and primer pair R16F2n/R16R2 (Gundersen and Lee, 1996) in nested PCR. The four phytoplasma infected samples were further used for phytoplasma molecular characterization using specifics primers pair of the *sec*A, *imp* and *SAP11* genes in nested PCR trials. Ready-To-Go' PCR beads (Pharmacia Biotech, Sweden) were used in PCR amplifications with 25 µl reaction volumes and 10 pM of each primer. The PCR products of the four genes were purified and sequenced at Macrogen Company (South Korea). Multiple sequence alignment of the 16S rDNA, *sec*A, *imp* and *SAP11* genes with 16S rDNA, *sec*A, *imp* and *SAP11* of phytoplasma GenBank available sequences (NCBI, Bethesda, MD, USA) (http://ncbi.nlm.nih.gov/ BLAST) were conducted using Clustal W (Thompson *et al*., 1994) and phylogenetic trees were made according to the neighbor-joining method using MEGA 6 software (Tamura *et al*., 2013). The bootstrap values are expressed as percentages of 1,000 replications. The trees of 16S rRNA and *sec*A genes were rooted using *Bacillus subtilis* GenBank accession numbers AB042061 and D10279, respectively.

Results and Discussion

The direct and nested PCR reactions of the 16S rRNA, *secA*, *imp* and *SAP11* genes showed positive results from all the symptomatic *V. encelioides* plants. The healthy *V. encelioides* plants and the water samples (negative control) did not show any amplification. The 16S rRNA, *secA*, *imp* and *SAP11* sequences of the four positive *V. encelioides* phytoplasma

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strains were deposited in GenBank under the accession numbers OQ623739 to OQ623742 for the 16S rRNA gene; OQ627208 to OQ627211 for *secA* gene; OQ603318 to OQ603321 for *imp* gene and OQ627204 to OQ627207 for *SAP11*. The sequences of each gene were 100% identical to each other. The BLAST search at NCBI of the 16S rRNA, *secA* and *imp* genes showed that the strains of *V. encelioides* phytoplasmas from this study had a 100% identity with 16SrII-D subgroup phytoplasma sequences. It is evident from the results of the phylogenetic trees of the 16S rRNA, *sec*A, *imp* and *SAP11* genes that *V. encelioides* phytoplasma from Oman clusters with *Scaevola taccada* phytoplasma from Oman (GenBank accession number AB257281) which is member of 16SrII-D subgroup (Figure 1).

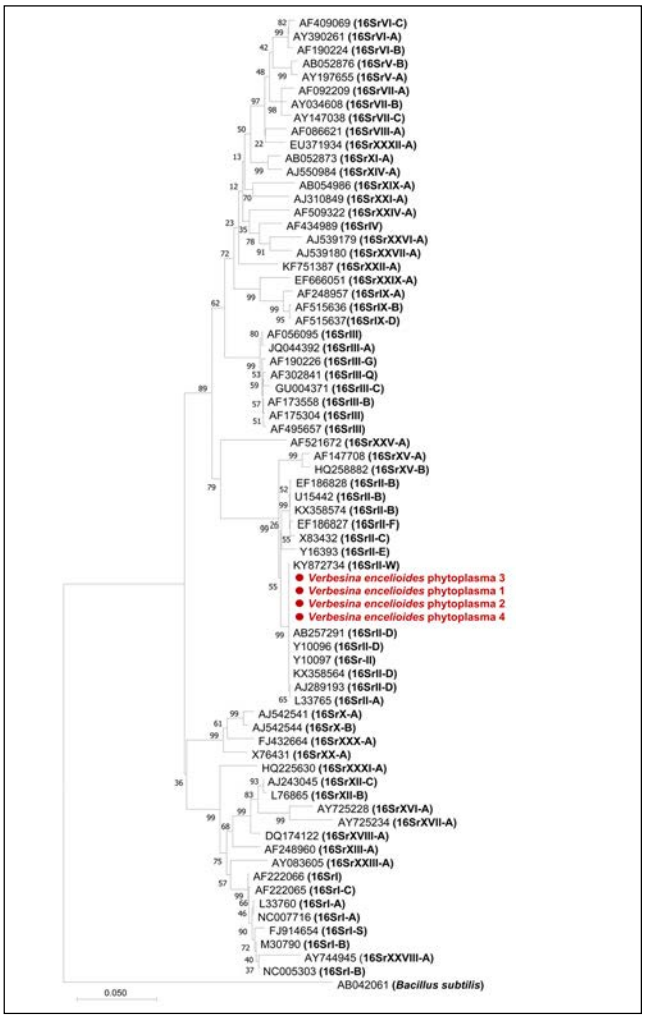


Figure 1. Phylogenetic tree constructed by neighbor-joining method of 16S rRNA sequences from *V. encelioides* phytoplasma.

The four *V. encelioides* phytoplasmas showed one mutation with 16SrII-D subgroup phytoplasma from Oman in the *SAP11* gene, the amino acid of SAP11 of all four *V. encelioides* phytoplasmas and 16SrII-D subgroup phytoplasmas from Oman strains were 100% identical (Figure 2).

```
16SrII-D          MQIKNKLYFLPLFLMSFLGLFLLININPVIAAPEKNDKGKKIASSEKQEKTTKKDISQYY
V. encelioides    MQIKNKLYFLPLFLMSFLGLFLLININPVIAAPEKNDKGKKIASSEKQEKTTKKDISQYY
                  ************************************************************

16SrII-D          ELYNTLENYSEEDRNKIIQMLSDSQTLKILQEEALKSKKKGSSSKKPDDSKK
V. encelioides    ELYNTLENYSEEDRNKIIQMLSDSQTLKILQEEALKSKKKGSSSKKPDDSKK
                  ***************************************************
```

Figure 2. Multiple sequence alignment of translated SAP11 sequences of *V. encelioides* phytoplasma along with the one of 16SrII-D subgroup phytoplasmas from Oman.

V. encelioides developed typical symptoms of phytoplasma infection. The symptoms of *V. encelioides* phyllody disease are similar to sesame phyllody disease (Al-Subhi *et al*., 2018) reported in Oman. The results of all four *V. encelioides* phytoplasma strains studied showed that they have almost the same DNA sequence of the 16S rRNA, *secA*, *imp* and *SAP11* genes sequences. Phylogenetic analysis clustered all *V. encelioides* phytoplasmas in the same subclade with phytoplasmas enclosed in 16SrII-D subgroup. Based on blast results of DNA sequences and phylogenetic analyses, the *V. encelioides* phytoplasma from Oman is member of 16SrII-D subgroup. This is the first report of a phytoplasma from 16SrII-D subgroup infecting *V. encelioides* plants.

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Worldwide

Phytoplasma diseases in Azerbaijan: an historical perspective

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Abstract

The review devotes to research on phytoplasma diseases in fruit trees and vegetables in Azerbaijan since 2003. In the frame of different projects, many surveys were undertaken and revealed various phytoplasmas that were identified and molecularly characterized. The use of molecular tools allowed to demonstrate that the Azerbaijanian phytoplasma strains were sometimes closely related if not identical to other strains detected in the Euro-Mediterranean basin. In the particular cases of '*Candidatus* Phytoplasma pyri' and '*Ca.* P. prunorum' strains with inter-species recombination were evidenced. Regarding the '*Ca.* P. solani' strains associated to grapevine "bois noir" cases in Absheron peninsula and Gabala, new and previous genotyping tests highlighted the high diversity of this phytoplasma in Azerbaïjan. Upon collections of Cixidae planthoppers conducted in northern Azerbaijan, *Hyalesthes obsoletus* and *Reptalus noahi* were shown to harbor two '*Ca.* P. solani' new genotypes phylogenetically distant from known genetic clusters.

Keywords: molecular epidemiology, solanaceous crop, fruit trees, insect vectors

Introduction

Phloem-limited bacteria had been observed by electron-microscopy since the early 1970s, in sieve-tube elements of declining or yellowing diseases affecting perennial and annual crops in the region of Ganja near the Georgian border. Since then, the advent of molecular techniques permitted to revisit former work.

Materials and Methods

A literature search was performed at the web address https://www.webofscience.com using all databases and the keywords phytoplasmas and Azerbaijan.

Results

In 2003, the first samples of fruit trees from the Quba region in the southern foothills of the Caucasus were collected and sent for analysis to INRA center in Bordeaux. First molecular detections of phytoplasmas were achieved and led to the initiation of a bilateral cooperation. The first studies have been devoted to the molecular detection, identification and genetic diversity of phytoplasmas associated with many different diseases in fruit trees and vegetable crops in Azerbaijan. The first field survey was conducted in the Absheron peninsula, Guba and Shaki regions of Azerbaijan. Phytoplasmas were detected in cherry, peach, pear, apricot, plum, cherry-plum and medlar tree samples and in vegetable crops, including eggplant, pepper and tomatoes using 16S rDNA nested PCR with universal primers for phytoplasmas R16mF2/R16mR1 and R16F2n/R16R2 (Gundersen and Lee, 1996). The identification realized through 16S rDNA RFLP and sequencing analysis demonstrated that '*Candidatus* Phytoplasma solani', '*Ca.* P. pyri', '*Ca.* P. prunorum', '*Ca.* P. brasiliense' were present in Azerbaijan (Balakishiyeva *et al.*, 2010; Aliyev *et al.*, 2015). In addition, '*Ca.* P. brasiliense' was reported for the first time in Eurasia. This phytoplasma, new for the old world, was detected in a peach tree in the Guba region of Azerbaijan. Detection and genotyping test were designed targeting the *dna*K gene previously cloned by comparative RAPD. Results showed the presence of two genetically distinct populations of '*Ca.* P. brasiliense', one being present in Azerbaijan (Balakishiyeva *et al.*, 2011).

'*Ca.* P. pyri' was detected in pear and '*Ca.* P. prunorum' in apricot, cherry-plum and plum trees (Balakishiyeva *et al.*, 2010). MLSA protocols based on the variability of *pnp*, *ace*F, *imp* and *sec Y* genes permitted to compare Azerbaijanian

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phytoplasma strains to other phytoplasmas detected in temperate fruit trees elsewhere in the Euro-Mediterranean basin (Danet *et al*., 2007; 2008; 2011). Genotyping revealed that Azerbaijanian '*Ca.* P. pyri' strains shared some alleles with '*Ca.* P. prunorum', suggesting the existence of inter-species recombination between '*Ca.* P. pyri' and '*Ca.* P. prunorum' (Danet *et al*., 2011).

In earlier surveys '*Ca.* P. solani' was detected in annual crops such as eggplant, pepper and tomato, and also in declining cherry, and common medlar trees. Genotyping on four non-ribosomal genes namely, *stamp*, *secY*, *vmp*l and *citS* was applied to these strains (Fabre *et al*., 2011; Balakishiyeva *et al*., 2018). The first grapevine "bois noir" (BN) cases in Gabala region and Absheron peninsula date 2015 (Balakishiyeva *et al*., 2016). Multilocus sequence analysis well as their common or different epidemiological cycles will help to control the impact of phytoplasmas and other phloem-limited bacteria in the context of global climate change.

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Worldwide

Important phytoplasma ribosomal subgroups distributed in Iran

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Abstract

So far, phytoplasmas enclosed in ribosomal groups and associated with phyllody, virescence, proliferation and sterility of flower, yellowing, little leaf, dwarfing, decline and witches' broom symptoms were identified in Iran. These groups are 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXIV, 16SXXIX and 16SrXXX. The most widespread phytoplasmas belong to 16SrI, 16SrII, 16SrVI, 16SrIX and 16SrXII groups, whilst the 16SrII phytoplasma group is the one with most numerous associated diseases. The most economically and environmentally spread phytoplasma ribosomal subgroups in Iran are reviewed.

Keywords: phytoplasma diseases, ribosomal groups, economic damage

Introduction

Phytoplasmas are wall-less plant-pathogenic bacteria, associated with a wide range of plant diseases worldwide. They are transmitted mainly by leafhoppers and psyllids. Currently 49 '*Candidatus* Phytoplasma' species were designed based on 16S rRNA gene sequences (Bertaccini *et al.*, 2022). In Iran, although phytoplasmas and their associated diseases have been observed in sesame since 1965 as "green flowering of sesame" the agent associated with the disease and its insect vector were reported for the first time in 1992.

Materials and Methods

Phyllody, virescence, proliferation and sterility of flower, yellowing, little leaf, dwarfing, decline and witches' broom were the main symptoms associated with phytoplasma in Iran reported. Based on biological and molecular studies (direct and nested PCR, real and virtual RFLP, phylogenetic and 16S ribosomal DNA and non-ribosomal gene comparison) in samples from various symptomatic plant species collected in different geographic areas of Iran, the phytoplasma subgroup identification was provided.

Results

The identified phytoplasmas enclosed in 16SrI, 16SrII, 16SrVI, 16SrIX and 16SrXII groups are summarized. The diseases are listed according with phytoplasma ribosomal subgroup identified (Esmaeilzadeh-Hosseini *et al.*, 2023; Siampour *et al.*, 2019).

Aster yellows, '*Ca.* P. asteris'. 16SrI-B: *Allium cepa* yellows; *Eruca sativa* phyllody; *Eucalyptus camaldulensis* little leaf; *Lactuca sativa* phyllody; *Morus alba* witches' broom; *Prunus armeniaca* decline, in mixed infection with 16SrXII-A subgroup; *Rosa canina* yellows; *Solanum tuberosum* purple top; *Sonchus oleraceus* phyllody; *Tamarix aphylla* and *Tragopogon dubius* witches' broom; *Vitis vinifera* yellows; *Ziziphus jujube* witches' broom and yellowing; 16SrI-F: *Catharanthus roseus* little leaf; *Lolium rigidum* dwarfing. 16SrI-R: *Aquilegia vulgaris* phyllody. 16SrI-S: *Calendula officinalis* phyllody.

Peanut witches' broom, '*Ca.* P. aurantifolia'. 16SrII-B: *Citrus aurantifolia* witches' broom. 16SrII-C: *Alhagi maurorum* witches' broom; *Aerva javanica* witches' broom and little leaf; *Artemisia sieberi* and *Citrofortunella floridana* witches'

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broom; *Citrus limettioides* phytoplasma associated with "huanglongbing"-like symptoms; *C. medica, C. paradisi, C. reticulata*, *C. sinensis*, *Convolvulus virgatus* and *Daucus carota* witches' broom; *Malus pumila* yellowing and leaf rolling; *Medicago sativa* and *Nicotiana tabacum* witches' broom; *Prunus armeniaca* leaf roll; *P. domestica* decline; *P. dulcis* and *P. persica* witches' broom; *Solanum lycopersicum* witches' broom; *Vicia faba* phyllody.

Papaya yellows, '*Ca.* P. australasia'. 16SrII-D: *Artemisia sieberi* witches' broom and little leaf; *Beta vulgaris* sbsp. *esculenta* and *B. vulgaris* sbsp. *vulgaris* witches' broom; *Calendula officinalis* phyllody; *Capsicum annuum* big bud; *C. roseus* phyllody; *Cosmos bipinnatus* phyllody; *Cucumis sativus*, *Cucurbita pepo* and *Heliopsis helianthoides* phyllody; *Medicago sativa, Peganum harmala*, *Petroselinum crispum*, and *Petunia hybrida* witches' broom; *Prunus dulcis* dieback; *Punica granatum* and *Saccharum officinarum* little leaf; *Sesamum indicum* phyllody; *Solanum lycopersicum* leaf roll; *Solanum melongena* phyllody; *Sophora alopecuroides* dwarfing and yellowing; *Tamarix aphylla, Taraxacum officinale* and *Taverniera cuneifolia* witches' broom; *Vicia faba* and *Zinnia elegans* phyllody. 16SrII-V: *Solanum melongena* phyllody. 16SrII-Z: *Helianthus annuus* phyllody.

Clover proliferation, '*Ca.* P. trifolii'. 16SrVI-A: *Brassica napus* witches' broom; *B. oleracea* yellows; *Calendula officinalis* and *C. roseus* phyllody; *Citrullus lanatus* and *Cota tinctoria* witches' broom; *Cucumis sativus* phyllody; *Erigeron canadensis* leaf malformation and witches' broom; *Glycine max* bud proliferation and aborted seed pods; *Juniperus procumbens* and *Medicago sativa* witches' broom; *Phoenix dactylifera* yellowing; *Pinus brutia* witches' broom; *Sesamum indicum* phyllody; *Solanum lycopersicum* big bud, flower virescence, phyllody and little leaf; *Solanum melongena* phyllody; *Sophora alopecuroides* dwarfing and yellowing; *Tamarix aphylla* witches' broom; *Vigna unguiculata* phyllody; *Vitis vinifera* yellows; *Zea mays* yellowing and dwarfing; *Ziziphus jujube* witches' broom and yellowing. 16SrVI-D: *Carthamus tinctorius* phyllody; *Prunus dulcis* witches' broom; *Rubia tinctorum* little leaf; *Solanum tuberosum* witches' broom. 16SrVI-F. *Sophora alopecuroides* dwarfing and yellowing.

Pigeon pea witches' broom. 16SrIX-A: *Conocarpus erectus* stem fasciation. '*Ca.* P. phoenicium'. 16SrIX-B: *Daphne mucronata* phyllody; *Prunus amygdalus* x *P. persica* (GF-677) witches' broom; *P. armeniaca* leaf roll and rosette; *P. domestica, P. dulcis*, *P. persica* witches' broom; *P. persica* witches' broom (variant of 16SrIX-B); *P. scoparia* witches' broom. 16SrIX-C: *Cannabis sativa* and *Prunus dulcis* witches' broom; *P. persica* yellowing; *Reseda lutea* phyllody; *Robinia pseudoacacia* yellows; *Sesamum indicum* phyllody; *Solanum lycopersicum* flower virescence; *S. melongena* phyllody; *Sophora alopecuroides* yellowing stunt and phyllody; *Vitis vinifera* yellows. 16SrIX-D: *Prunus dulcis* and *P. scoparia* witches' broom. IX-E: *Cicer arietinum* witches' broom. IX-H: *Sophora alopecuroides* dwarfing and yellowing. 16SrIX-I: *Coreopsis grandiflora* phyllody; *Convolvulus glomeratus* witches' broom; *Onobrychis viciifolia* leaf yellowing; *Lactuca sativa* phyllody; *L. serriola* phyllody; *Solanum melongena* big bud and phyllody.

"Stolbur", '*Ca.* P. solani' 16SrXII-A: *Alhagi maurorum* witches' broom; *Capsicum annuum* "stolbur"; *Convolvulus arvensis* witches' broom; *C. arvensis* witches' broom (mixed infection with 16SrXXIX-B subgroup); *Cucumis sativus* phyllody; *Eucalyptus camaldulensis, Juglans regia* and *Medicago sativa* witches' broom; *Narcissus tazetta* phyllody; *Pistachia vera* yellowing and scorch; *Prunus armeniaca* decline (mixed infection with 16SrI-B subgroup); *Solanum lycopersicum* and *S. melongena* big bud; *S. tuberosum* purple top; *Sophora alopecuroides* dwarfing and yellowing; *Vitis vinifera* yellows.

Discussion

So far, phytoplasma studies in Iran were mainly based on electron microscopy, transmission assays, host range and symptomatology, PCR and RFLP analysis. The study on 16S rRNA gene as well as of non-ribosomal genes and sequencing lead to identification of phytoplasmas in 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXIV, 16SXXIX and 16SrXXX groups in some cases in mixed infection. The most numerous phytoplasma subgroups detected are 16SrI-B, 16SrII-D, 16SrVI-A, 16SrIX-C and 16SrXII-A. Among the reported phytoplasma groups, 16SrII (peanut witches' broom) is the most important and widespread in Iran. The most reported areas in which phytoplasmas of this group were identified are the central and southern regions, which have tropical and subtropical climates. Lime and alfalfa witches' broom harboring 16SrII phytoplasmas are very destructive and economically the most important phytoplasma diseases in Iran. The other subgroups reported in Iran (16SrIII, 16SrVII, 16SrX, 16SXI, 16SrXIV, 16SXXIX and 16SrXXX), seem to be of limited or localized presence.

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Worldwide

Occurrence of a '*Candidatus* Phytoplasma asteris' strain associated with onion yellows disease in Iran

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Abstract

In a 2022 survey for phytoplasma diseases, onion yellows symptoms were observed in seed production fields of Zarghan, Beedzard and Kavar areas (Fars province, Iran). The main symptoms were virescence, phyllody, yellowing, witches' broom and sterility. The disease incidence was recorded up to 4%. Nested polymerase chain reaction using P1/P7 followed by R16F2n/R16R2 primer pairs was carried out with DNAs extracted from twelve symptomatic and four symptomless onion plants for phytoplasma detection. DNA fragments of about 1.8 and 1.25 kbp respectively were obtained from symptomatic onion plants but not from the symptomless ones. The R16F2n/R16R2 sequences of twelve samples showed 100% identity with each other and a representative of these sequences from Zarghan (strain ZOY) was deposited in GenBank under accession number OQ332407. BLASTn analysis of this 16S rRNA sequence showed 100% identity with '*Candidatus* Phytoplasma asteris'. Phylogenetic analysis using neighbor-joining method showed that ZOY phytoplasma strain clustered within members of subgroup 16SrI-B. Computer-simulated analysis using *i*PhyClassifier also showed that the RFLP pattern of the ZOY phytoplasma 16S rRNA gene was identical to the reference strain of 16SrI-B subgroup. This is the first detection of a 16SrI-B phytoplasma strain in onion yellows in Iran.

Keywords: aster yellows, PCR, RFLP, Fars province, phytoplasmas

Introduction

Phytoplasmas are wall-less plant-pathogenic bacteria, associated with a wide range of important plant diseases worldwide. In nature, they are transmitted mainly by leafhoppers and psyllids. Currently, 49 '*Candidatus* Phytoplasma' species have been proposed based on 16S rRNA gene sequence comparison (Bertaccini *et al*., 2022). The aster yellows phytoplasma group (16SrI), with numerous subgroups, is the prevalent phytoplasma group, as it infects a broad range of host plants, including monocotyledonous and dicotyledonous species of herbaceous and woody plants. Onion (*Allium cepa*) is an important crop in Iran. The present work reports onion yellows disease association with a 16SrI-B subgroup phytoplasma in Iran.

Materials and Methods

In 2022, a survey was conducted in Fars province (Iran) onion seed production fields for phytoplasma diseases. Twelve symptomatic and four symptomless onions were selected for DNA extraction and phytoplasma detection. Extracted DNA samples were subjected to direct and nested PCR using primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996). Virtual RFLP, phylogenetic and DNA identity analyses of partial 16S ribosomal sequences were used to identify the detected phytoplasma.

Results and Discussion

In 2022 surveyed areas of Fars province, a disease tentatively named yellows was observed in seed production fields of onion in Zarghan, Beedzard and Kavar areas (Fars province, Iran). The main symptoms were virescence, phyllody, yellowing, witches' broom and sterility (Figure 1). The disease incidence in a 3-ha field was recorded up to 4%. After 35 cycles of direct and nested PCR, DNA fragments of about 1.8 and 1.25 kbp respectively were obtained from symptomatic onion but not from symptomless ones. R16F2n/R16R2 sequences of amplicons from twelve samples showed 100% identity to each other and a representative of these sequences

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from Zarghan (ZOY) was deposited in GenBank (accession number OQ332407). BLASTn (www.ncbi.nlm.nih.gov) analysis of ZOY phytoplasma sequence showed 100% identity with '*Candidatus* Phytoplasma asteris' (GenBank accession number M30790), *Tragopogon dubius* phyllody phytoplasma strain Bajgah (GenBank accession number KR262955) and members of subgroup B of 16SrI group.



Figure 1. Symptoms of onion yellows including flower virescence, phyllody and witches' broom in a diseased inflorescence of an onion plant from Zarghan area on the right. Healthy inflorescence on the left.

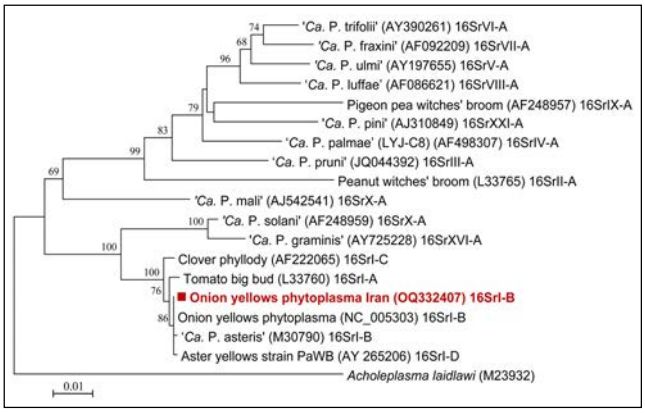


Figure 2. Phylogenetic tree of partial 16S rDNA sequences from the onion yellows phytoplasma from Iran (in red) and selected phytoplasmas from GenBank. The tree was constructed by the neighbour-joining method. GenBank accession numbers are shown in parentheses. Numbers on branches are bootstrap values of 1,000 replicates. *Acholeplasma laidlawii* was used as outgroup.

Phylogenetic analysis using neighbor-joining method (MEGA7) showed that ZOY phytoplasma strain clustered within 16SrI group closest to onion yellows phytoplasma (NC_005303), a 16SrI-B subgroup strain (Figure 2). Computer-simulated analysis with 17 restriction endonucleases using *i*PhyClassifier program showed that the RFLP pattern of the ZOY phytoplasma 16S rRNA gene was identical to the one of '*Ca.* P. asteris' reference strain (16SrI-B subgroup, GenBank accession number M30790) with a similarity coefficient of 1. This is the first report of a '*Ca.* P. asteris' strain associated with onion yellows disease in Iran. Formerly *Allium cepa* flower malformation was reported from Isfahan province (Vali Sichani *et al.*, 2014) but the phytoplasma subgroup was not determined. *Eruca sativa* phyllody (Esmailzadeh Hosseini *et al.*, 2017), *Eucalyptus camaldulensis* little leaf (Salehi *et al.*, 2016), *Vitis vinifera* leaf yellowing and reddening (Babaie *et al.*, 2019) and *Ziziphus jujube* witches' broom and yellowing (Babaei *et al.*, 2020) are the most important diseases associated with 16SrI-B phytoplasma diseases in Iran.

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Worldwide

Multilocus sequence analysis of phytoplasmas associated with cucurbitaceous crops in Kerman province of Iran

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Abstract

The cucurbitaceous fields in Kerman province of Iran were surveyed for phytoplasma diseases. The phytoplasmas associated with symptomatic plants were detected and molecularly characterized by PCR tests using amplification of 16S rRNA, *rp* and *secY* genes carried out with phytoplasma universal and group specific primers. BLAST searches and *i*PhyClassifier analyses on the obtained sequences indicated that the phytoplasmas associated with cucumber, cantaloupe and zucchini plants showing phyllody, yellowing, little leaf and fruit malformation symptoms were identical and belonged '*Candidatus* Phytoplasma australasia', subgroup 16SrII-D.

Keywords: cucurbits, melon, cucumber, phyllody, yellowing

Introduction

About 32% of Iran's agricultural products belong to vegetables including cucumber, watermelon, cantaloupe, pumpkin and so on. Kerman, located in south of the country, is ranked first for vegetable production among the provinces of Iran with a production of more than 495,000 tons per year (Ahmadi *et al*., 2022). Cucurbitaceous crops are widely grown throughout the province under low or high plastic tunnels, under greenhouse conditions or in open fields during all the different seasons. Yellowing, little leaf, phyllody, virescence, proliferation, witches' broom and stunting are the predominant symptoms observed in phytoplasma infected cucurbitaceous plants in the world.

Materials and Methods

Surveys were conducted for phytoplasma diseases of cucurbitaceous crops in Kerman province of Iran. Total DNA was extracted from leaf midrib tissues of the collected samples by a CTAB method (Zhang *et al*., 1998). PCR assays were performed to amplify the 16S rRNA, *rp* (ribosomal protein) and *secY* genes using universal and group specific phytoplasma primer pairs P1/P7 (Deng and Hiruki 1991; Schneider *et al*. 1995) and R16F2n/R16R2 (Gundersen and Lee 1996), rp(II)F1/rp(II)R1 (Martini *et al*., 2007) and SecYF1(II)/SecYR1(II) (Foissac *et al*., 2013), respectively. The data obtained from nucleotide sequences were edited by BioEdit (Hall, 1999), and analysed using BLAST (http://www.ncbi.nlm.nih.gov/blast) and *i*PhyClassifier (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) tools for phytoplasma 16Sr group and subgroup determination.

Results

Cucumber, zucchini and Cantaloupe plants showing phyllody, little leaf, yellowing, fruit cracking and malformation were observed in Kerman province (Figure 1). The expected PCR products of 1.8 kb and 1.25 kb were amplified in direct and nested PCR assays using P1/P7 and R16F2n/R16R2 primers, respectively for the symptomatic samples but not in the healthy one.



Figure 1. Little leaf, phyllody and fruit malformation of cucumber (a); yellowing and fruit malformation of zucchini (b); yellowing and fruit malformation of Cantaloupe (c) in Kerman province of Iran.

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Analysis of the obtained 16S rDNA sequences by *i*PhyClassifier indicated that the phytoplasmas detected in cucumber, zucchini and cantaloupe were identical and showed the highest similarity (99%) to 16S rRNA gene sequence of subgroup D of 16SrII reference strain (GenBank accession number Y10097). Sequence analyses of the *rp* (1.2 kb) and *secY* gene (1.6 kb) amplicons confirmed that the phytoplasmas associated with cucumber, zucchini and Cantaloupe plants are clustering with phytoplasmas enclosed in the 16SrII-D subgroup.

Discussion

Greenhouse cucumber phyllody was the first cucurbitaceous phytoplasma disease reported from Iran. Although the phytoplasma group was not determined the associated phytoplasma was experimentally transmitted by *Orosius albicinctus* (Azadvar *et al*., 2005). Sesame, tomato, eggplant and alfalfa seem to be the most important non-cucurbitaceous and alternative hosts for 16SrII-D subgroup phytoplasmas in the Kerman province. Diseases associated with phytoplasmas of group 16SrII are the prevalent in the south of Iran (Siampour *et al*., 2019). Distinct phytoplasma groups/subgroups including 16SrI-B, 16SrI-X, 16SrII, 16SrII-M, 16SrII-V, 16SrIII and 16SrIV-A have been reported in cucurbitaceous plants in different countries (Montano *et al*., 2006, 2007; Dehghan *et al*., 2015; Salehi *et al*., 2015; Tripathi *et al*., 2017; Rao *et al*., 2017; Venkataravanappa *et al*., 2017; Ghayeb Zamharir and Azimi, 2018; Kumari *et al*., 2019; Weng *et al*., 2021; Xu *et al*., 2021).

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Worldwide

Detection of 16SrII-D phytoplasmas associated with tomato little leaf in Eastern Uttar Pradesh

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Abstract

During field surveys in Rajahi village, Gorakhpur, Uttar Pradesh little leaf symptoms were noticed in cultivated tomato varieties with an incidence of 3-5%. With PCR and nested PCR assays using phytoplasma-specific primer pairs P1/P7 and R16F2n/R16R2, 1.2 kb amplicons were obtained and in symptomatic samples phytoplasma presence was confirmed. *In silico* RFLP analysis of the 16Sr RNA sequence directed its assignment to the 16SrII-D subgroup. This is the first report of tomato little leaf disease from Eastern Uttar Pradesh confirming the widespread and increased incidence of phytoplasma diseases in the tomato growing areas of the Country causing severe yield loss.

Keywords: tomato, molecular identification, PCR, RFLP, phylogenetic analysis, yield loss

Introduction

Tomato (*Solanum lycopersicum* L.), a crop of agricultural significance, originated from South America, is grown widely around the globe. India being its second largest producer after China at global level, with 19.32 t/ha production. As every crop experiences abiotic and biotic stresses, there are various constraints affecting tomato yield and quality as well, among which phytoplasma presence is emerging as a major threat (Kumari *et al*., 2019). In tomato six ribosomal subgroups of phytoplasmas have been reported: 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrXII (Bertaccini *et al*., 2021). Among all these groups, the 16SrII has proven to be most devastating for tomato with repeated disease incidence (Rao, 2021). Tomato little leaf is emerging as an alarming disease in Uttar Pradesh region. It has been reported from western Uttar Pradesh (Singh *et al*., 2012) with incidences of 5-8%. During a survey in Gorakhpur district of eastern Uttar Pradesh severe symptoms of little leaf were discovered in tomato fields of Rajahi village. The disease incidence recorded was 3-5%. In the present study tomato plants showing little leaf symptoms were tested for phytoplasma detection and identification.

Materials and Methods

Tomato crops exhibiting little leaf symptoms were sampled from Rajahi village fields near Gorakhpur, Uttar Pradesh, India in 2022. Sample collection was done from three symptomatic and two asymptomatic plants. Leaf samples (100 mg) were used to extract DNA utilizing a CTAB method (Ahrens and Seemüller, 1992). PCR assay was performed using universal primer pairs for phytoplasmas P1/P7 (Deng and Hiruki 1991; Schneider *et al*., 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996) for nested PCR. Electrophoresis of the nested PCR products was performed in a 1% (w/v) agarose gel and results were observed under UV transilluminator (REMI India), ethidium bromide was used for staining the gel. Three nested PCR amplicons were purified (Wizard SV gel kit, Promega) and sequenced (Eurofins, India). The sequences were assembled using BioEdit software (Hall, 1999), aligned using CLUSTAL W and deposited in NCBI GenBank after editing. The aligned sequence and those of phytoplasma strains retrieved from GenBank were utilised for phylogenetic tree construction using Neighbour-Joining method with 1,000 replicates using MEGA 11 software (Tamura *et al*., 2021). *Acholeplasma laidlawii* (GenBank accession number NR074448) served as outgroup for rooting the tree. Aligned sequences were used for *in silico* RFLP analysis and 16Sr subgroup attribution using the *i*PhyClassifier online tool (Zhao *et al*., 2009).



Figure 1. Infected tomato plant showing little leaf symptoms (right).

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Results

Extensive little leaf symptoms (Figure 1) were observed in tomato crops at Rajahi village, Gorakhpur. After DNA extraction, nested PCR in all the symptomatic samples yielded amplicons of 1.25 kb. Meanwhile no amplification was recorded from asymptomatic samples.

To further characterize the tomato little leaf (TLL) phytoplasma strain, the obtained 1.25 kb amplicons were sequenced and the sequence was deposited to GenBank under the accession number OQ519880. In BLASTn search comparison, the obtained sequence showed 98% identity with 16S rRNA gene sequence of tomato witches' broom strain TWB-Eg from Egypt (GenBank accession number KT225548). Phylogenetic analysis confirmed this clustering (Figure 2). In *in silico* RFLP analysis, TLL sequence showed restriction profiles most similar is the reference pattern of the 16Sr group II, subgroup D (GenBank accession number Y10097), with a similarity coefficient of 0.71.

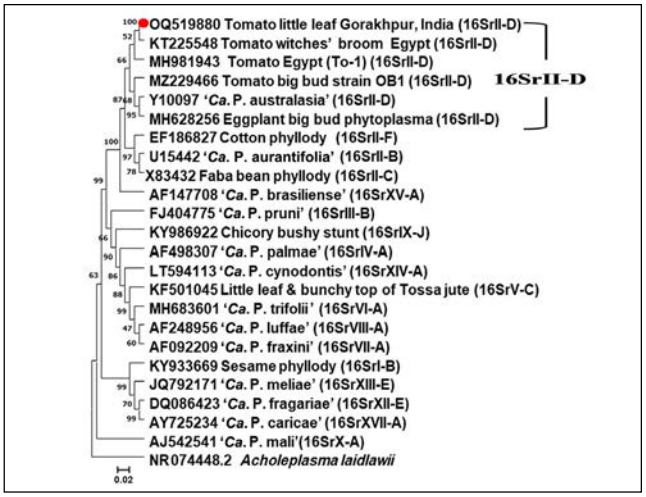


Figure 2. Phylogenetic tree showing the relationship of tomato little leaf sequence with other phytoplasma sequences. Red circle: sequence obtained in this study.

Discussion

In this study virtual RFLP analysis suggested a tentative assignment to 16SrII-D subgroup of the phytoplasma infecting tomatoes, however more phytoplasma strains should be sequenced in order to cross verify the phytoplasma identity. This is first report of tomato little leaf disease from eastern Uttar Pradesh. Phytoplasmas belonging to subgroup 16SrII-D are the most widespread among vegetable crops (Reddy *et al.*, 2021) and were reported from various parts of India namely, western Uttar Pradesh, Karnataka and Telangana (Singh *et al.*, 2012; Swarnalatha and Reddy, 2014; Kumari *et al.*, 2018; Venkataravanappa *et al.*, 2018). The rapid expansion of this disease to new geographical areas demands detailed investigation regarding the distribution, identification of associated phytoplasma strains, epidemiological factors involved and its sources to adopt well focused management measures.

Acknowledgements

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Detection

Development of multilevel monitoring systems for the identification of phytoplasma diseases in German viticultural areas

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Abstract

"Flavescence dorée" (FD) and "bois noir" (BN) are the most important phytoplasma diseases of grapevine. While BN is widespread in German winegrowing regions, only one single grapevine plant infected with FD phytoplasma has been reported and eradicated in 2020. Although the main vector of FD, *Scaphoideus titanus*, is not present in Germany, the FD phytoplasma is classified as quarantine pest because of its epidemic potential and a systematic monitoring of the disease and the vector is mandatory in Germany. Furthermore, a non-epidemic strain Palatinate grapevine yellows (PGY), with the same symptomatology as FD and BN is present in Germany. For large scale monitoring digital multilevel monitoring systems for grapevine yellows (GY) are being developed. Reference plots with infection by either BN or PGY in risk zones in southwestern Germany and Württemberg have been visually monitored and symptomatic grapevines were analysed by molecular means. In parallel, the same samples were examined with different sensor-based methods to define a correlation between phytoplasma presence and specific spectral signatures. A spectral distinction to similar symptoms due to virus infection, insect damage, and nutritional deficiencies was investigated for the system validation.

Keywords: grapevine yellows, multispectral analysis, hyperspectral analysis, remote sensing

Introduction

Phytoplasmas associated with severe diseases of grapevine are responsible for an important economic loss in many vine-growing areas worldwide. "Bois noir" (BN) is associated with '*Candidatus* Phytoplasma solani' and transmitted by cixiid vectors. The most devastating phytoplasma disease in Europe, "flavescence dorée" (FD), is associated with subgroups 16SrV-C and -D and epidemically transmitted by *Scaphoideus titanus*. While BN is widespread in German vineyards, so far only a single grapevine in a commercial vineyard was found infected with FD in Germany and it was immediately eradicated in 2020 (Jarausch *et al.*, 2021). Probably, the phytoplasma was transmitted from an infected European alder nearby as proposed by Malembic-Maher *et al.* (2020). As *S. titanus* is not present in Germany, the risk for an epidemic outbreak of the disease is negligible. The current situation in German vineyards is composed of a spread of BN, a limited appearance of PGY (Palatinate grapevine yellows) and no known presence of FD. Nevertheless, since FD phytoplasma is a regulated quarantine pest in the European Union (EU, 2019/2072), a systematic monitoring of FD is required.

Sensor-based methods have been widely applied for the non-invasive analysis of different plant diseases as leaf spectral patterns change upon an infection and during symptom development (Mahlein *et al.*, 2016). Thereby, either the entire spectral region (hyperspectral) or selected spectral bands (multispectral) can be used for disease detection. The development of spectral disease indices for field detection of FD was successfully achieved by Al-Saddik *et al.* (2019). In addition, Bendel *et al.* (2020) identified significant wavelengths for the differentiation symptomatic leaves of BN or PGY infected grapevines from those of healthy ones. Albetis *et al.* (2017) tested the air-borne detection of FD symptoms under field conditions using multispectral imaging. Based on those promising approaches, the aim of

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this study is to develop a feasible multilevel monitoring system to facilitate the detection of phytoplasma infection under field conditions.

Materials and Methods

Selection of BN- and PGY-infected reference vineyards was done with the help of geo-data based risk maps of vineyards adjacent to alder stands (Jarausch *et al*., 2018). Sampling symptomatic grapevines was carried out in late summer of 2021 and 2022. Total nucleic acids were extracted as previously described (Maixner *et al*., 1995), and group-specific primers were used for detection of phytoplasmas. The differentiation between FD and PGY strains was based on the amplification and sequencing of the secY-map locus (*map* gene) according to Malembic-Maher *et al*. (2020).

Hyperspectral images of grapevine leaves were taken in the visible-near infrared range VIS-NIR (400-1000 nm). The images were taken of 100 control and 100 symptomatic leaves from six phytoplasma-infected vineyards, two with virus infection, two leaf hoppers-infested vineyards, two Mg- and two Fe-deficient vineyards. Half of the vineyards were white varieties and the rest were red varieties. For hyperspectral imaging the camera FX10 (Specim) was used.

For the acquisition of air-borne hyperspectral data, an integrated system consisting of the Headwall Co-Aligned VNIR-SWIR Sensor and the DJI M600 pro drone was used. In addition, to two hyperspectral sensors in the VNIR range (400 -1000 nm) and SWIR range (900 – 2500 nm), a GPS system (Applanix APX 15 AV) and a LiDAR (Velodyne Puck Lite) are built-in. GPS and LiDAR data were processed to a DEM (digital elevation model) to orthorectify the hyperspectral images. This data is used to train a machine learning algorithm using the perClass Mira software to identify vines showing phytoplasma symptoms in real time.

For ground-based imaging a quad was equipped with a 5-channel MSI camera system (FS-3200T-10GE-NNC) which provides RGB channels and two channels in NIR range (400-1000nm) and a time-of flight camera (Helios 2+HTP003S-001). An inertial navigation system (SBG, Ellipse-D) was installed for localization. In combination with two antennas, the unit is able to estimate the position and the orientation of the vehicle with an accuracy of a few cm. This enables precise georeferencing of the acquired multispectral data and derived parameters.

Results and Discussion

During the years 2021 and 2022, a total of 595 symptomatic grapevines have been sampled in reference plots in the Palatinate region and 110 in Württemberg, respectively. The molecular analysis resulted in the detection of 20 grapevines infected with 16SrV-group phytoplasmas in the Palatinate region. Based on *map* gene differentiation all of them were PGY genotypes. A total of 514 samples were infected with 16SrXII-group phytoplasmas. For Württemberg, all samples were infected with BN, no FD-related phytoplasmas were detected. This indicates a very low incidence of 16SrV-group phytoplasmas despite adjacent alder trees harbouring FD-related agents. On the other hand, despite low populations of *Hyalesthes obsoletus*, BN is spreading in the examined areas. Current studies are focused on other host plants as reservoir for '*Ca*. P. solani' phytoplasmas and alternative insect vectors.

Hyperspectral imaging in the laboratory has been performed on infected and control grapevines directly after sampling and prior extraction. Annotation of the images and the following image analysis is in progress. Air-borne hyperspectral data via drone were recorded from ten vineyards with phytoplasma infections in Württemberg and from two sites in the Palatinate region. Ground-based multispectral data via quad were taken in 13 vineyards with a total of 10 varieties. Image analysis is in progress.

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Detection

Digital phytoplasmology: remote sensing of fruit tree phytoplasma diseases

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Abstract

Apple proliferation (AP) and pear decline (PD) are important diseases of fruit trees in Europe that are difficult to control. Timely uprooting of infected trees is a successful measure to limit the spread of the diseases and is therefore mandatory in some production areas. Large-scale monitoring strategies are required. AP and PD induce in infected trees an early leaf reddening in late summer or early autumn, which is suitable to be monitored by remote sensing. Investigations were carried out to find applicable strategies for remote sensing of AP and PD in the heavily affected fruit-growing region of Palatinate in Southwest Germany. In 2019-2022, more than 20,000 apple trees and more than 6,300 pear trees were monitored for partial or entire leaf reddening. A very high correlation to AP was found according to specific symptom recording and PCR testing of '*Candidatus* Phytoplasma mali' and a high correlation to PD according to PCR testing of '*Ca.* P. pyri'. Hyperspectral images from infected and healthy leaves of apple and pear were analysed and specific spectral differences were identified. Remote sensing of AP- and PD-infected trees, respectively, was achieved by drone flights with hyperspectral or multispectral cameras in reference orchards. To upscale the remote sensing, multispectral satellite images covering the region of the reference orchards were analysed and AP- and PD-infected trees, respectively, could be identified. The accuracy of remote sensing relies on the timing when phytoplasma-induced leaf reddening appears before leaf fall in autumn.

Keywords: apple proliferation, pear decline, hyperspectral analyses, drone, satellite

Introduction

Apple proliferation (AP) and pear decline (PD) are among the economically most important diseases of apple and pear in Europe. Different psyllid vectors transmit both '*Candidatus* Phytoplasma mali' and '*Ca.* P. pyri' very efficiently. Therefore, timely uprooting of infected trees is an efficient strategy to limit the spread of the diseases. In some apple growing regions, it is even mandatory. For this, large-scale monitoring strategies are required, *e.g.* based on spectral data. Spectral disease indices for field detection of "flavescence dorée" were achieved by Al-Saddik *et al.* (2019). The strategy to use unmanned aerial vehicles (UAV) for remote sensing of grapevine yellows-infected grapevine was applied by Albetis *et al.* (2017).

While symptoms of AP like enlarged stipules are very specific but difficult to observe, PD symptoms were considered so far unspecific. However, both diseases induce characteristic early reddening of leaves in late summer to early autumn. These reddening was used for remote sensing detection of AP and PD infected plants. The experimental area was the historically heavily AP- and PD-affected fruit-growing region of Palatinate in Southwest Germany. After confirmation of a high correlation of red-coloured leaves of apple or pear and the presence of the respective pathogen, hyperspectral images were taken from infected and healthy leaves. In addition, AP- or PD-infected reference orchards were analysed by drone flights with a hyperspectral camera. As infected and healthy trees could be differentiated by their spectra, first applications of remote sensing by drone flights with a multispectral camera were evaluated. In a larger scale, multispectral satellite images were used to identify AP- or PD-infected trees in the reference orchards. Finally, for optimal use of the remote sensing strategies the best timing to identify AP-infected trees or PD-infected trees was analysed.

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Materials and Methods

Monitoring AP and PD was done in four local fruit-growing regions of Palatinate in 2019 – 2022. To find a high percentage of infected trees, monitoring was focused on older orchards ranging from approximately 10 – 50 years of age. AP-infection was confirmed either by specific symptom recording or PCR testing. PD-infection was exclusively confirmed by PCR. Standard PCR using primers fO1/rO1 in total nucleic acid extracts of branch phloem preparations as described in Jarausch *et al.* (2011) was applied to detect both pathogens.

Laboratory hyperspectral analyses on apple and pear leaves were done with a dual hyperspectral camera system consisting of a HySpex VNIR-1800 and a HySpex SWIR-384 from Norsk Elektro Optikk AS with wavelength ranges of 400 – 1,000 nm and 950 – 2,500 nm, respectively. The reflectances of the leaves were determined relative to a reference target. Using patch-wise sampling across the leaves, several averaged samples were pulled for each leaf and their spectra were investigated. Machine learning was used to discriminate between infected and healthy leaves and a relevance analysis of the complete spectral signatures based on feature importance of the underlying machine learned model was performed for further usage in drone measurements.

For the transfer from laboratory measurements to field applications, hyperspectral drone measurements on reference orchards were done using a specifically designed drone in combination with a S185 camera from Cubert GmbH, measuring a spectral range of 450 950 nm. Thereby, challenges by the transfer to field measurements could be evaluated, as for example spectral mixing induced by resolution, light variations, atmospherically influences and the varying distribution of symptoms in a whole tree. In the end, multispectral measurements were done with the DJI P4 Multispectral drone including an RGB camera as well as five separate cameras for 450 nm (blue), 560 nm (green), 650 nm (red), 730 nm (red edge) and 840 nm (NIR). Additionally, the DJI P4 includes an RTK-functionality increasing the GNNS accuracy so that an easy assignment to single trees is possible. The data was processed photogrammetrically by the software Agisoft Metashape and further investigated by using suitable spectral indices and machine learning. Tree-wise PCR tests served as target values for the underlying classification task.

Satellite data with different spectral and spatial resolutions were used with the aim of detecting the typical symptoms of the diseases in the apple and pear orchards. The temporal prognosis of the leaf reddening determined the start of the satellite monitoring in the years 2019-2022. Images from the PlanetScope and SkySat satellites (Planet Labs PBC) as well as WorldView-2 and -3 (Maxar Technologies), acquired specifically for the project by the satellite operating companies, were included in the investigations. The images provided data from visual and near-infrared spectral channels at spatial resolutions between 15 cm and 3 m for the analyses.

Results and Discussion

Leaf reddening of apple in early autumn was correlated to 96% to AP-specific symptoms. Furthermore, '*Ca.* P. mali' was detected by PCR in 82% of red-leaved trees (W. Jarausch *et al.*, unpublished). Thus, leaf reddening in apple is a sufficiently reliable symptom usable for remote sensing. PD-infected pear trees do not show specific symptoms. However, infection with '*Ca.* P. pyri' was detected by PCR in more than 80% of pear trees with leaf reddening in September (Jarausch *et al.*, 2023). Therefore, premature leaf reddening can also be used as symptom for remote sensing of PD.

This is also evident in the evaluation of the spectra generated by the laboratory measurements. High classification performances were achieved to distinguish infected from healthy leaves (P. Menz *et al.*, 2023, unpublished). Relevance analysis also indicates a high importance of the red edge. Therefore, multispectral drone spectra were promising, but also showed limitations compared to the hyperspectral data. It was challenging to reliably detect symptomatic leaves when they were only present on single branches of the tree, as in case of AP.

Reliable spectral indicators for the detection of the disease-typical leaf reddening could be developed from all satellite images. Images from WorldView-3, which have a very high spatial resolution of 15 cm, proved to be most suitable for localizing the symptomatic small-growing trees planted closely in rows. Satellite images with a lower spatial resolution were applicable for identifying symptomatic rows, zones or orchards. The satellite data guaranteed both, high accuracy in the localization of the "hot spots" as well as high spectral quality. The accuracy of the remote sensing of AP and PD relies on the timing which was best in the beginning of October for AP and in the middle of September for PD in the study area.

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Detection

Leaf reddening as suitable symptom of pear decline for remote sensing

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Abstract

Pear decline (PD) is a devastating disease of pears in Europe and North America associated with the presence of '*Candidatus* Phytoplasma pyri'. Reduction of insecticide treatments against the pear psyllid vectors and drought stress due to climate change increase its spread and severity in central Europe. Timely uprooting of infected trees is one of the best measures to limit the spread of the disease. However, monitoring of PD is hampered by the lack of specific symptoms and molecular detection is often less reliable due to irregular distribution of the phytoplasma in the tree. Thus, for large-scale monitoring new strategies are needed. The early leaf reddening in late summer was evaluated as suitable symptom for remote sensing of PD. In 2019 – 2022, more than 6,300 pear trees in 13 older orchards in the Palatinate region of southwest Germany were monitored. Trees with leaf reddening were analysed in August, September and October by PCR for '*Ca.* P. pyri' presence. More than 80% of reddening trees were shown to be infected with PD. However, latent infection was common as 40% of asymptomatic trees tested positive as well. The correlation of phytoplasma detection and leaf reddening was highest in September. Testing green and red leaves of single infected trees showed that phytoplasmas could only be detected in the red-coloured leaves. However, quantitative PCR analysis indicated that there is no tight relationship between phytoplasma concentration in the leaf and intensity of the reddening. In conclusion, early leaf reddening in pear is a sufficiently reliable indicator of PD to be used in remote sensing approaches.

Keywords: '*Candidatus* Phytoplasma pyri', quantitative PCR, phytoplasma symptomatology

Introduction

Pear decline (PD) is one of the most important diseases of pear (*Pyrus communis*) in Europe and North America (Seemülller *et al*., 2011). It is associated with the presence of '*Candidatus* Phytoplasma pyri' which is transmitted by pear psyllids. PD does not induce specific symptoms but is often associated with reddening of the foliage accompanied with premature leaf drop (Seemüller *et al*., 2011). In central Europe, PD-affected trees usually show a slow decline with progressive weakening and poor fruit set and size. Quick decline is reported from southern Europe when trees are under stress because of hot and dry summer. Climate change, however, leads also in central Europe to an increasing decline of PD-infected trees and, thus, to an increased economic impact of the disease. Regular insecticide treatments against the psyllid vectors have been the most important control measures. Reduced treatments, however, have led to increased vector populations and, thus, to an increased spread of the disease. In this regard, timely uprooting of diseased trees is one of the best measures to limit the spread of PD. The objective of the work was, therefore, to investigate if large-scale monitoring of PD is possible by remote sensing. For this, it was evaluated if phytoplasma-induced leaf reddening is a suitable symptom for spectral identification of PD. The work was part of a larger project which aimed to identify specific spectral signatures of PD-infected leaves and to apply them in remote sensing of PD either with drones equipped with hyperspectral and multispectral cameras or by using multispectral satellite images (Jarausch *et al*., 2023).

Materials and Methods

Thirteen 10 to 50 year's old pear orchards were monitored in four consecutive years (2019 – 2022) in August, September and October for leaf reddening symptoms associated with PD in the Palatinate region in southwest Germany. The major cultivar in the orchards was Williams but additional data were also obtained from more than 100 varieties in a pear cultivar collection. '*Ca.* P. pyri' infection was confirmed by PCR using primers fO1/rO1 (Lorenz *et al*., 1995) for total nucleic acid extracts of branch phloem preparations as described in Jarausch *et al.* (2011). Phytoplasma quantification was done in total nucleic acid extracts of leaf petioles with a SYBR-Green assay according to Jarausch *et al.* (2011) using forward and reverse primers of Nikolic *et al.* (2010). The phytoplasma titer was expressed as phytoplasmas per ng of total nucleic acid extract.

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Table 1. Correlation of reddened leaves with '*Ca.* P. pyri'-infection (number of PCR-positive/total number of tested).

Pear trees tested	2019	2020	2021	2022	Total
Number of orchards	5	13	7	5	13
Number of trees monitored	617	3,363	1,666	752	6,398
Number of reddened trees	54 / 72 (75.0%)	320 / 374 (85.6%)	165 / 202 (81.7%)	82 / 106 (77.4%)	621 / 754 (82.4%)
Number of asymptomatic trees	3 / 7 (42.9%)	58 / 111 (52.3%)	21 / 94 (22.3%)	17 / 30 (56.7%)	99 / 242 (40.9%)

Table 2. Reliability of '*Ca.* P. pyri' detection in trees with reddening at different periods in 2019 – 2022.

	PCR-positive	Total tested	Positive (%)
August	121	155	78.1%
September	432	500	86.4%
October	88	138	63.8%



A	1	2	3	4	5	6	7	8	9	10
PCR	-	+	-	+	-	+	+	+	-	-
Titer		2,4		26,5		14,5	3,3	71,8		

Figure 1. PCR detection and quantification of '*Ca.* P. pyri' (phytoplasma/ng DNA extract) in reddened leaves of the same pear tree cultivar Williams. All green-coloured leaves were negative.

Results and Discussion

A total of 6,398 pear trees was monitored in 2019 – 2022 and a total of 754 trees with reddening were tested (Table 1). The correlation of this symptom with the presence of '*Ca.* P. pyri' was between 75% and 85% in the different years. The best reliability of PD detection in red-leaved trees was in September with more than 86% positive plants (Table 2). As autumn progresses the natural chlorophyll breakdown leads to symptoms similar to those of PD. This reduces the reliability of PD detection. Both a single infected tree and different cultivars show a variety of different reddish leaf colours, ranging from green red to bright red to completely red. In total of the data obtained in September 2019 – 2022, the correlation to PD was for green-red leaf coloration 83%, for bright red 81% and for completely red leaves 91%.

Regarding cultivars, red-leaved trees were tested from more than 100 different pear cultivars for PD-infection and found a 90% correlation to the presence of '*Ca.* P. pyri'. Thus, a good correlation of PD with trees showing all kinds of reddish leaves was obtained. In addition, the test was performed on four infected trees of cultivar Williams in 10 green and 10 red leaves each and the phytoplasma was only detected in petioles of red leaves (Figure 1). Furthermore, only part of the reddish leaves was colonised by the phytoplasma which is in agreement with the known irregular distribution of '*Ca.* P. pyri' in the tree. Quantification of the phytoplasma in the reddish leaves showed no correlation to the intensity of the red colour (Figure 1). Thus, reddish leaves in pear trees in late summer are a reliable indicator of PD. In the work performed it can even compete with molecular detection as the irregular distribution of '*Ca.* P. pyri' in the tree limits its reliability leading to false negative results. On the other hand, the high amount of latent infected trees (Table 1) limits the possibilities of non-invasive PD detection either by visual inspection or remote sensing.

Acknowledgements

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Detection

Unravelling the puzzle of 16SrV phytoplasmas in hazelnuts: a systematic study of sampling and detection

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Abstract

The presence of 16SrV phytoplasmas led to a decline in hazelnuts and caused serious problems in hazelnut production in Slovenia. Sampling of various plant parts revealed that 16SrV phytoplasmas are more reliably detected in roots than in shoots. A sufficiently high concentration is important for downstream analysis.

Keywords: *Corylus avellana*, phytoplasma disease, roots, shoots, concentration

Introduction

Phytoplasmas from different ribosomal groups were found in declining hazelnuts (*Corylus avellana*), *i.e.*, 16SrI, 16SrIII, 16SrX, 16SrXII and 16SrV (Marcone *et al.*, 1996; Jomantiene *et al.*, 2000; Cieslińska and Kowalik, 2011; Hodgetts *et al.*, 2015; Mehle *et al.*, 2018, 2019). Molecular examination of the partial 16S rRNA, *secY*, *map*, and ribosomal protein gene locus of 16SrV phytoplasma strains from hazelnut shows that they are identical to strains associated with grapevine "flavescence dorée" (FD) disease (Mehle *et al.*, 2019). Symptoms of 16SrV infection on cultivated hazelnuts are manifested by curling and yellowing of leaves, wilting of branches or the entire shrub (Mehle *et al.*, 2019). The 16SrV phytoplasmas were also detected in asymptomatic wild hazelnuts located near a vineyard infected with FD phytoplasma (Casati *et al.*, 2017). *Orientus ishidae* has been proposed as a vector and has been shown to harbour phytoplasmas of the 16SrV group (Casati et al., 2017; Mehle *et al.*, 2010).

Different approaches can be used to identify phytoplasma, but in the first stage, the sampling is very important. In woody plants, titers are low and vary depending on the season or plant organ (Prezelj *et al.*, 2016). Therefore, the aim of this study was to systematically show, which part of the hazelnut shrub is more reliable for the sampling and the detection of 16SrV phytoplasmas, which is important for further analysis (*e.g.* molecular genotyping).

Materials and Methods

Symptomatic hazelnut samples were collected from two intensive orchards: one at northeast (NE) of Slovenia (in October 2017, September 2018, October 2020, and July 2021) and one at southwest (SW) (in July 2021). Hazelnuts belonged to three cultivars Istrska Dolgoplodna, Istrska Okrogloplodna and Giffoni. The sample consisted of shoots or roots from three different parts of a symptomatic shrub. For the shoot samples, 1 g of tissue was taken from the leaf mid-vein tissue and in some cases also from the branch phloem. Roots were washed extensively to remove soil, and 1 g of phloem tissue was collected. Samples were homogenised and total DNA was extracted as described in Mehle *et al.* (2013).

The tenfold diluted total DNA was tested for the 16SrV phytoplasma group using a specific quantitative PCR (Hren *et al.*, 2007). Each run included a positive and a negative amplification control as well as an extraction control - the 18S rRNA assay (Applied Biosystems, Massachusetts, USA). Samples that had a sufficiently high concentration of 16SrV phytoplasma were further analysed using nested PCR on the *secY*-map locus (Arnaud *et al.*, 2007) with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Massachusetts, USA). These samples were subjected to Sanger sequencing (Macrogene) and analysed using CLC Genomic Workbench (version 21.0.1, Qiagen) and MEGA.

Results

The results of shoot and root sampling of 32 16SrV-positive hazelnut shrubs are shown in Figure 1. In 26 shrubs (81%), testing of roots proved more reliable than testing of shoot samples: in 16 of these shrubs, no phytoplasma was detected

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in the shoot samples, whereas the root samples were clearly positive, and in 10 shrubs the phytoplasma concentration was estimated to be higher in the roots (lower Ct values) than in the shoots. Only six shrubs (19%) were estimated to have higher phytoplasma concentrations in shoots than in roots, and five of these shrubs were collected in July. However, 58% of the samples collected in July had higher concentrations in the roots.

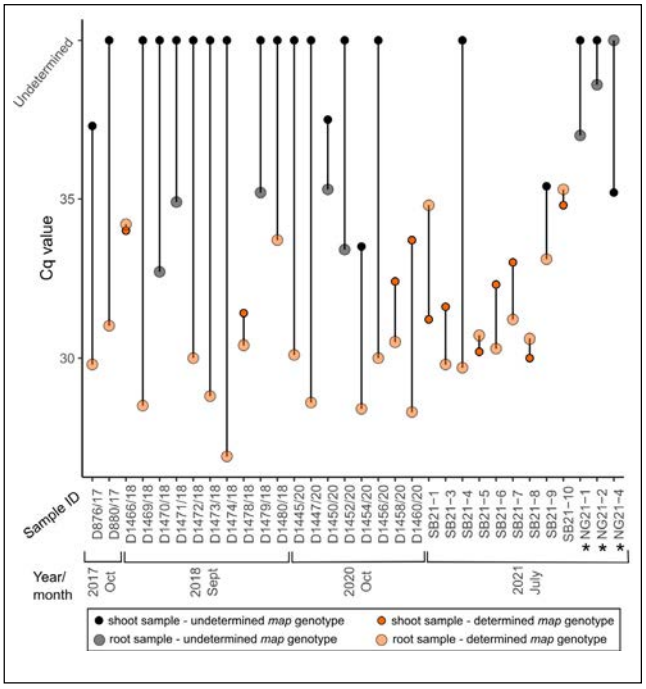


Figure 1. Results of 16SrV specific quantitative PCR for shoot and root samples of symptomatic hazelnut shrubs. All samples were collected at NE Slovenia, except the ones marked with *, which are from SW Slovenia. Samples, for which *map* gene genotype could be determined, are marked with orange colour.

Map gene genotypes could be determined in 24 of 32 16SrV-positive shrubs. Eight different map genotypes were detected (Z. Kogej Zwitter *et al.*, unpublished). In 11 shrubs, the map genotype was determined in both shoot and root samples, and in all of these 11 cases, the map genotype determined in shoot and root samples from the same shrub was identical.

Discussion

Phytoplasmas are known to be unevenly distributed throughout the plant (Christensen *et al.*, 2004; Prezelj *et al.*, 2016). In the cultivated hazelnuts tested the analysis for 16SrV phytoplasma presence resulted more reliable in root than in shoot samples, so although digging roots is tedious, it is recommend using this part of the shrub for analysis. The season of sampling could also have an influence on the change in titers (Prezelj *et al.*, 2016). The higher concentration of 16SrV phytoplasma in the July 2021 shoot samples, is likely related to this phenomenon, but more structured monthly sampling should be conducted to draw conclusions.

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Detection

Exploring practical applications of metabarcoding with MinION to support the surveillance of the phloem bacteria '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter'

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Abstract

Anticipating the huge potential of ONT long read sequencing in plant health diagnostics, the METAMINSURV project will evaluate the feasibility to use MinION metabarcoding as a fast and accurate diagnostic tool for detection and identification of the phloem bacteria '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter'.

Keywords: phytoplasma, liberibacter, MinION, metabarcoding, detection, identification, classification, MLST

Introduction

'*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter' are wall-less phloem bacteria. They are linked to various plant diseases which can cause severe economic losses (Satta *et al*., 2016). Fast and correct identification of pathogens is crucial in the surveillance of plant diseases. For phloem inhabiting bacteria, however, this is anything but straightforward.

Phytoplasmas have a broad host range and can infect hundreds of cultivated plants. The identification of these bacteria is very complex, with the taxon enclosing 49 '*Candidatus* Phytoplasma' species, strains, ribosomal groups and subgroups, and is currently based on the 16S rRNA gene. Usually, samples are processed starting with a generic phytoplasma analysis through (nested) PCR, followed by Sanger sequencing of the obtained 16S rRNA fragment, and finally subsequent RFLP analysis and (sub)group assignment. However, for both reliable detection and correct identification often multiple nested PCR assays are needed. For '*Ca.* Phytoplasma' species differentiation, recent updated guidelines suggest the use of a nearly full 16S rRNA gene sequence, corresponding to amplicon length of 1.5 kb (98.65% sequence identity threshold). Even so, assignment based on 16S rRNA only can, in some cases, still remain challenging. In such cases, it is suggested to consolidate the results by the whole genome average nucleotide identity (ANI; 95% sequence identity threshold) and/or the gene identity of at least two, alternative housekeeping genes by performing a multi-locus analysis (Bertaccini *et al*., 2022; Wei and Zhao, 2022).

The liberibacter consists of four relevant phytopathogenic '*Candidatus* Liberibacter' species. While '*Ca.* L. asiaticus', '*Ca.* L. africanus' and '*Ca.* L. americanus' are associated with "huanglongbing" (HLB, citrus greening) in citrus, '*Ca.* L. solanacearum' is mainly known for its impact on *Solanaceae* (zebra chip in potato) and *Apiaceae* (carrots and celery yellows). Phylogenetic analysis of '*Ca.* Liberibacter' species revealed the existence of many distinct lineages linked to their geographic distribution. For '*Ca.* L. solanacearum', ten distinct haplotypes have so far been identified: A-H, H1, U (Haapalainen *et al*., 2020). '*Ca.* Liberibacter' species detection and identification is also based on 16S rRNA (Li *et al*., 2006, 2009). The haplotyping, however, requires additional analyses based on single nucleotide polymorphisms (SNPs) on the 16S, 16S/23 intergenic spacer region (ISR) and 50S rRNA genes (*rplJ* and *rplL* ribosomal protein genes) (Nelson *et al*., 2011).

Phloem inhabiting bacteria are difficult to recognize in the field because they can be associated with slowly developing and variable symptoms. In addition, liberibacters and phytoplasmas can occur together and produce similar symptoms, such as foliage discoloration, proliferation of shoots and roots, stunting and vascular discoloration of tubers (Ermacora and Osler, 2019; Katsir *et al*., 2018). Simultaneous presence of liberibacter and phytoplasmas was reported in carrots and citrus (Satta *et al*., 2016; Luis-Pantoja *et al*., 2021; Yu *et al*., 2022). This of course complicates the selection of the appropriate molecular diagnostic test and in routine diagnostics, the limited available time often also forces plant pathologists to run several molecular tests in parallel. As previously indicated, this is not a one-step procedure but a cascade of tests for both types of bacteria, being very complex, time-consuming and expensive. An untargeted testing method, such as the use MinIon metabarcoding, could be

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more appropriate in these cases. Due to its fast turnaround time, possibility to obtain long read lengths and ability to allow a lower level of sample pooling than Illumina sequencing it has a high potential as a surveillance tool for routine diagnostics of pathogens, such as for phloem bacteria. By combining different amplicons in one PCR reaction or one metabarcoding library preparation, a higher taxonomic resolution might be obtained, and/or multiple species of phloematic bacteria could be detected, without the need to run multiple tests in parallel. In the METAMINSURV project MinION metabarcoding targeting multiple *loci* will evaluate the combination of broad detection of '*Ca.* Phytoplasma' and '*Ca.* Liberibacter' in one test.

Materials and Methods

The project consists of 4 work packages (WPs) (Figure 1). In WP1 suitable primers and reference databases will be set up for the target organisms by reviewing existing literature combined with *in silico* analysis. WP2 deals with the preparation of samples in the form of mock communities, spiked samples and real samples. In WP3 MinION metabarcoding protocol(s) and accompanying data analysis workflows will be developed. WP4 will explore which steps are transferable to other pathogen/barcode combinations for future protocol development.

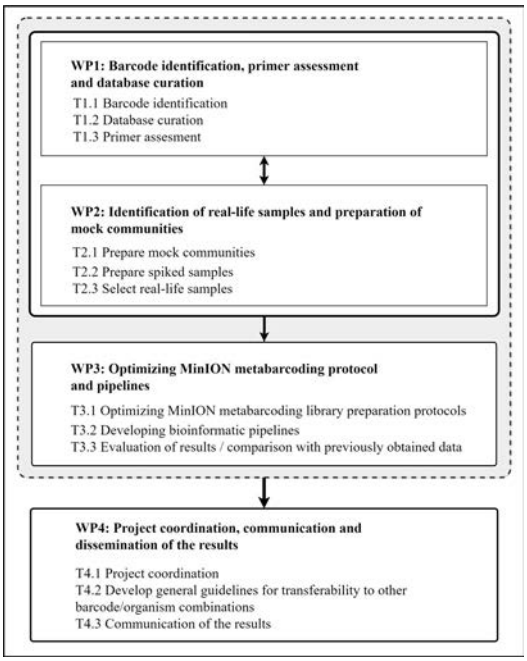


Figure 1. Overview of the different work packages in the project.

Expected Results

The main outcomes of the METAMINSURV project will be protocols and accompanying bioinformatics workflows to detect and identify phloem bacteria using MinION metabarcoding. Since the project will use mock communities, spike-ins and real samples of different case studies, and will benchmark with alternative methodologies, it will allow to assess the potential of MinION metabarcoding for routine plant pathogen diagnostics and surveillance. Research results will be disseminated through scientific publications in peer-reviewed journals, and protocols and bioinformatics pipelines will be shared with the authorities and the scientific community. By developing general guidelines for the optimization of MinION metabarcoding protocols and analysis pipelines, a spill-over effect to other plant pathogens, in particular for the detection of multiple (closely related) pathogens at once it is expected.

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Detection

Validation of a modified quantitative PCR and synthetic DNA gBlocks control for detection of '*Candidatus* Phytoplasma mali'

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Abstract

A primer set targeting the *imp* gene was duplexed with an 18S rRNA plant internal control primer set and validated for specific detection of '*Candidatus* Phytoplasma mali.' The assay was compared to a modified 23S/18S rRNA (23S) duplex universal phytoplasma assay that was validated previously. The *imp* duplex assay produced comparable results to the 23S duplex assay for all metrics, demonstrating high linearity, repeatability, and intermediate precision. The amplification efficiency and limit of detection were 97-98% and C_t=36 - 37, respectively. The assay showed 100% analytical specificity, selectivity, and diagnostic specificity. Assays metrics were consistent across two platforms, the ABI QuantStudio™ 5 and Bio-Rad CFX96™. A synthetic gBlocks™ control was designed and validated to work with both duplex assays and a semi-nested PCR assay. The *imp* duplex assay shows increased specificity over current EPPO protocols, and the addition of an internal control increases confidence in test results. Together, the assay and synthetic control can be successfully deployed to aid in quarantine and eradications efforts of '*Ca.* P. mali.'

Keywords: apple proliferation, detection, quantitative PCR, synthetic nucleic acid

Introduction

Apple proliferation is one of the most economically important apple diseases in Europe (Valasevich and Schneider, 2017). '*Candidatus* Phytoplasma mali' (16SrX-A) (Seemüller and Schneider, 2004), the associated agent, is classified as an A2 quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO). Currently, '*Ca.* P. mali' has been reported only in Europe. Introduction of this plant pest into the United States of America (USA) could have huge economic consequences. Therefore '*Ca.* P. mali' is, in USA and Canada, a quarantine pest. Controlling disease and spread of this quarantine organism requires accurate and rapid detection methods.

Three published assays (Aldaghi *et al.*, 2007; Nikolic *et al.*, 2010; Valasevich and Schneider, 2017) were evaluated and the *imp* assay was selected for in-depth validation. Successful implementation of diagnostic assays requires use of appropriate controls. Incorporation of an internal control (IC) allows quality assessment of each reaction; therefore, *imp* primers from Valasevich and Schneider (2017) were duplexed with host IC primer set. Additionally, as '*Ca.* P. mali' is not known to occur in the USA, access to and availability of source material for use as positive controls is limited. A synthetic DNA gBlocks was designed and validated for ease of distribution, as well as to ensure traceability and integrity of test results.

Materials and Methods

Valasevich and Schneider (2017) primer/probes (*imp*) were duplexed with a modified 18S rRNA (18S) plant IC set (Christensen *et al.*, 2004). Fluorophore/quencher pairs for *imp* and 18S probes were FAM/QSY and ABY/QSY, respectively. Final concentrations were 100 nM forward (F)/probe (P) and 500 nM reverse (R) for *imp* and 48 nM F/R and 24 nM P for 18S. Reactions were run in a QuantStudio™ 5 (Applied Biosystems, USA) (QS5) or CFX96 (Bio-Rad Laboratories, USA), using PerfeCTa® qPCR ToughMix®, Low ROX™ (QuantaBio, US), under the following conditions: 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds and 58°C for 40 seconds. For the CFX96, the 18S fluorophore/quencher pair was labeled with Cy3/IAbRQSp. This assay was compared to a 23S rRNA (23S) assay (Hodgetts *et al.*, 2009), also duplexed with 18S and validated previously; the reaction conditions were identical. Thresholds for *imp*/18S (and 23S/18S) were set at 0.23/0.09 and 270/25 for the QS5 and CFX96, respectively.

A synthetic gBlocks was designed based on a 1,793 bp 16S base sequence, incorporating *imp*, 23S and 18S amplicons, and two unique identifiers: no amplification with 16S assay

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described by Christensen *et al.* (2004) and translated amino acid sequence reads "APHIS SCIENCE." DNA for 16SrX-group phytoplasma strains were obtained and tested. Serial dilutions (10-fold) of '*Ca.* P. mali' DNA (from *Catharanthus roseus*) in healthy apple DNA, and gBlocks in water, were used to establish reaction metrics (N=3 per dilution); linearity and precision were based on data from three operators (N=9 per dilution). Semi-nested PCR was performed on low-titer dilutions using published primers (Deng and Hiruki, 1991; Lee *et al.*, 2004). Several non-16SrX phytoplasmas, other organisms of apple and stone fruit, and biocontrol agents, were used for selectivity (N=41). Non-16SrX-positive diagnostic samples (N=29) were used for diagnostic specificity.

Results

Preliminary screening eliminated two assays from further testing, as they produced cross-reactions with '*Ca.* P. prunorum,' with C_t values similar for '*Ca.* P. mali.' This corroborated *in silico* data that showed that the specificity was dependent solely on probe binding sites, which had one or three mismatches for 16S rRNA (Aldaghi *et al.*, 2007) and ITS (Nikolic *et al.*, 2010), respectively. The *imp* gene target itself was found specific to '*Ca.* P. mali' strains used.

The *imp* duplex assay demonstrated good repeatability, as indicated by low standard deviations (StDev) (<1) across all dilutions (C_t range ~23-37). The assay demonstrated acceptable intermediate precision (<2%) across all dilutions for three operators and different QS5s, with an average total variance of about ~1%. The amplification efficiency was 97-98% and the limit of detection was determined to be C_t ~37. This dilution produced a band by semi-nested PCR and amplicon sequence analysis confirmed '*Ca.* P. mali' showing adequate assay sensitivity confirmable by sequencing. The *imp* duplex assay showed 100% analytical specificity, selectivity, and diagnostic specificity, as only the five strains of '*Ca.* P. mali' tested yielded positive results; no cross-reactions were observed with other phytoplasmas, other non-target organisms, or host tissues from apple, peach, pear and plum. Assay data were comparable to the 23S/18S duplex assay for both the QS5 and CFX96.

The synthetic gBlocks positive control was compatible with both qPCR (*imp*/18S and 23S/18S) and semi-nested PCR assays. A 4 fg/µl working stock solution resulted optimal; *imp* and 18S C_t values were approximately $25 < x < 28$ and $27 < x < 30$, respectively. Amplicons were obtained by semi-nested PCR that produced quality sequence data; unique "APHIS SCIENCE" marker was visible when the sequence was translated as a protein.

Discussion

This assay provides high specificity, excellent repeatability and precision, and comparable linearity and sensitivity to the 23S/18S duplex assay, at a reasonable price point (<$1 USD/reaction); in both QS5 and CFX96. The *imp* duplex assay improves the original published assay by including a plant IC, and the *imp* target demonstrated improved specificity over the ITS assay used by EPPO (Nikolic *et al.*, 2010). This assay can be run in parallel with the 23S/18S duplex assay, as reaction parameters are identical, allowing general detection of phytoplasmas and discrimination of '*Ca.* P. mali' simultaneously, if needed. The gBlocks positive control worked as designed. It is compatible with *imp* and 23S duplexed assays, as well as semi-nested PCR. Testing laboratories can purchase the synthetic control, obviating their dependency on others for source material and/or DNA. Together, the validated assay and synthetic control benefits USA Cooperative Agricultural Pest Survey (CAPS) efforts to prevent introduction of and/or eradicate '*Ca.* P. mali' in the USA. Additionally, improved specificity of *imp* and the addition of an IC can benefit quarantine efforts.

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Detection

Phytoplasmas in papaya: detection and identification

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Abstract

Papaya diseases associated with phytoplasmas enclosed in diverse ribosomal groups have been reported all over the world except in Europe. In this study, '*Candidatus* Phytoplasma solani' was detected for the first time in symptomatic papaya plants cultivated in Italy. The identification was performed through nested-PCR/RFLP and sequencing on the 16S ribosomal gene. The characterization of the phytoplasma strains was obtained from their *secY*, *tuf*, *stamp* and *vmp1* gene sequences. Isolation of endophyte microorganisms from symptomatic tissues led to obtain colonies in which phytoplasmas were detected.

Keywords: papaya bunchy top disease, MLST analysis, RFLP, sequencing, microorganism isolation

Introduction

Carica papaya L. is originated in the tropical areas of America and nowadays is grown also in India and other parts of the world. The crop is susceptible to infections by virus, fungi and bacteria and since the nineties papaya bunchy top disease associated with the presence of phytoplasmas was reported in several countries. Phytoplasmas of groups 16SrI, 16SrII, 16SrXII, 16SrXIII, 16SrXVII were identified from different geographic areas (Table 1), however in Europe there have been no reports until now. Recently a severe disease similar to papaya bunchy top was observed in a trial plantation in Italy where the yellowing symptoms were often accompanied with premature falling of fruits and ooze production (Figure 1). Symptomatic plants started to be observed during the fruit production season and the disease incidence was about 1% with a slow increase overtime. Molecular and biological tests were performed to verify the phytoplasma presence.

Table 1. Phytoplasma ribosomal groups reported in papaya.

Continent	16Sr group	Literature
Australia	16SrI; 16SrII; 16SrXII	Gibb *et al.*, 1998. *Plant Pathology*, 47: 325-332.
Asia	16SrI; 16SrII; 16SrXII	Gera *et al.*, 2005. *Plant Pathology,* 54: 560; Rao *et al.*, 2011. *Bulletin of Insectology*, 64(Suppl.): S105-S106.
Africa	16SrII; 16SrXII	Arocha *et al.*, 2007. *Plant Pathology*, 56(6): 1039; Kazeem *et al.*, 2021. *Crop Protection*, 148: 105731.
America	16SrI; 16SrII 16SrXIII; 16SrXV; 16SrXVII	Arocha *et al.*, 2005. *International Journal of Systematic and Evolutionary Microbiology*, 55(6): 2451-2463; 2006. *Plant Pathology*, 55: 821; Acosta *et al.*, 2011. *New Disease Reports*, 24: 2044-0588; Melo *et al.*, 2013. *European Journal of Plant Pathology*, 137: 445-450; Wei *et al.*, 2017. *Crop Protection*, 92: 99-106.



Figure 1. Bunchy top-like symptoms of papaya: small fruits and yellow leaves.

Materials and Methods

Total nucleic acid was extracted using a chloroform/phenol method (Prince *et al.*, 1993) from 1 g of midribs and lamina tissues of five symptomatic and one asymptomatic papaya plant samples. PCR amplifying the 16S rRNA gene was carried out with 20 ng of extracted DNA using the phytoplasma universal primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by nested PCR using primers pair 3F/3R (Manimekalai *et al.*, 2010) on amplicons diluted 1: 30. PCR products were analyzed in a 1% agarose gel electrophoresis and stained with ethidium bromide. Subsequently *stamp*, *secY*, *vmp1* and *tuf* genes were amplified using published primers and protocols (Fabre *et al.*, 2011; Fialová *et al.*, 2009; Schneider *et al.*, 1997). The strain STOF (Bertaccini, 2023) was used as a positive control. Restriction fragment length polymorphism (RFLP) analysis was performed on PCR amplicons. In particular 3F/3R and *secY* amplicons were digested with *Tru*1I, *vmp1* gene amplicons with *Rsa*I and *tuf*

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amplicons with *Hpa*II. Restriction products were analyzed in a 6.7% polyacrylamide gel electrophoresis stained with ethidium bromide. Furthermore, selected amplicons were directly sequenced in both directions with the same primers used for the amplification. In addition to molecular analysis, isolation was performed from symptomatic papaya tissues (leaves, stems and fruits) using the artificial media and following procedures described for phytoplasmas (Contaldo *et al*., 2016, 2019). Colonies obtained on solid CB agar medium were subjected to PCR analysis with primers GPO3F/MGSO (Satta *et al*., 2017) and amplicons were digested with *Tru*I and sequenced with the primer GPO3F.

Results and Discussion

All papaya symptomatic samples yielded amplification for the 16Sr phytoplasma gene. The RFLP profiles obtained with *Tru*I were identical to each other and to the control STOF, indicating the presence of 16SrXII-A phytoplasmas (Figure 2).

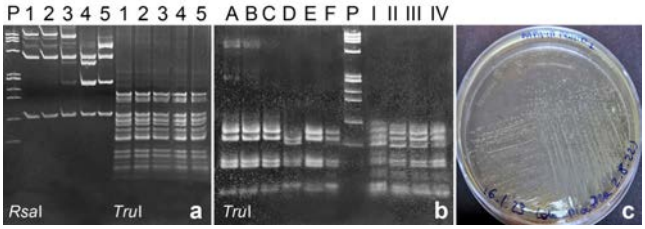


Figure 2. RFLP of amplicons from papaya samples: *vmp1* and *secY* genes (a); GPO3F/MGSO profiles on different phytoplasma groups (left) and mixed profiles of papaya colonies (right) (b); colonies isolated from papaya tissues (fruit) in CB agar medium (c). Samples 1, 2, 3, 4 papaya; 5, STOF; A, B, C, D, E, F phytoplasma control strains; I, II, III, IV amplicons from colonies. P, marker phiX174 DNA digested with *Hae*III.

RFLP identical profiles were obtained for the *secY* and *tuf* amplicons, while those corresponding to the *vmp1* gene produced diverse restriction profiles (Figure 2). The 16Sr gene sequence shared an identity of 99.67% (4 SNPs) with that of '*Ca*. P. solani' (GenBank accession number AF248959) under 100% coverage. The comparison (Bertaccini *et al*., 2022) with the '*Ca*. P. solani' reference strain (GenBank accession number JQ797668) showed that the *secY* gene sequences shared a 99.59% identity (coverage 88%; 5 SNPs) and the *tuf* gene 100% identity (coverage 100%) with the reference strain (GenBank accession number JQ797670). Moreover, the strains from papaya grouped as tuf type b1 while the *stamp* gene sequences were identified as variant St10. The isolation on artificial media was successful from most of the samples in both liquid and solid media. Molecular analysis on colonies generated mixed RFLP banding enclosing phytoplasma profiles (Figure 2). Sequencing results confirmed the presence of different bacteria among which *Enterococcus* sp. and *Pantoea* sp. were identified. From amplicons with mixed RFLP profiles, the presence of short 16Sr phytoplasma gene sequences corresponded to '*Ca*. P. solani'. This is the first report of '*Ca*. P. solani' in diseased papaya in Italy. Molecular analysis on different genes confirmed this identification. The variability of RFLP profiles for *vmp1* gene amplicons support the hypothesis that phytoplasmas in different samples may be different strains for either insect vectors and/or alternative host plants presence in the environment. The isolation of colonies containing both endophyte microorganisms and phytoplasmas confirmed the low selectivity of the media used as reported from other infected crop species, such as palm and citrus (Contaldo *et al*., 2019; Luis-Pantoja *et al*., 2021).

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Detection

Multigene analyses for identification of phytoplasma strains infecting *Dimorphandra gardneriana* and *Turnera ulmifolia* in Brazil

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Abstract

Turnera ulmifolia, the yellow alder, and *Dimorphandra gardneriana*, known as "fava d'anta" are plant species distributed in Brazil. In several locations these plants exhibited witches' broom growths, reduced leaf and yellowing. Phytoplasma characterization based on the 16S rDNA gene by RFLP and sequencing analyses allowed the identification of phytoplasmas of the 16SrXIII-A ribosomal group. However, their assignment to a '*Candidatus* Phytoplasma' species was not defined. RFLP analyses and sequence comparison of non-ribosomal gene sequences (*leu*, *tufC*, *secA* and *rp*) provide however a good resolution of their molecular diversity suggesting their differentiation from '*Ca.* P. meliae' and their possible assignment to '*Ca.* P. hispanicum' or to a new phytoplasma taxon.

Keywords: witches' broom, phytoplasma, Brazil, 16Sr gene, multigene analysis

Introduction

Dimorphandra gardneriana Tul., known as "fava d'anta" and "faveiro", is a Brazilian native leguminous tree, naturally found in the Cerrado biome. *Turnera ulmifolia* L., the yellow alder, is a perennial, dense, compact shrub native to Mexico and Central America and naturalized in Brazil. Both species are distributed in Brazil, and in previous works it was demonstrated the presence of phytoplasmas belonging to the 16SrXIII in diseased plants showing witches' broom and yellowing (Montano *et al.*, 2011, 2015). The molecular characterization of these phytoplasma strains following revised guidelines on '*Candidatus* Phytoplasma' species designation (Bertaccini *et al.*, 2022) for appropriate taxon assignment was performed to clarify possible epidemiological implications.

Materials and methods

Samples from *D. gardneriana* and *T. ulmifolia* exhibiting witches' broom, reduced leaf and yellowing were collected in various locations in Brazil and DNA was extracted according to Montano *et al.* (2000). Phytoplasma universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and 3Fwd/3Rev (Manimekalai *et al.*, 2010) were used in nested PCR for amplification of 16S rDNA. Extracted DNAs diluted 1: 30 were used as template in all the PCR assays and nested-PCR assays were performed on 1: 30 diluted PCR products. Furthermore, the amplification of isoleucine (*leu*), *secA*, *tuf* and *rp* genes was carried out according to published protocols (Abeysinghe *et al.*, 2016; Hodgetts *et al.*, 2008; Contaldo *et al.*, 2011; Martini *et al.*, 2007). DNA from '*Ca.* P. meliae' strain ChTY-Mo3 (Fernández *et al.*, 2016) was employed as positive control. The amplicons were subjected to RFLP analysis with *Tru1*I restriction enzyme (Fermentas, Lithuania). The PCR and RFLP products were analysed by electrophoresis on 1% agarose and in 6.7% polyacrylamide gels, respectively, stained with ethidium bromide and visualized under UV transilluminator. Selected amplicons were sequenced in both directions. The obtained sequences were assembled and aligned using PreGap4 and Gap4 software (Staden Package) and compared with reference nucleotide sequences available with BLAST in NCBI database.

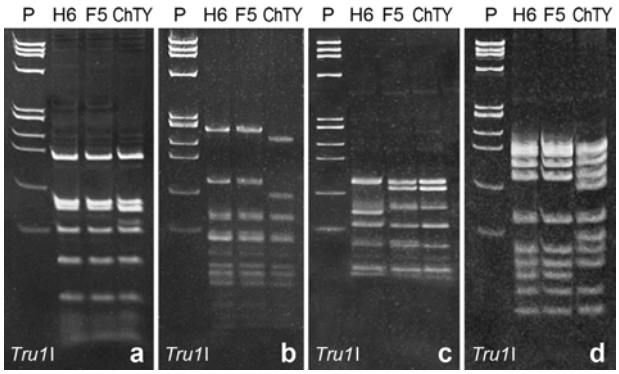


Figure 1. RFLP profiles of *sec*A (a), rp (b), tuf (c) and leu (d) amplicons digested with *Tru1*I. M: Marker phiX174 *Hae*III digested. H6, *D. gardneriana*; F5, *T. ulmifolia*; ChTY, '*Ca.* P. meliae'.

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Table 1. Results from the amplification, RFLP digestion patterns and sequencing of the samples analysed.

Sample	Sequence identity*	Gene 16Sr (1100 nt)	Gene *tufC Tru*1I RFLP – sequence identity	Gene *rp Tru*1I RFLP – sequence identity	Gene *secA Tru*1I RFLP – sequence identity	Gene *leu Tru*1I RFLP – sequence identity
D. gardneriana H6	'*Ca*. P. hispanicum'	99.36%	A, 377 nt – na	A, 1152 nt – 96.53%	A, 563 nt – 95.98%	A, 921 nt – na
	'*Ca*. P. meliae'	99.00%	96.29%	94.54%	95.56%	95.61%
T. ulmifolia F5	'*Ca*. P. hispanicum'	99.55%	B, 312 nt – na	A, na – na	A, 565 nt – 95.98%	A, 912 nt – na
	'*Ca*. P. meliae'	99.00%	97.12%	na	95.40%	95.60%
ChTY-Mo3	'*Ca*. P. hispanicum'	98.91%	C, 441 nt – na	B, 1259 nt – 95.07%	A, 588 nt – 95.16%	B, 912 nt – na
	'*Ca*. P. meliae'	100.00%	100.00%	100.00%	100.00%	100.00%

*, The sequence similarity is calculated based on the comparison with 16S ribosomal and other available gene sequences of '*Ca*. P. meliae' strain ChTY-Mo3 and '*Ca*. P. hispanicum ' strain MVP. na, sequence not available

Results and Discussion

Amplifications of the selected genes allowed the molecular characterization of the phytoplasma strains. The comparison of the 16S ribosomal sequences showed difficulty of '*Candidatus* Phytoplasma' species identification since both strains had sequences with identity percentages above 98.65% compared with both '*Ca*. P. hispanicum' and '*Ca*. P. meliae'. According to *i*PhyClassifier (Zhao *et al*., 2013) the virtual RFLP pattern based on the 16Sr gene sequence from the *D. gardneriana* strain is a variant of 16SrXIII-A subgroup, with a pattern similarity coefficient of 0.98. As reported in Table 1, *Tru*1I RFLP analyses based on the non-ribosomal genes amplified, show no differentiation for the *secA* gene, while RFLP profiles based on the *rp* and *leu* genes differentiate *T. ulmifolia* and *D. gardneriana* phytoplasma strains from '*Ca*. P. meliae'. Moreover, the restriction analyses of *tuf* gene allowed differentiation among the three phytoplasma strains (Figure 1). The *leu* gene sequences showed 3 GAPs between *T. ulmifolia* and *D. gardneriana* strains. The comparison of *secA* gene sequences indicated a closer identity to '*Ca*. P. hispanicum' of both studied strains also confirmed from the *rp* gene sequence of the *D. gardneriana* strain. The overall molecular comparison seems however not sufficient to substantially differentiate the two Brazilian strains from '*Ca*. P. meliae' and clearly assign them to '*Ca*. P. hispanicum' suggesting the need of better focused molecular tools to support '*Ca*. Phytoplasma' taxon designation in case of border-line strains as those studied.

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Detection

Volatile signatures of phytoplasma presence from marigold plants showing witches' broom and their potential as biomarkers for diagnostics

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Abstract

Phytoplasmas often induce phyllody symptoms at late stage of flowering. Other symptoms of infections like little leaf, stunting and witches' broom are often confused with virus infection or nutrient deficiency. In order to search for some early signals of infections in vegetatively propagated marigold plants, volatilome profiling of infected and healthy marigold plants were undertaken through GC*GC-ToF/MS analysis. Differential emission of volatilome compounds have been observed in infected plants compared to healthy plants. Compounds like linalool, isobemeol, β-bisabolene and methyl salicylate were emitted in high amounts in infected samples. Biogenic volatile based detection of plant diseases is emerging as a promising field noninvasive technology. The volatile organic compounds can be potentially exploited as biomarkers for phytoplasma diagnostics in the early stages of the crop production.

Keywords: volatilome, *Tagetes erecta*, aster yellows, GC*GC-ToF/MS

Introduction

Phytoplasma is a plant pathogenic mollicute living in the phloem tissues of plants causing economic losses to the crops by inducing various symptoms including phyllody, witches' broom, little leaves. Their infection in ornamental plants and flower crops are widely reported (Prabha *et al*., 2022). Marigold is one of the most important flower crops grown in India which is used for loose flowers, dried petals for "agarbatti", food colour, pesticides and lutein extraction. Incidence of phyllody affects the flower production in the major flower growing areas. Up to 30% incidence of phytoplasma has been observed in major marigold growing areas. The major marigold variety cultivated for flower production is "Calcutta", which is of high demand due to its flower texture and composure. It is vegetatively propagated through rooted cuttings which leads to the spread of phytoplasmas into the field from infected mother stock. Often the symptoms of infection appear in a later growing stages as phyllody at the flowering time. Till that the plants looks normal making the identification of disease at an early stage difficult. Pathogens, their host plants and vectors communicate through various volatile chemical signals. To identify a biomarker for disease detection at an early stage, the volatilome of phytoplasma infected plants in vegetative phase showing little leaf and witches' broom symptoms was analysed in comparison with healthy plants of same growth stage and the observations are reported here.

Materials and Methods

Marigold cuttings from phytoplasma infected mother plants were collected and rooted cuttings were grown under insect proof polytunnels under controlled conditions as the source of infected plant samples. Healthy rooted cuttings were grown under insect proof conditions similar as infected samples. Presence of phytoplasma infection in both mother plants and cuttings were confirmed through nested PCR using P1/P7 and R16F2n/R16R2 primer combinations (Prabha and Rao, 2022). For volatile organic compound (VOCs) analysis, sampling was done when plants were in full vegetative growth at 40 days after planting. The top shoot along with 3-4 leaves from each plant were used for analysis. The symptomatic and healthy branches were placed in three replications in 20 ml SPME headspace GC vials, sealed with a polypropylene screw-cap with a PTFE/ silicone septum and kept air tight for an hour before injection for release of the volatiles. VOC was extracted by a pre optimized extraction protocol and analysed using GC*GC-ToF/ MS (Amrapali *et al*., 2020).

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Table 1. Volatile compounds differentially emitted from marigold plants phytoplasma infected *versus* healthy plants.

Compounds	Molecular formula	RT*
1,3,6-Octatriene, 3,7-dimethyl-(Z)-	C10H16	985
1,3,8-p-Menthatriene	C10H14	1105
1H-Indene, 1-methylene-	C10H8	1265
4-Hexen-1-oi, acetate	C8H14O2	870
β-Bisbolene	C15H24	1690
α-Cubebene	C15H24	1565
β-Myrcene	C10H16	835
β-Ocimene	C10H16	970
α-Phellandrene	C10H16	860
α-Pinene	C10H16	695
α-Terpineol	C10H18O	1290
Benzaldehyde	C7H6O	760
Benzaldehyde, 4-(1-methylethyl)-	C10H12O	1390
Benzeneacetaldehyde	C8H8O	950
Camphene	C10H16	730
Caryophyllene	C15H24	1635
cis-β-Farnesene	C15H24	1650
γ-Terpinene	C10H16	990
Dimethyl sulfide	C2H6S	130
Eugenol	C10H12O2	1575
Germacrene D	C15H24	1565
Humulene	C15H24	1660
Indole	C8H7N	1495
Isoborneol	C10H18O	1240
Linalool	C10H18O	1085
Methyl salicylate	C8H8O3	1295
Neophytadiene	C20H38	1855

*RT: retention time

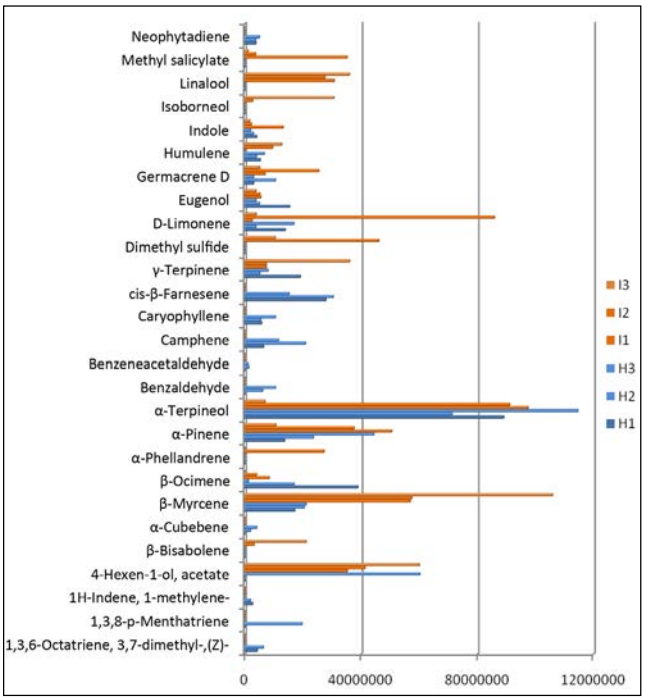


Figure 1. Major compounds differentially emitted from healthy and phytoplasma infected marigold plants represented by area under the peak (X-axis, detector response).

Results

The phytoplasmas associated with witches' broom and little leaf has been confirmed through nested PCR and sequencing as '*Candidatus* Phytoplasma asteris'. The volatile profile of phytoplasma infected and healthy marigold was found to be different from each other with differential release of the compounds from each sample type (Table 1).

The release profile of the volatile compounds based on the area of three replicates is given in the Figure 1. The α-terpineol has been found in abundance in both healthy and infected plants. Compounds like linalool, isobemeol, β-bisabolene and methyl salicylate were emitted in high amounts in infected samples, while neophytadiene, caryophyllene, camphene, benzaldehyde, benzeneacetaldehyde, 1,3,8-p-menthatriene, 1,3,6-octatriene, 3,7-dimethyl-(Z) were released in high amounts in healthy plants and were absent in volatile samples from plants showing witches' broom.

Discussion

Chemical communication between the pathogens, plants and its vectors are well known (Gross, 2016). In this study a difference in the profile of the volatiles released by the infected and healthy plants have been observed in the early stage of the plant development before reaching the reproductive phase. Use of biogenic volatiles for non-invasive diagnostics of plant diseases are emerging as a promising technology (Sharifi and Ryu, 2018).

The compounds released at the early stage from infected marigold plants identified in this study can be used as biomarkers for diagnostics of phytoplasma infection.

Acknowledgements

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