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Welcome by the editors

We are delighted to edit the extended abstracts of the "Fourth International Phytoplasmologist Working Group Meeting" (IPWG) that will be held in Valencia, Spain. 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 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 and organizing a meeting in different countries every four years. So far, IPWG has successfully organized three meetings in Italy (2007), Germany (2011) and Mauritius (2015).

The 4th IPWG is held in Valencia, Spain September 8-12, 2019 with expected attendance of more than 140 scientists from about 40 countries worldwide. Two round tables on very update topics such as phytoplasmas and other pathogens mixed infection and quarantine are planned. There will be also some special presentations illustrating the phytoplasma taxonomy situation and the most recent finding in phytoplasma pathogenicity and management. The meeting will also have two sessions dedicated to EUPHRESCO network projects that are dialing with important topic for agriculture such as "Modelling the epidemiology of "flavescence dorée" in relation to its alternate host plants and vectors" (Flavid), "Study on the diversity of phytoplasmas detected in European forests" (PhyFor).

All the presentations of the meeting are included in this issue of Phytopathogenic Mollicutes, which is a half yearly official internationally known publication of the Technology Society of Basic & Applied Sciences (TSBAS) and is published by Indianjournals.com, New Delhi, India. This issue contains 120 extended abstracts printed in the journal format.

These extended abstracts cover different aspects of phytoplasma research and are really imperative and valuable in redefining the research and development needs of this branch of the plant pathology and challenges all over the world. Different aspects of phytoplasma-associated diseases are covered: detection methods and new disease reports worldwide, epidemiology and management, insect vectors, phytoplasma-host interaction and omic applications, mixed infection of phytoplasmas and other pathogens and quarantine relevance of phytoplasma associated diseases. These topics will provide an authoritative scientific backdrop for informed discussions and debates on recent achievements on phytoplasma research and also share the vision for a scientifically driven growth of phytoplasma research globally.

We are sure that this Phytopathogenic Mollicutes issue 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. Unintentional substantial mistakes will be corrected adding *an errata corrige*, as a last page of the "electronic reprint", on the online version of the issue.

Online information concerning these IPWG proceedings will be 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 timing in preparing their submissions. We apologize for errors that could have arisen during the editing process despite our careful attention. We wish to thank all the meeting participants, the IPWG scientific committee members and Fabio Montanari for their consistent support to the delivery of this issue of the Journal that is a very special one for the whole phytoplasma scientific community.

Assunta Bertaccini and Govind Pratap Rao
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Introduction

The phytoplasma classification between '*Candidatus* species' provisional status and ribosomal grouping system

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Abstract

The two available phytoplasma classification systems are suffering for the inability to accommodate all the taxa that are described with an always faster pace. There are still problems in fulfilling taxonomic rules for binomial nomenclature in spite the advances in knowledge of phytoplasma molecular and physiological aspects. For the plant pathology perspective the most important taxon is the strain that is usually providing information about specific epidemiological features of diseases. The finding of suitable molecular markers is very important to verify phytoplasma epidemiological routes and pathogen relevance.

Keywords: '*Candidatus* Phytoplasma solani', tomato, susceptibility, molecular detection, optimal timing and sampling

Introduction

Fifty one years after their discovery, the role of phytoplasmas as plant pathogens is still based on indirect biological or molecular proofs, such as electron microscopy observation, detection of pathogen nucleic acid, symptom elimination after tetracycline treatments (Ishiie *et al.*, 1967; Kirkpatrick *et al.*, 1987). Two provisional classification systems were however developed both based on the 16S ribosomal gene: the '*Candidatus* Phytoplasma' species (IRPCM, 2004) and the ribosomal grouping and subgrouping (Lee *et al.*, 1998). These phytoplasma classification schemes were adopted in the IOM meeting held in Bordeaux (France) in 1992 when the trivial name of phytoplasma was proposed (Figure 1).

The naming of phytoplasmas is the basis for their study, therefore almost all the available molecular tools were and still are, employed toward their identification and differentiation. The phytoplasma strains which may warrant designation of a new taxon but fail to meet the requirement of sharing <97.5% 16Sr sequence similarity with existing '*Ca.* Phytoplasma', can be differentiated and classified using additional unique biological properties such as antibody, host range and vector transmission specificity (Seemüller and Schneider, 2004) or geographic isolation (Win *et al.*, 2013). However still some phytoplasmas associated with relevant and/or quarantine diseases such as lethal yellowing (16SrIV group) and "flavescence dorée" (16SrV group) cannot be officially named as '*Ca.* Phytoplasma'. A barcode screening system using a short fragment of the *tuf* gene resulted able to differentiate the phytoplasma strains in agreement with the 16S rDNA classification. This tool allows a fast phytoplasma detection and identification that must be however verified and confirmed by amplification of the 16Sr gene sequences (Makarova *et al.*, 2012).

Classification

The high number of '*Ca.* Phytoplasma' species and ribosomal groups and subgroups described (Table 1) indicate the presence of great biodiversity in these bacteria intensified by the frequent report of 16Sr interoperon heterogeneity and/or mixed phytoplasma infection in both insects and plants. While both classification systems are greatly helping in epidemiology and management studies discrimination among strains and for pathogenicity characteristics is still not available. The '*Ca.* Phytoplasma' species designation have limitations imposed by the high degree of rDNA sequence conservation across diverse phytoplasma lineages and by the presence in a genome of two, sometimes sequence-heterogeneous, copies of the 16S rRNA gene.

Figure 1. The early phytoplasmologists at the IOM meeting in Bordeaux. On the right the name proposed by Barbara Sears (white arrow in the picture).

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Table 1. Grouping of officially described 'Ca. Phytoplasma' species.

16Sr subgroup	Disease name	'Candidatus' sp.
I-B	Aster yellows	'Ca. P. asteris'
I-Y	Tomato "brote grande"	'Ca. P. lycopersici'
II-B	Lime witches' broom	'Ca. P. aurantifolia'
II-D	Papaya mosaic	'Ca. P. australasia'
III-A	Peach X-disease	'Ca. P. pruni'
V-A	Elm yellows	'Ca. P. ulmi'
V-B	Jujube witches' broom	'Ca. P. ziziphi'
V-E	Rubus stunt	'Ca. P. rubi'
V-F	*Balanites* witches' broom	'Ca. P. balanitae'
VI-A	Clover proliferation	'Ca. P. trifolii'
VI-I	Passionfruit	'Ca. P. sudamericanum'
VII-A	Ash yellows	'Ca. P. fraxini'
VIII-A	Loofah witches' broom	'Ca. P. luffae'
IX-B	Almond witches' broom	'Ca. P. phoenicium'
X-A	Apple proliferation	'Ca. P. mali'
X-B	European stone fruit yellows	'Ca. P. prunorum'
X-C	Pear decline	'Ca. P. pyri'
X-D	Spartium witeches' broom	'Ca. P. spartii'
XI-A	Rice yellow dwarf	'Ca. P. oryzae'
XI-E	Cirsium phytoplasma	'Ca. P. cirsii'
XII-A	"Stolbur"	'Ca. P. solani'
XII-B	Australian grapewine yellows	'Ca. P. australiense'
XII-D	Japanese hydrangea phyllody	'Ca. P. japonicum'
XII-E	Yellows diseased strawberry	'Ca. P. fragariae'
XII-H	Bindweed yellows	'Ca. P. convolvuli'
XIII-A	Mexican periwinkle virescence	'Ca. P. hispanicum'
XIII-G	Chinaberry yellowing	'Ca. P. meliae'
XIV-A	Bermudagrass white leaf	'Ca. P. cynodontis'
XV-A	Hibiscus witches' broom	'Ca. P. brasiliense'
XVI-A	Sugarcane yellow leaf	'Ca. P. graminis'
XVII-A	Papaya bunchy top	'Ca. P. caricae'
XVIII-A	American potato purple top wilt	'Ca. P. americanum'
XIX-A	Chestnut witches' broom	'Ca. P. castaneae'
XX-A	*Rhamnus* witches' broom	'Ca. P. rhamni'
XXI-A	*Pinus* phytoplasma	'Ca. P. pini'
XXII-A	Lethal yellowing	'Ca. P. palmicola'
XXIX-A	*Cassia* witches' broom	'Ca. P. omanense'
XXX-A	Salt cedar witches' broom	'Ca. P. tamaricis'
XXXI-A	Soybean stunt	'Ca. P. costaricanum'
XXXII-A	Malaysian periwinkle virescence	'Ca. P. malaysianum'
XXXIII-A	Allocasuarina phytoplasma	'Ca. P. allocasuarinae'
XXXVI-A	Foxtail palm phytoplasma	'Ca. P. wodyetiae'
n.a.	Bogia coconut syndrome	'Ca. P. noviguineense'

Several molecular tools were developed allowing the finer phytoplasma differentiation. In particular non-ribosomal single-copy genes less conserved such as ribosomal protein (*rp*), *secY*, *secA*, *rpoB*, *tuf*, *groEL* have been extensively used for differentiation across the majority of the phytoplasmas. Moreover the differentiation of phytoplasmas in the diverse ribosomal groups should be carried out using specific multiple genes, including housekeeping genes, and possibly also variable genes encoding surface proteins (Martini *et al.*, 2019). These studies showed that in several cases the housekeeping genes have more phylogenetically informative characters than the 16S rRNA gene, and significantly increase the resolving power for discriminating genetic closely related but distinct phytoplasma strains within a given 16Sr group or 'Ca. Phytoplasma' species. However while the work for the

first 22 identified ribosomal groups was exploited according to their epidemiologic relevance in some areas of the world, for the remaining ribosomal groups these information even if potentially very relevant are still lacking.

Conclusions

It is very important to continue to exploit phytoplasma genes for their relevance in molecular epidemiology paying strong attention to their genetic conservation in order to avoid producing unreliable taxa with no phytopathological relevance. At the end biology represents still the missing knowledge for phytoplasmas therefore while continuing the work of phytoplasma classification it is also necessary to be aware that"...we must make an attempt to species definition. In doing so we are confronted by the paradoxical incongruity of trying to establish a fixed stage in the evolutionary stream. If there is evolution in the true sense of the word, as against catastrophism or creation, we should find all kind of species – incipient species, mature species, incipient genera, as well as all intermediate conditions" (Ernst Mayer, 1942).

Acknowledgements

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Worldwide

Molecular identification of phytoplasmas in ornamental pomegranates in Turkey

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Abstract

Leaves, flowers and shoots of ornamental pomegranates showing yellowing and leaf deformation were collected from symptomatic and non-symptomatic plants between 2015 and 2017 from Hatay, Sanliurfa and Gebze provinces of Turkey. Among the 20 tested pomegranate samples, 10 were positive for phytoplasmas using 16R758f/16R1232r and U5/U3 primers amplifying 16S ribosomal RNA gene. The sequences of 8 strains shared the closest nucleotide identity (99%) with phytoplasmas in group 16SrIX and the remaining 2 strains had 98% identity with the 'Candidatus Phytoplasma solani' (subgroup 16SrXII-A). The present study represents the first evidence of phytoplasma association with yellowing symptoms in ornamental pomegranates in Turkey.

Keywords: ornamental pomegranate, phytoplasma, 16SrIX-C, 16SrXII-A, PCR, sequencing, Turkey

Introduction

Pomegranates (*Punica granatum* L.) are originated from central Asia but they are adaptable to a wide range of soil and climates. Turkey is one of the main pomegranate producers among the Mediterranean countries. Beside the cultivated pomegranates, some *P. granatum* have an ornamental value. In Turkey, the ornamental pomegranates have been commonly used in parks and botanical gardens. Recently there have been some studies on virus and virus-like diseases of cultivated pomegranates in Turkey, but very limited data is available on ornamental pomegranates (Çaglayan *et al.*, 2016). Moreover phytoplasmas belonging to 16SrI-B, 16SrI-F, 16SrII-D and 16SrXII-A subgroups have been reported in cultivated pomegranates in Turkey, Iran, China and Guadeloupe (Gazel *et al.*, 2015; Karimi *et al.*, 2015; Salehi *et al.*, 2016, Gao *et al.*, 2018 and Castaneda-Alvarez *et al.*, 2018). Phytoplasma symptoms have recently been observed in ornamental pomegranate trees and the aim of this study was the detection and identification of phytoplasmas in those plants.

Materials and Methods

During 2015–2017 ornamental pomegranates showing yellowing symptoms were sampled from symptomatic and non-symptomatic plants in Hatay, Sanliurfa and Gebze provinces of Turkey. DNA samples from diseased and healthy plants were prepared from leaf midribs using a previously published method (Doyle and Doyle, 1990). Nested-PCR assays were performed using universal primer pair P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al.*, 1995) in the direct reaction. Two sets of primers: U5/U3 (Lorenz *et al.*, 1995) and 16R758f/16R1232r (M1/M2) (Gibb *et al.*, 1995) were then used in nested PCR to detect 16S rRNA gene sequences from phytoplasmas amplifying a 509 bp and 865 bp fragments, respectively. The PCR cycling conditions and the parameters were used as described by Matus *et al.*, (2008). The amplified PCR products were directly sequenced by Iontek Biotechnology Company (Istanbul, Turkey). BLAST search and CLC Main software was used to analyze the sequences.

Results

Among the 20 tested pomegranate samples, 2 out of 3 from Hatay, 4 out of 4 from Sanliurfa and 4 out of 13 from Gebze were found positive for phytoplasmas by using both M1/M2 and U5/U3 (Figure 1). The phytoplasma strains in ornamental pomegranate plants were identified by comparing their sequences with the 16S ribosomal DNA sequences of different phytoplasma groups. They clustered with phytoplasmas classified in the pigeon pea witches' broom phytoplasma group 16SrIX (Figure 2). Among phytoplasma positive samples, two strains exhibited identity of 98% with the 'Candidatus Phytoplasma solani'-related strains (subgroup 16SrXII-A) when 16S ribosomal DNA sequences were subjected to BLAST analysis.

Figure 1. Nested PCR amplification of phytoplasma ribosomal DNA from symptomatic pomegranates by using the universal primers P1/P7 followed by primers M1/M2 and U5/U3. Marker: SMO331 (MBI Fermentas); C: pear decline phytoplasma; W: water control.
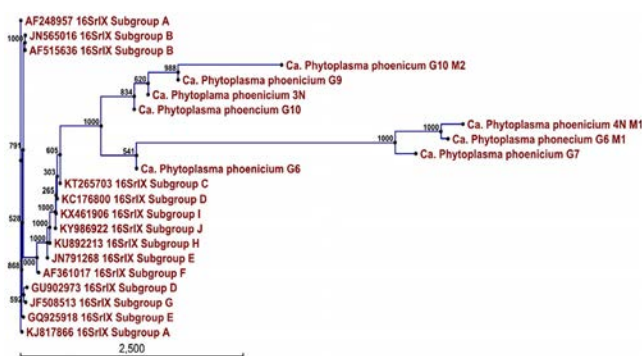
Figure 2. Phylogenetic relationships of 16SrIX phytoplasmas from ornamental pomegranate with other phytoplasmas in 16SrIX subgroup retrieved from NCBI Genbank, constructed by the neighbour-joining algorithm. The numbers on the branches indicate bootstrap values.

Discussion

The first report of 16SrI-B and 16SrXII-A phytoplasmas in mixed infection in cultivated pomegranate trees from Turkey (Gazel *et al.*, 2015) has led the current study to further verify the phytoplasma presence in this species.

In this study 16SrIX phytoplasmas were detected for the first time infecting ornamental pomegranates. Prior to this study, different plant species have been described as natural host for phytoplasmas in group 16SrIX. In Turkey it has been reported associated with sesame phyllody (Ikten *et al.*, 2014). It has been shown that some wild plants in Lebanese *Prunus* orchards harbour the 16SrIX-C phytoplasmas which genetically distinct from the agent associated with almond witches' broom (AlmWB) disease (16SrIX-B) '*Candidatus* Phytoplasma phoenicium' (Zirak *et al.*, 2009; Casati *et al.*, 2016). '*Ca*. P. solani' (subgroup 16SrXII-A) was also detected in ornamental pomegranates. A wide range of wild and cultivated hosts for this phytoplasma was reported and cultivated pomegranates were also detected as a natural host for this phytoplasma in Turkey (Gazel *et al.*, 2015); this study confirmed the presence of the pathogen in ornamental pomegranates. This is the first report for the presence of phytoplasmas in group 16SrIX and '*Ca*. P. solani' (subgroup 16SrXII-A) in ornamental pomegranates.

Acknowledgements

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Worldwide

Molecular identification of 'Candidatus Phytoplasma oryzae' in Garcinia gummi-gutta in India

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Abstract

Garcinia gummi-gutta is a medicinal plant growing in Asia and Africa. During a survey in 2017, witches' broom and little leaf symptoms were observed on *G. gummi-gutta* (GaLL&WB) plants at Kumarakom Kottayam district of Kerala, India. The disease incidence recorded was 2%. An investigation was carried out to identify the phytoplasma associated with the GaLL-WB. Amplicons of ~1.25 kb were consistently amplified from DNA extracted from all the GaLL-WB affected plants in a nested PCR assays using primer pairs P1/P7 and R16F2n/R16R2, however, no amplification was observed with any of the symptomless plant samples. The 16S rDNA sequence of the GaLL&WB phytoplasma revealed 98.2% of sequence identity to those of 'Candidatus Phytoplasma oryzae' strains and computer simulated RFLP analysis of the GaLL-WB 16S rDNA sequence confirmed it as a member of the group 16SrXI, subgroup B. This is the first report of 'Ca. P. oryzae' associated with little leaf and witches' broom disease of *G. gummi-gutta*.

Keywords: *Garcinia gummi-gutta*, 'Ca. P. oryzae', 16SrXI-B subgroup, Kerala, India

Introduction

Garcinia gummi-gutta (Clusiaceae), commonly known as brindle berry and "malabar tamarind" is grown because of its important medicinal properties in Southeast Asia and west and central Africa (Hart *et al.*, 2016). The economic part of the plant is its mature fruit and the extract obtained from the mature fruit rind attracts foreign markets for its use in medicines controlling obesity (Sethi, 2011). Major factor that limits *G. gummi-gutta* productivity is its extreme susceptibility to various abiotic and biotic factors like *Curvularia lunata* and *Pestalotiopsis* sp. which were associated with necrotic lesions on leaf margins. So far, no report is available of phytoplasma infection in *G. gummi-gutta* from any part of the world.

Materials and Methods

During 2017 (October-November) survey in the Kumarakom, Kottayam District of Kerala, India, phytoplasma-like symptoms of little leaf and witches' broom were observed in *G. gummi-gutta* (GaLL&WB) plants. DNA was extracted from leaf tissues of symptomatic and non-symptomatic *G. gummi-gutta* by using a CTAB method (Ahrens and Seemüller, 1992). The DNA concentrations and quality were measured with a nanodrop spectrophotometer (ND1000UV/VIS, USA). The DNA extracted from symptomatic sugarcane infected with the grassy shoot disease phytoplasma (SCGS, 16SrXI) (Rao *et al.*, 2014) maintained in the greenhouse was used as positive control. Amplification of phytoplasma ribosomal DNA was performed with the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by nested primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). PCR reactions were performed in a Mastercycler (Eppendorf Germany) and the cycling protocols used for direct and nested PCR were as described earlier (Rao *et al.*, 2014). Five microlitres of nested PCR products were subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and observed under a UV transilluminator. The R16F2n/R2 amplified 16S rDNA fragments were purified using the WizardR SV Gel and PCR Clean-up System (Promega, Madison, USA). Purified products were directly sequenced in both directions at the AgriGenome, Kakkanad, Kerala, India. The R16F2n/R2 sequences were assembled using DNA Base V.4 (http://www.dnabaser.com) and aligned with phytoplasma ribosomal group/subgroup representatives available in GenBank using ClustalW software (Hall, 1999) and the consensus sequence was submitted to the GenBank. A

phylogenetic tree was constructed using the neighbor joining method with MEGA 7.0 (Kumar *et al.,* 2016) using 1,000 bootstrap replications. *Acholeplasma laidlawii* 16S rDNA sequence was used to root the trees for the phylogeny. The phytoplasma sequences corresponding to the R16F2n/R16R2 fragments detected in *G. gummi-gutta* were subjected to *in silico* RFLP comparison using the *i*PhyClassifier online tool and compared with representative sequences of the phytoplasmas in the 16SrXI group (Zhao *et al.,* 2009).

Figure 1: Symptoms of witches' broom on *Garcinia gummi-gutta*
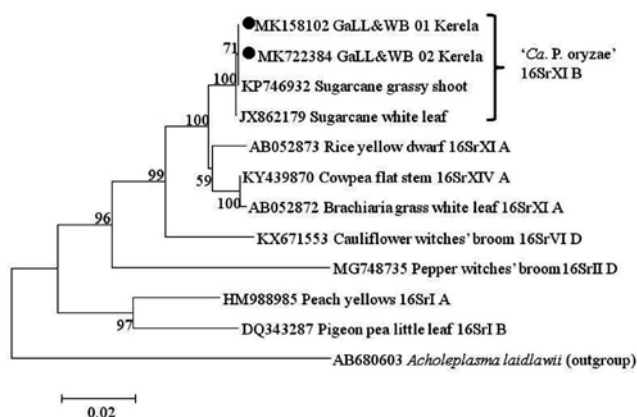
Figure 2: Phylogenetic tree constructed by the neighbor-joining method of 16SrDNA gene sequence of the garcinia little leaf and witches' broom (GaLL&WB) isolates (black circles).

Results and Discussion

During the survey phytoplasma-like symptoms of little leaf and witches' broom were recorded on *G. gummi-gutta* plants in the fields of the Kottayam district with an incidence of 2% (Figure 1). The phytoplasma presence was confirmed by PCR amplification of ~1.8 kb products and ~1.25 kb amplicons in nested PCR. The comparison of the R16F2n/R16R2 sequences deposited in GenBank under the accession numbers MK158102 and MK722384 revealed the highest identity (98.2%) of the GaLL&WB phytoplasma strains to

that of a member of the 16SrXI group ('*Candidatus* Phytoplasma oryzae', GenBank accession number AJ550984) (Figure 2). The virtual RFLP analysis classified the GaLL&WB phytoplasma strains within the 16SrXI-B subgroup (data not shown). Earlier, around 200 medicinal plants including alfalfa, Bermudagrass, bitter gourd, garlic, lemongrass, *Plantago* spp., thyme, *Tylophora asthamatica*, senna, Indian ginseng, *Ocimum* sp., *Cannabis sativa* were reported as hosts of different groups of phytoplasmas in India and abroad (Rao *et al.,* 2017). This is the first world report of '*Ca.* P. oryzae' subgroup B strain associatiation with the GaLL&WB disease.

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First report of a '*Candidatus* Phytoplasma aurantifolia'-related strain in *Citrus macrophylla* in Oman

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Abstract

Citrus macrophylla is one of the most commonly used citrus rootstocks and it was found exhibiting witches' broom disease symptoms. Polymerase chain reaction (PCR) was used to amplify the 16S ribosomal gene of phytoplasmas with the universal primers P1/P7 as a direct PCR and R16F2n / R16R2 primers for the nested PCR. The amplifications from all the symptomatic plants yielded a product of 1.8 kb and 1.2 kb in the direct and the nested PCR, respectively. Sequence identity results from BLAST and phylogenetic analysis of the 16S rRNA gene sequences confirmed that the phytoplasma identified in *C. macrophylla* in Oman is a '*Candidatus* Phytoplasma aurantifolia'-related strain.

Keywords: phytoplasma, WBDL, *Citrus macrophylla*, witches' broom, Oman

Introduction

Citrus macrophylla is one of the most commonly used citrus rootstocks worldwide. It has been reported as salt-tolerant and has resistance to many fungal, bacterial and viral diseases (Fernández-Ballester *et al*., 2003). Ten-year old *C. macrophylla* trees grown in a field at the Agricultural Experimental Station, Sultan Qaboos University (N: 23.600955, E: 58.164136), Al-Seeb, Muscat, Oman, were observed with symptoms of witches' broom (WB), which are typical of phytoplasma diseases. The *C. macrophylla* symptoms were similar to those shown by the acid lime witches' broom disease (WBDL) (Garnier *et al*., 1991) reported previously in Oman and associated with the presence of '*Candidatus* Phytoplasma aurantifolia' (16SrII-B subgroup) (Zreik *et al*., 1995).

Materials and Methods

Leaf samples from five WB-symptomatic and two asymptomatic *C. macrophylla* trees were sampled for total nucleic acid DNA extraction as described by Al-Subhi *et al*. (2017). The WB phytoplasma DNA was used as positive control and sterile distilled water instead of template, and asymtomatic *C. macrophylla* DNA as negative controls. Specific phytoplasma primers for the 16S rRNA gene P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) were used in direct PCR and the primer pair R16F2n/R16R2 (Gundersen and Lee, 1996) in nested PCR. Ready-To-Go' PCR beads (Pharmacia Biotech, Sweden) were used in PCR amplifications with 25 µl reaction volumes and 10 pmoles of each primer. The PCR products were purified and sequenced at the Macrogen Company (South Korea). The DNA sequences obtained were deposited at GenBank (NCBI, Bethesda, MD, USA) (http://ncbi.nlm.nih.gov). Multiple sequence alignment of the 16S rDNA gene sequences with 38 16S rDNA phytoplasmas retrieved from the GenBank sequences (NCBI, Bethesda, MD, USA) (http://ncbi.nlm.nih.gov/ BLAST) was performed using Clustal W (Thompson *et al*., 1994) and phylogenetic trees were constructed according to the neighbor-joining (NJ) method using MEGA 6 software (Tamura *et al*., 2013). The sequence of *Bacillus subtilis* 16S rRNA (GenBank accession number AB042061) was used as an outgroup to root the phylogenetic trees.

Results

The direct and nested PCR reactions yielded 1.8 kb and 1.2 kb fragments, respectively from the symptomatic *C. macrophylla* trees as well as from the WB phytoplasma. Asymtomatic *C. macrophylla* trees and water samples did not show any amplification. The 16S rDNA sequences (about 1,440 bp) of the five *C. macrophylla* phytoplasma strains were deposited in GenBank under the accession numbers MK518321 to MK518325. They were 100% identical to each other. The BLAST search at NCBI showed that the phytoplasma strains from *C. macrophylla* from this study had a more than 99.5% of sequence identity to that of the WBDL phytoplasma (16SrII-B) (GenBank accession numbers

KX348042, U15442 and LN866569). The *C. macrophylla* phytoplasma clusters with a citrus witches' broom phytoplasma from Oman (Figure 1).
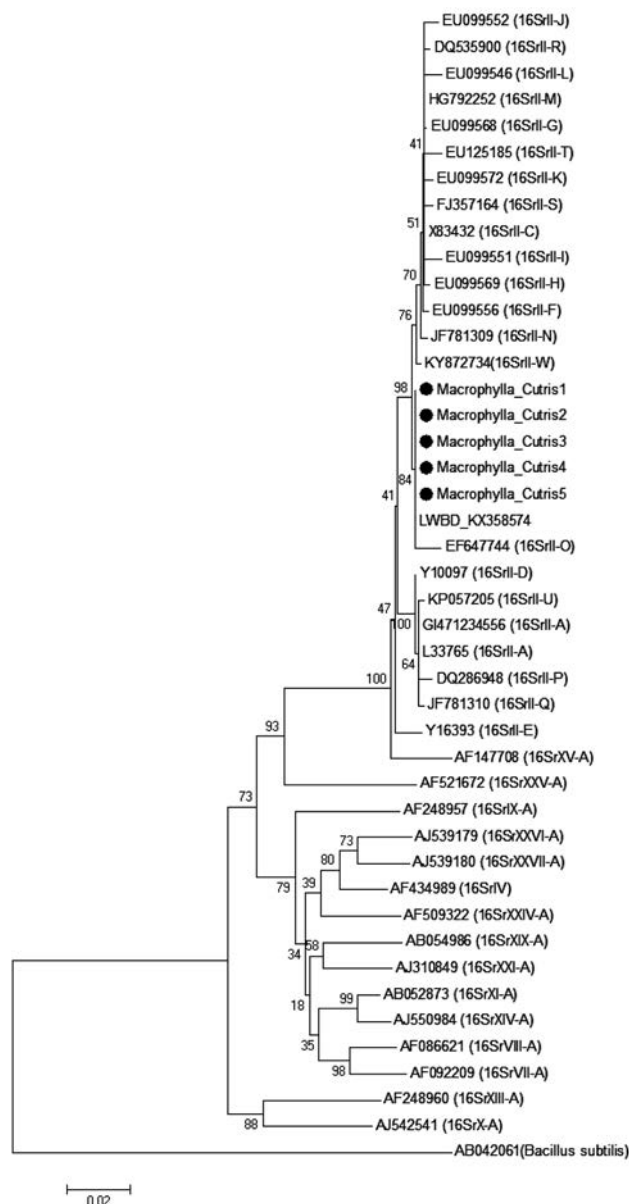
Figure 1. Phylogenetic tree based on 16S rDNA sequences of the *C. macrophylla* phytoplasma strains.

Discussion

C. macrophylla developed typical symptoms of phytoplasma infection similar to those described for the lime witches' broom disease (Garnier *et al.*, 1991) in Oman. The results of all five *C. macrophylla* phytoplasmas included in this study showed that they have the same 16S rRNA gene sequences. Phylogenetic analysis clustered all *C. macrophylla* phytoplasma strains in the same subclade with a strain from citrus. This is the first report of a '*Ca.* P. aurantifolia'-related strain naturally infecting *C. macrophylla* trees in Oman.

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Worldwide

Molecular detection of phytoplasmas infecting date palm in Saudi Arabia and Jordan

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Abstract

Date palm is one of the most important cash crops in Saudi Arabia and Jordan. Symptoms of mild leaf stunting, yellow streaking and a reduction in fruit and stalk size had been observed in date palm orchards in both countries. In Jordan a total of 262 healthy and symptomatic samples were collected from different date palm cultivars, weeds growing under date palm trees and other different plant species possible phytoplasma hosts growing in the orchards in addition to leafhoppers, while 567 leaf samples were collected from Saudi Arabia. Nested PCR was performed using phytoplasma P1/P7 and R16F2n/R2 primer pairs. BLAST analysis of the obtained sequences of samples from Jordan revealed over 98% sequence identity with different reference phytoplasma strains deposited in the GenBank and 98-100% sequence identity with 'Candidatus Phytoplasma ulmi'-related strains. A 16SrII phytoplasma, 'Candidatus Phytoplasma aurantifolia'-related strain was identified in the samples from Saudi Arabia.

Keywords: *Phoenix dactylifera*, '*Candidatus* Phytoplasma ulmi', date palm, phytoplasma, 16S rDNA

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most important cash crops in Saudi Arabia and Jordan. The "Al-Wijam" diseases symptoms include stunting and yellow streaking of the leaves, with fruits and fruit stalks reduced in size (around 30%); intensity of symptoms varies with the variety. Phytoplasmas have been associated with diseases in date palm such as white tip dieback and slow decline in North Africa (Cronjé *et al.*, 2000), yellowing in Kuwait (Al-Awadhi *et al.*, 2002), and lethal decline in Texas (Harrison *et al.*, 2002). Phytoplasmas are vectored by Auchenorrhyncha insects: leafhoppers, planthoppers and psyllids. Thus the objective of this study was to conduct identification of phytoplasmas associated with "Al-Wijam" in Saudi Arabia and Jordan including putative vector of the disease. The results of this work provide important contributions to the knowledge of the disease in Saudi Arabia and open new understanding of the epidemiology of this disease in both Countries.

Materials and Methods

Different samples of date palm, weeds and other plant species and crops that grow under the date palm trees were collected from different locations in Saudi Arabia and Jordan. The samples were kept between plastic sheets in ice boxes and each sample was identified by a code number before processing in the laboratory. In addition, leafhopper insects were collected by placing traps at the base of date palm trees. Date palm tissue culture was used in all experiments as negative control. Universal primers P1/P7 and R16F2n/R2 (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Gundersen and Lee, 1996) were used in the direct and nested PCR amplifications respectively. Direct sequencing of the obtained products was then carried out together with sequence assembling and alignment in order to verify the phytoplasma identity.

Results and Discussion

The phytoplasma universal primer pairs P1/P7 yielded a sharp band of about 1,784 bp from symptomatic tomato from Jordan and another sharp band of about 1,293 bp with R16F2n/R16R2 (Figure 1). The BLAST analysis of the obtained sequence revealed over than 98% of identity when compared to different reference phytoplasma strains deposited in the GenBank and 98-100% of identity with the sequence of '*Candidatus* Phytoplasma ulmi'-related strains. In Saudi Arabia, the percentage of infection in all tested plants was 7.3% while in Jordan was 57.2%. PCR products were sequenced and sequences were subjected to phylogenetic analysis for

the Jordanian strains and compared with different phytoplasma strains available in GenBank. So far this is the first study in Jordan concerning phytoplasma disease possibly related to palm trees. These results will help researchers to understand the epidemiology of the disease and to further implement integrated pest and disease management.
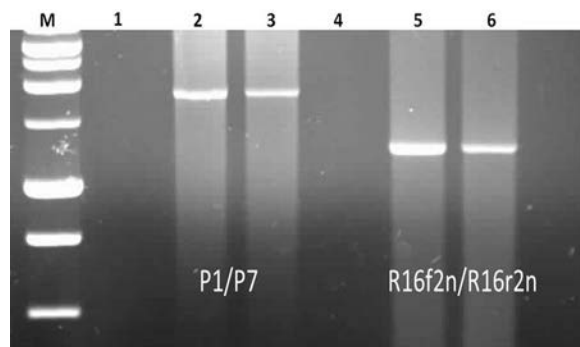
Figure 1. Agarose gel electrophoresis analysis showing P1/P7 and R16F2n/R16R2 PCR amplification products from tomato from Jordan. M: 1 kb DNA ladder, 1, 4: negative controls; 2, 3: bands with the set of primers P1/P7, 5,6: bands from the nested reaction with the set of primers R16F2n/R16R2.

In Saudi Arabia, two phytoplasma groups were identified; 16SrI ('*Candidatus* Phytoplasmas asteris') in Alhasa, and 16SrII ('*Ca.* P. aurantifolia') in other locations. These findings are supporting previous reports of a 16SrI phytoplasma associated with "Al-Wijam" in Saudi Arabia identified in the Alhasa oasis in the Eastern Province (Alhudaib *et al.*, 2007). Both 16SrI and 16SrII phytoplasmas were indistinctly found in date palms affected by the disease in six different locations of Saudi Arabia. The group 16SrII was identified from Alkharj, Qassim and Riyadh regions where it was confirmed in date palms, basil (*Ocimun basilicum*) and alfalfa (*Medicago sativa*) grown nearby the date palms. Therefore, either basil or alfalfa may play a role as secondary hosts for the 16SrII phytoplasma currently infecting date palms in these two neighboring regions, which may also have common polyphagous Hemiptera vectors. *M. sativa* is a

very well-known host for the 16SrII group since it has been widely reported also from Oman (Khan *et al.*, 2001; 2002).

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Worldwide

A new elm yellows phytoplasma strain associated with leaf yellowing disease of *Tamarindus indica* in India

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Abstract

Tamarind is an economically important tree species; its fruit pulp is widely used in kitchen in India. A symptomatic tamarind tree with yellow leaf symptoms found in the western part of India was assayed for phytoplasma presence. The 16S rDNA sequence of the detected phytoplasma (TM01) was found clustering with sequences in the elm yellows (16SrV) phytoplasma group. The virtual RFLP analysis of obtained 16S rDNA sequence TM01 revealed that it could be tentatively classified into a novel phytoplasma subgroup, 16SrV-L since its *Alu*I, *Hae*III and *Mse*I restriction profiles of R16F2n/R16R2 sequenced amplicons generated were unique as compared to the earlier reported profiles of this phytoplasma subgroup. This is the first phytoplasma report in *Tamarindus indica*.

Keywords: elm yellows; tamarind, India, disease, detection

Introduction

Tamarind (*Tamarindus indica* L., Fabaceae) is a leguminous tree originating in tropical Africa, but now widespread through the tropical subcontinent of India. The genus *Tamarindus* is known for its use in medicine since the ancient times. It yields 150-500 kg fruits per tree and its fruit pulp is extensively used in culinary preparations in India, especially in the southern states, it is also the most abundant natural source of tartaric acid (Shankaracharya, 1998). Tamarind usually grows as a massive tree, and its wood can be used for woodworking; the tamarind seed oil is used in medicine. Though seldom diseases are reported in tamarind plant, no phytoplasma associated diseases have been reported. A tamarind plant showing leaf yellowing was suggestive of phytoplasma infection and samples were collected for phytoplasma detection assays.

Materials and Methods

Tamarind tree showing symptoms of leaf yellowing was observed at Baramati district, Maharashtra, India in November 2017. The leaf yellowing was restricted to a branch of the tree. The usual symptoms of phytoplasma infection like a little leaf, brooming of tree branches or phyllody were not observed. To verify the presence of phytoplasmas, 2 g of leaves from one each of symptomatic and asymptomatic plant parts were used for total DNA extraction using the CTAB method (Doyle and Doyle, 1990). The phytoplasma 16S rRNA gene was amplified from 50 ng of DNA using primers P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al.*, 1995) followed by nested primers R16F2n and R16R2 (Gundersen and Lee, 1996). For nested PCR reaction, either 5-, 10- or 20-fold diluted template generated by P1/P7 primers was used. Each PCR reaction was performed with 1X PCR buffer, 1.5 mM MgCl$_2$, 200 µM of dNTPs, 1 U of Invitrogen Brazilian Taq DNA Polymerase (Cat. No. 10342020), 2 µg BSA and one µM of primers. PCR products were purified and sequenced directly using bacterial universal primers 343R, 704F, 907R, 1028F and 1492R (Baker *et al.*, 2003) on ABI® 3730xl DNA analyser. The obtained 16S rDNA sequences were curated and analysed using EzTaxon database (Yoon *et al.*, 2017) trimmed and subjected to further analysis using nBLAST, EzBiocloud. Computer-simulated RFLP analysis of the 16S rRNA gene was performed on obtained sequences using the *i*PhyClassifier (Zhao *et al.*, 2009) to identify the phytoplasma at the ribosomal group and subgroup level. Each sequence was digested *in silico* with 17 restriction enzymes and separated on 3% virtual gels. The virtual RFLP patterns were compared, and the similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula F52Nxy/ (Nx+Ny), (Wei *et al.*, 2007). Fragment profiles obtained for the phytoplasmas associated with tested samples were compared with published RFLP pattern of phytoplasma representatives of available ribosomal groups and 16SrV subgroups (Lee *et al.*, 1998).

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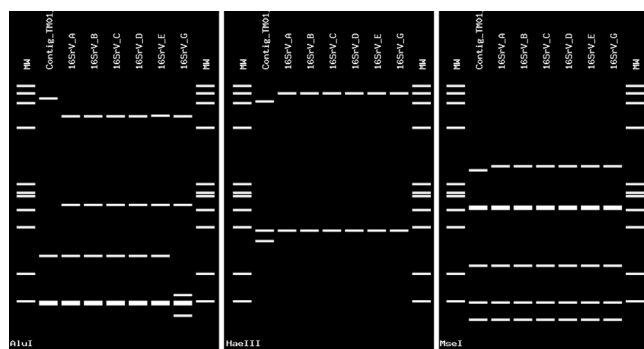
Figure 1. The virtual RFLP gel images showing the unique RFLP profiles of *AluI* (left), *HaeIII* (middle) and *MseI* (right) digested 16S rRNA gene fragments of the phytoplasma infecting *Tamarindus indica* (sample TM01) compared to subgroups of the 16SrV group in the *i*PhyClassifier.

Results

The collected symptomatic leaf samples of tamarind resulted positive to phytoplasma presence while no amplification was observed in the asymptomatic samples. The obtained sequence of 16S rRNA gene was found matching with the phytoplasma strains in the elm yellows (16SrV) group. The 16S rDNA sequence of strain TM01 was 99.53% similar to a strain from *Balanites triflora* (GenBank accession number AB689678) when analysed using EzTaxon database. Computer-simulated RFLP analysis of the 16S rRNA gene was performed with the obtained about 1,250 bp of R16F2n and R16R2 generated PCR product sequences. The TM01 scored the virtual RFLP similarity coefficient of 0.91 with earlier published subgroups of the 16SrV group of phytoplasmas. The restriction profiles of *AluI*, *HaeIII* and *MseI* (Figure 1) were found different from all those available in the *i*PhyClassifier and also from the other reported patterns of recently published subgroups (Fránová *et al.*, 2016).

Discussion

Several diseases with typical phytoplasma symptoms on various shrub and tree species reported were associated with phytoplasmas of elm yellows (16SrV) group. The tree and shrub species of *Prunus, Ziziphus, Pluchea* and *Corchorus* were reported infected with 16SrV phytoplasma strains from various parts of India (Rao *et al.*, 2017). This is the first report of a previously undescribed subgroup, tentatively named 16SrV-L associated with yellow leaf disease of *T. indica* in India. Its collective RFLP pattern is infact also differentiable not only from the strains available in the *i*PhyClassifier but also from other published strains enclosed in subgroup 16SrV-I detected in Portuguese blackberry (GenBank accession number KR233473) (Fránová *et al.*, 2016), and in subgroup 16SrV-H detected in while *Bischofia polycarpa* witches' broom from China (GenBank accession number KJ452547) (Lai *et al.*, 2014; Fránová *et al.*, 2016). Notably, very few herbaceous plant or crop species were found susceptible to 16SrV phytoplasma strains, such as tomato in Italy and Mauritius (Martini *et al.*, 2019). This indicates a limited plant host range which is very likely restricted by polyphagous

but host-specific insect vectors. Further studies are required to investigate the origin of this phytoplasma strain and its possible spread through insect vectors among the tree and shrub species in this area of India.

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Worldwide

New host plants and distribution areas of 'Candidatus Phytoplasma omanense'-related strains in Iran

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Abstract

During 2015-2018, total DNAs extracted from symptomatic and symptomless fruit and weed plant species in Yazd and Esfahan provinces, Iran, were subjected to direct and nested PCR using P1/P7, R16mF2/R16mR2 and R16F2n/R16R2 primer pairs. Expected amplicons were obtained only from the symptomatic plants. Real and virtual RFLP, phylogenetic and DNA analyses of partial 16S ribosomal sequences of the detected phytoplasma strains showed the presence of phytoplasmas referable to 16SrXXIX group (Cassia witches' broom phytoplasma) in *Prunus persica*, *Prunus domestica*, *Diospyros kaki* and *Sophora alopecuroides* with yellowing, reddening, dwarfing, die-back and decline symptoms. This phytoplasma was often detected in mixed infection with others. The need for a precise survey of phytoplasmas associated to symptomatic fruit trees in Iran and a further investigation of the possible role of '*Candidatus* Phytoplasma omanense' strains as a threat to Iranian fruit orchards are urgently needed.

Keywords: *Diospyros kaki, Prunus persica, Prunus domestica, Sophora alopecuroides*

Introduction

Phytoplasmas are wall-less plant-pathogenic bacteria, associated with a wide range of important plant disease worldwide. In nature, they cause significant yield losses worldwide in more than one thousands plant species and are transmitted mainly by leafhoppers and psyllids (Bertaccini *et al.*, 2014). Phylogenetic analysis of 16S rRNA gene sequence has revealed that phytoplasmas constitute a coherent genus of the *Mollicutes* (Lee *et al.*, 1998). Currently, they are divided into 36 groups based upon RFLP analysis of 16S rRNA gene sequences, and a total of 43 members of '*Candidatus* Phytoplasma' have been proposed (IRPCM, 2004; Bertaccini and Lee, 2018). '*Candidatus* Phytoplasma omanense' (*Cassia* witches' broom, 16SrXXIX-A) was identified as associated with witches' broom disease of *Cassia italica* in Oman (Al-Saady *et al.*, 2008). In 2014, bindweed witches' broom and dwarfing symptoms were observed in plants grown in alfalfa fields in Bafg (Yazd province, Iran) and identified as a new host plant of the phytoplasma that resulted to be assigned to the 16SrXXIX-B subgroup (Esmailzadeh Hosseini *et al.*, 2014). A '*Ca*. P. omanense'-related strain was later detected in grapevine yellows, stunted bindweed and Cixiidae planthoppers in Lebanon (Foissac *et al.*, 2019). The present work reports new host species and distribution areas of '*Ca*. P. omanense'-related strains in Iran.

Materials and Methods

During 2015-2018, total DNAs were extracted from symptomatic and symptomless fruit and weed plant species in Yazd and Esfahan provinces, Iran using the method described by Zhang *et al.* (1998). Samples collected from 28 symptomatic and four symptomless plants 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. Real and virtual RFLP and DNA homology analyses of partial 16S ribosomal sequences were used to identify the phytoplasmas. Phylogenetic analysis using neighbour-joining method (MEGA software version 7.0) (Kumar *et al.*, 2016) were employed for phylogenetic assessments.

Results and Discussion

The results of molecular analyses allow to confirm the phytoplasma presence in *Diospyros kaki, Prunus persica, Prunus domestica* and *Sophora alopecuroides*. The prevalent disease symptoms were yellowing, reddening and dieback

in *D. kaki*; yellowing, reddening, dieback and decline in *P. persica* and *Prunus domestica*, and yellowing and dwarfing in *S. alopecuroides* (Figure 1). DNA fragments of approximately 1,800, 1,400 and 1,250 bp were amplified from all the symptomatic plants in direct PCR using P1/P7 and nested PCR using P1/P7 followed by R16mF2/R16mR2 and R16F2n/R16R2, respectively. No amplification was observed in asymptomatic samples and water negative control. Results of RFLP analyses indicate the presence of phytoplasmas enclosed in the 16SrXXIX group ('*Ca.* P. omanense') and these results were confirmed by the RFLP analyses of the 1.25 kbp 16S rDNA. This phytoplasma was often detected in mixed infection with other phytoplasmas in the symptomatic plants (Figure 2).

Figure 1. The prevalent disease symptoms were yellowing, reddening and dieback in *Prunus domestica* (a), decline in *Prunus persica* (b), yellowing, reddening and dieback in *Diospyros kaki* (c), and yellowing and dwarfing in *Sophora alopecuroides* (d).
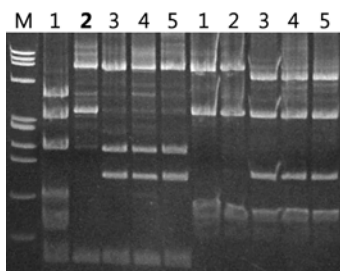
Figure 2. RFLP analysis in 6.7% polyacrilmide gel showing the results of restriction with *Hpa*II (left) and *Taq*I (right) of five R16F2n/R2 amplicons from the same P1/P7 product of *S. alopecuroides*. The profile referable to 16SrXXIX is the one of sample 2 in *Hpa*II (in bold); other profiles are referred to phytoplasmas still under study. M, marker phiX174 *Hae*III digested.

Sequence comparison by BLAST analysis (www.ncbi.nlm.nih.gov) showed the highest sequence identity with phytoplasmas in group 16SrXXIX. Phylogenetic analysis confirmed that one of the associated strain is a '*Ca.* P. omanense'-related strain. Formerly 16SrXXIX-A and -B subgroups were detected in *Prunus persica* and *Convolvulus arvensis* witches' broom in Iran respectively (Esmailzadeh

Hosseini *et al.*, 2016, 2017). This finding is enclosing the '*Ca.* P. omanense' among the phytoplasma strains infecting also fruit trees and indicates that it is a threat in diverse areas of their cultivation in Iran.

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New diseases associated with 16SrIII-J phytoplasmas in Chile

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Abstract

Several plants of potato, cactus pear and carrot showing symptoms of presumable phytoplasma infection, were collected in Metropolitana and Lagos regions of Chile. Nucleic acid was extracted from the leaf midribs and used for phytoplasma detection. The amplification products of the expected size with nested polymerase chain reaction on *tuf* and 16S rRNA genes were obtained only from symptomatic samples. Cloning, sequencing and virtual restriction fragment length polymorphism analysis, allowed the identification of the 16SrIII-J phytoplasma presence in all plant species tested.

Keywords: potato, cactus pear, carrots, nested-PCR, cloning, RFLP, sequencing

Introduction

The phytoplasmas belonging to the ribosomal subgroup 16SrIII-J are endemic in Chile and South America. They infect many commercial, herbaceous and woody species, as well as spontaneous plants (González *et al.*, 2011; Zamorano *et al.*, 2015; Quiroga *et al.*, 2017). The known vectors of the phytoplasma 16SrIII-J are *Bergallia valdiviana* Berg 1881 (Quiroga *et al.*, 2015) and *Paratanus exitiosus* Beamer (Quiroga *et al.*, 2019a). Symptoms possibly due to phytoplasma presence were observed in potato (*Solanum tuberosum* L.) cactus pear [*Opuntia ficus-indica* (L.) Mill., 1768] and carrot (*Daucus carota* L.). Potato plants showed leaf curl, witches' broom and yellowing. In cactus pear, phloem necrosis and bud deformation were observed. The carrots plants showed reddening of the leaves, deformation of the floral buds and proliferation of the lateral roots (Figure 1).

Materials and Methods

Samples of asymptomatic and symptomatic plants were collected during late spring: potato plants in the Lagos region; cactus pear and carrot in the Metropolitana region. From each sample, 150 mg of leaf midribs were macerated with the extraction buffer and processed according to the silica capture method (Rott and Jelkmann, 2001). The PCR amplification was carried out using 20 ng/µl of nucleic acid; direct and nested PCR on *tuf* gene were carried out according

to the protocol of Makarova *et al.* (2012); direct PCR with primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and nested PCR with R16F2n/R2 primers on the 16S rRNA gene (Gundersen and Lee, 1996) were performed following Schaff *et al.* (1992). Amplicons from nested PCRs for both genes were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania). Putative recombinant clones were analysed by colony PCR and selected fragments were sequenced in both directions in Macrogen USA Corp (Rockville, MD, USA). The sequences were then aligned with those of phytoplasma strains deposited in GenBank and in EppoQ-Bank, using BLAST engine for local alignment (version Blast N 2.2.12). Phytoplasma identification was done using *in silico* restriction fragment length polymorphism (RFLP) analysis with *Mse*I, *Nde*II, *Hha*I, *Bst*UI, and *Rsa*I restriction enzymes. The first two enzymes were used for the *tuf* gene and the last three for the 16S rRNA gene sequences.

Results

PCR amplification was only obtained from symptomatic plants for both genes. The nucleotide sequence identity of the cloned fragments obtained from the three species, showed 100% and 99.9% to 100% identity for *tuf* (438 bp) and 16S rRNA (1,250 bp) genes, respectively.

Figure 1. Symptoms associated with the 16SrIII-J phytoplasma. A) Carrot: reddening and proliferation of lateral roots. B) Cactus pear: proliferation and bud deformation. C) Potato: witches' broom and yellowing.

The nucleotide sequence identity in the 16S rDNA gene amplicons, showed a close correlation (99.8%) with the strain Ch10 (GenBank Accession number, Acc. No., AF147706), corresponding to the chayote witches' broom phytoplasma (16SrIII-J) from Brazil. When compared the *tuf* gene amplicons, the nucleotide sequence identity was 100% with the strain Hort72, detected in sugar beet in Chile (GenBank Acc. No. KM658259). The *tuf* and 16S rDNA amplicons were also subjected to *in silico* RFLP analysis, which confirmed the assignment of phytoplasmas to the ribosomal subgroup 16SrIII-J.

Discussion

This is the first report of a 16SrIII-J phytoplasma infecting carrot, cactus pear and potato plants. In the orchards inspected, the number of symptomatic plants was less than 1%. However, it has been shown that 16SrIII-J is able to infect different host species and, apparently, it is transmitted by several species of leafhoppers that can easily be found in the Chilean fields (Quiroga *et al.*, 2019b). Based on this, a constant surveillance must be maintained to prevent spreading and epidemic outbreaks associated with the presence of 16SrIII-J phytoplasmas in Chile.

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The first steps towards investigating the molecular epidemiology of *Stylosanthes* little leaf phytoplasma in Australia

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Abstract

Stylosanthes little leaf (StLL) phytoplasmas were first identified from two Fabaceae plant species in Queensland Australia in 1999. More recently, StLL was detected in a potato crop in Victoria, southern Australia, with a small percentage (<1%) of plants showing stunted and deformed growth, including little leaf symptoms. StLL had not been previously detected in potato or in this region of Australia. The 16S rRNA gene of StLL in potatoes was 99.2% identical to the rRNA gene sequence of the 1999 Queensland strain. Phylogenetic analyses of this gene shows that StLL from potato samples shares the highest sequence homology with 'Candidatus Phytoplasma malaysianum' (16SrXXXII-A; 97.2%). Phylogenetic analyses that incorporated representative 'Ca. Phytoplasma' taxa as well as StLL 16S rDNA sequences showed that all the StLL strains form a well-supported cluster away from the other phytoplasma taxa. Nucleotide and phylogenetic analyses of genes more variable than 16S rRNA, such as *secA*, *secY*, *tuf* and *rp*, are being investigated for their suitability as molecular markers for subgrouping within StLL samples. The results may allow for the proposal of a new phytoplasma taxon and to test the usefulness of various genes in subgrouping these phytoplasmas. Importantly, the results may provide a better understanding of the epidemiology of StLL disease in Australia.

Keywords: 'Candidatus Phytoplasma' species, MLSA, taxonomy, ribosomal grouping, barcoding

Introduction

Stylosanthes little leaf (StLL) phytoplasmas were first identified from two Fabaceae plant species in Queensland Australia in 1999 (Schneider *et al.*, 1999). In a mixed infection of StLL with other phytoplasmas, diseased *Arachis pintoii* plants showed little leaf symptoms, while *Stylosanthes scabra* showed little leaf and witches' broom symptoms. More recently, a StLL-like phytoplasma was detected in a potato crop in Victoria in southern Australia with a small percentage (<1%) of plants showing stunted and deformed growth, including little leaf symptoms. StLL phytoplasma had not been previously detected in potato or in this region of Australia. The aim of this study was to determine the relationship between StLL phytoplasmas detected in Fabaceae plants in Queensland and the phytoplasma detected in potato plants in Victoria.

Materials and Methods

DNA was extracted from potato tissues using using a DNeasy® plant DNA extraction kit (QIAGEN) as previously described by Green *et al.* (1999). Platinum® *Taq* DNA Polymerase (Invitrogen) was used for PCR according to the manufacturer's instructions except that the total reaction volume was 25 µl and 1.0 unit of Platinum® *Taq* DNA Polymerase was used in each reaction. Primers developed for bacterial phylogenetic studies (Weisberg *et al.* 1991) were used in PCR to determine the quality of DNA extracts from plants. Detection of the region containing the 16S rRNA gene, the 16Sr/23Sr intergenic spacer (ITS) region and part of the 23S rRNA gene was done as described previously (Gibb *et al.*, 1999). PCR fragments were purified and then cloned using a pGEM-T Easy kit (Promega). Three clones per PCR product were sequenced to generate a consensus sequence. All

sequences were aligned using the MAFFT online program (https://mafft. cbrc.jp/alignment/server/), maximum likelihood inferences were performed using PhyML using the bootstrap method with 1,000 replicates (Guindon *et al.*, 2010). The tree topology was visualized using MEGA 7.0 (Kumar *et al.*, 2016).

Results and Discussion

The 16S rRNA genes of the StLL strain from the potato crop in Victoria shared approximately 99.2% identity with the strain from *A. pintoii* and *S. scabra* plants from Queensland.

Figure 1. Maximum likelihood tree of 16Sr rRNA sequences. The taxa used in the analysis included 42 '*Candidatus* Phytoplasma', *Acholeplasma palmae* as the out group, and StLL (blue bar) detected from a potato crop in Victoria in 2019 and from Fabaceae plants in Queensland (detection years are indicated in brackets). Heterogeneous 16S rDNA sequences were included and are labelled with the suffix *rrnA* or *rrnB*. Boostrap support for each node are shown as a value out of 1,000 replicates. The bar represents the number of nucleotide substitutions per site.

This is, therefore, the first report of StLL in potato and in southern Victoria, representing an extensions of host and geographical ranges.

Sequence homology analyses of the 16S rRNA gene confirmed that StLL from potato is a phytoplasma strain that shares the highest sequence identity with '*Candidatus* Phytoplasma malaysianum' (16SrXXXII-A; 97.2%). The 16S rRNA gene of StLL from the Fabacae plants shared the highest sequence identity with '*Ca.* P. ulmi' (16SrV-A; 96.0%) and shared 95.4% sequence homology with '*Ca.* P. malaysianum'. Since all StLL phytoplasma strains used in this study shared less than 97.5% 16S rRNA gene sequence similarity with previously described '*Candidatus* Phytoplasma' species, this group may represent a novel taxon. The phylogenetic tree reconstruction based on 16S rDNA sequences of previously described '*Ca.* Phytoplasma' taxa, *Acholeplasma palmae* and StLL phytoplasmas (Figure 1) showed that all StLL 16S rDNA sequences clustered together with well supported branches.

Signature sequences unique to the StLL 16S rRNA gene will be identified. Nucleotide and phylogenetic analyses of the *secA*, *secY*, *tuf* and *rp* genes, will be investigated for their suitability as molecular markers to further differentiate this phytoplasma in Australia.

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Unveiling subgroups of 16SrIII phytoplasmas affecting *Trillium* sp. and *Melothria pendula* in Brazil and Canada

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Abstract

Melothria pendula and *Trillium* spp. are important weed species in Brazil and Canada. *M. pendula* plants showing symptoms of chlorosis, little leaf and leaf malformation and *T. grandiflorum* and *T. erectum* exhibiting virescence were tested for phytoplasma presence by nested PCR with primers that amplify the 16S rRNA, ribosomal protein, and *secA* genes, and subjected to RFLP and sequencing analyses. A phytoplasma of group 16SrIII was identified from *M. pendula* and classified as a new variant of subgroup 16SrIII-U. The 16SrIII phytoplasmas, first reported in *T. grandiflorum* and *T. erectum* in Ontario in 2016 were classified as members of the subgroup 16SrIII-F. Besides, one strain from each *Trillium* species examined, corresponded to a new variant of subgroup 16SrIII-F (for *T. grandiflorum*) and a new variant of subgroup 16SrIII-J (for *T. erectum*). Results refer to the first report of a phytoplasma affecting *M. pendula*, and the identification of new variants of 16SrIII phytoplasma subgroups in *Melothria* and *Trillium* species, which may pose a threat for the spread of 16SrIII phytoplasmas to other plant species grown nearby.

Keywords: *Melothria, Trillium*, 16SrIII phytoplasma, subgroup variants

Introduction

The X-disease group (16SrIII) of phytoplasmas is one of the most economically important and widely spread groups across Europe, East Africa, and the Americas. They affect many different plants, particularly *Prunus* species and differ greatly in their geographical origin, plant host/insect vector relationships, and symptoms (Saccardo *et al.*, 2012). In Canada, 16SrIII phytoplasmas were reported in 1941 in the Ontario province associated with peach X-disease, while there is a vast list of plant species in Brazil known to host this phytoplasma group, which include cassava (Oliveira *et al.*, 2014), *Melia azedarach* (Duarte *et al.*, 2009), citrus (Wulff *et al.*, 2019) and others. *Trillium* species (family Melanthiaceae) are commonly used as medicinal plants, and particularly *T. grandiflorum* serves as the emblem and official flower of the Ontario. In Brazil, *Melothria pendula* (Cucurbitaceae family), known as "pepininho" is a weed with laxative properties and grows wild in household gardens and backyards. This study presents the results on the characterization of phytoplasmas of group 16SrIII found in *Trillium* sp. in Canada, and *M. pendula* in Brazil.

Materials and Methods

Surveys for phytoplasma presence were conducted in private gardens of the Seropédica area in Rio de Janeiro, Brazil, during 2016. Leaves, petioles and flowers were collected from *M. pendula* plants showing symptoms of chlorosis, little leaf and leaf malformation (Figure 1A). In Canada, *Trillium* species (*T. grandiflorum* and *T. erectum*) exhibiting typical virescence symptoms (Figures 1C and 1D), collected from a private property in Ontario in 2016 were subjected to multigene characterization. Total DNA extracted by the FAST DNA Spin Kit (MP Biomedicals, USA) was tested by nested PCR (Illustra Ready-to-go-PCR, GE Healthcare, UK) with universal phytoplasma 16S rDNA primers R16mF2/R1 and R16F2n/R2 (Gundersen and Lee, 1996). The phytoplasma secretion protein gene, *secA* was amplified with primers SecAfor1/SecArev3 nested with SecAfor5/SecArev2 (Dickinson and Hodgetts, 2013), and the ribosomal protein genes were amplified with rp(III)F1/R1 primers (Martini and Lee, 2013). PCR amplicons were purified (Omega Bio-Tek, USA) and cloned (pGEM-T Easy Vector, Promega). Plasmid DNA was extracted, purified (GenElute Plasmid Miniprep Kit, Sigma-Aldrich, USA) and sequenced with primers M13F/M13R (Centre for the Analysis of Genome Evolution and Function, University of Toronto, Canada). The consensus sequences were compared to GenBank phytoplasma sequences (https://blast.ncbi.nlm.nih.gov) and used in phylogenetic analysis with MEGA version 7.0. The

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R16F2n/R2 sequences were subjected to *i*PhyClassifier (https://plantpathology.ba.ars.usda.gov) for phytoplasma group and subgroup affiliation while *secA* and *rp* gene sequences were analysed with pDRAW32 (http://www.acaclone.com). Ten microliters of PCR amplicons were digested with appropriate restriction endonucleases (New England Biolabs, Canada). RFLP profiles were visualized in 3% agarose gels stained with SYBR Safe (Invitrogen, USA) in an UV gel documenter (Alpha Innotech, USA).
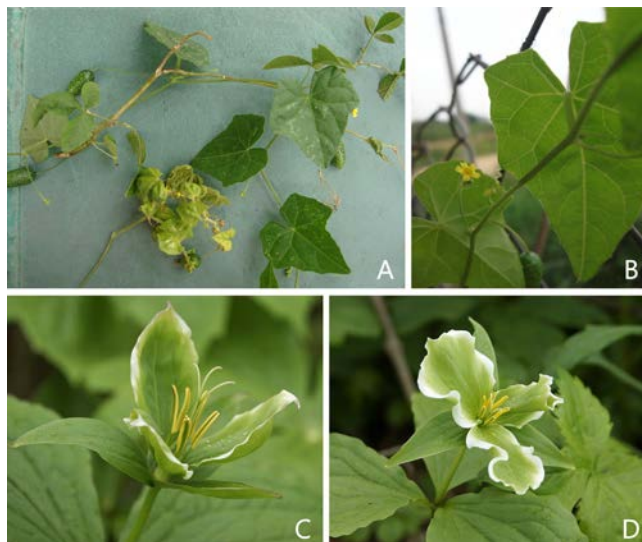
Figure 1. Chlorosis, little leaf and leaf malformation (A) in *M. pendula* versus symptomless plants (B). Virescence in *T. erectum* (C) and *T. grandiflorum* (D) flowers.

Results

The 16S rDNA sequences from the four symptomatic *M. pendula* plants were identical. The consensus sequence deposited in GenBank (accession number MK108032) of the *M. pendula* phytoplasma shared a 99% identity with those of the 16SrIII phytoplasma group (X-disease), and was placed within the 16SrIII cluster as a '*Candidatus* Phytoplasma pruni'-related strain. The *i*PhyClassifier analysis indicated that the virtual R16F2n/R2 RFLP pattern of the *M. pendula* phytoplasma was most similar to that of the group 16SrIII, subgroup U (GenBank accession number HM589213), with a similarity coefficient of 0.98, which suggested that this strain is a variant of the phytoplasmas in subgroup 16SrIII-U. The *i*PhyClassifier analysis from the *T. grandiflorum* (GenBank accession number KX470428) and *T. erectum* (GenBank accession numbers KX470427, KX470429 and KX470430) ribosomal sequences supported their 16SrIII-F subgrouping as '*Ca*. P. pruni'-related strains with a 99% sequence identity. Based on 0.98 RFLP similarity coefficients, the phytoplasma from *T. grandiflorum* was suggested as a new variant of the 16SrIII-F subgroup, and that from *T. erectum*, as a new variant of the 16SrIII-J subgroup. Results from *i*PhyClassifier were confirmed by the analysis of *secA* and *rp* gene sequences and phylogeny.

Discussion

Weeds are proven to be one of the best means for phytoplasma survival, particularly in crop systems. Therefore, knowing those secondary/alternative plant hosts is critical to support an effective management and control of phytoplasma diseases, and to help mitigating the impact of chemical use to crops reducing the environmental impacts. *M. pendula* was confirmed as a new host for phytoplasmas worldwide and was also added to the Brazilian inventory of alternative phytoplasma host species (Montano and Arocha Rosete, 2019). Results suggest that there may be epidemiological factors associated with the presence of mixed infection between the subgroups 16SrIII-F and 16SrIII-J in *T. erectum*, as well as with the occurrence of new variants related to the two subgroups in both *T. grandiflorum* and *T. erectum*. The presence of '*Ca*. P. pruni'-related subgroups and their variants in *M. pendula* in Brazil and *Trillium* species in Canada poses a threat for other weed species grown in nearby private gardens in Rio de Janeiro and in the province of Ontario, which may contribute to the spread of diseases associated with 16SrIII phytoplasmas.

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'*Candidatus* Phytoplasma asteris' host species in Costa Rica

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Abstract

In Costa Rica a growing number of plants wild species and cultivated crops during the last 18 years have been detected harboring phytoplasmas. This report resumes the most common phytoplasmas detected in Costa Rica, where natural infections by these bacteria in 30 plant species belonging to 15 botanical families resulted to be associated with the presence of '*Candidatus* Phytoplasma asteris'-related strains (aster yellows).

Keywords: aster yellows, witches' broom; little leaf disease, yellows

Introduction

Phytoplasmas are responsible for devastating damages to several hundred plant species worldwide, including many economically important crops, fruit and woody trees and ornamental plants (Bertaccini *et al.*, 2014). The majority of phytoplasmas found in Costa Rica belongs to 16SrI group, following the worldwide distribution of representatives of this ribosomal group. The aster yellows group is the most diverse and widespread phytoplasma in all plant species and is also named '*Candidatus* Phytoplasma asteris' (Lee *et al.*, 2004). Surveys to detect phytoplasmas were carried out across Costa Rica during 2001 to 2018, different plants species with symptoms reminiscent of diseases associated with the presence of these pathogens were collected. This report resumes findings of natural infections of '*Ca.* P. asteris' in different plant species in Costa Rica.

Materials and Methods

Sampling was done to plants, shrubs and trees exhibiting symptoms associated to phytoplasma presence such as axillary proliferation, little leaf and/or leaf malformation, chlorosis or yellowing, virescence, phyllody, flower or fruit abortion from January 2001 to December 2018. Total DNA from all samples collected were obtained from small veins and midribs using DNeasy plant Mini kit (Qiagen) or a protocol modified from Lee *et al.* (1993), and analyzed by nested PCR using phytoplasma universal primer pairs P1/P7 and R16F2n/R16R2 (Duduk *et al.*, 2013). After visualization of positive amplification of 1.25 kb fragments by agar gel electrophoresis, these were purified and directly sequenced (both directions) using services at Macrogen (Korea). A contig sequence was generated (Bioedit software) to each sample, and a BLASTn searching for phytoplasmas group identification was carried out. *In silico* RFLP analyses (*i*PhyClassifier platform) and phylogenetic analysis (Mega software) were done.

Results and Discussion

Thirty plant species from 15 botanical families have been identified as phytoplasmas hosts in Costa Rica (Table 1). Before 2001 only two phytoplasmas disease were reported: the maize bushy stunt and the *Gliricidia* little leaf (Kenyon *et al.*, 1998). After 2002, eight outbreaks of phytoplasma diseases have been detected, five of these associated to '*Ca.* P. asteris' (Moreira *et al.*, 2010, Villalobos *et al.*, 2002, 2003, 2018, 2019), one to '*Ca.* P. costaricanum' (Lee *et al.*, 2011), '*Ca.* P. brasiliensis' and '*Ca.* P. pruni' (Villalobos *et al.*, 2011, 2019). Alternate host plants were found for chayote witches' broom, and *Erythrina* witches' broom diseases (Table 1).

Some phytoplasmas were detected only in one or two plants of one species, however those represent new host opening new ecological niches for a broader spread of this pathogen in the country. Phytoplasma presence in *Alstroemeria* sp., *Aralia* sp., *Carica papaya*, *Citrus sinensis*, *Croton niveus*, *Euphorbia heterophylla*, *Heliocarpus appendiculatus*, *Impatiens walleriana*, *Melampodium perfoliatum*, *Sacharum officinarum*, *Solanum lycopersicum*, are here reported for the first time in Costa Rica.

These findings suggest that phytoplasma diseases are spreading in Costa Rica and represent an important threat for its agriculture and biodiversity.

Table 1. Plant host species naturally infected with '*Ca.* P. asteris' detected in Costa Rica from 2001 to 2018.

Plant species (family)	Symptoms	Frequency	Reference
Ageratina anisochroma (Asteraceae)	LL, WB	outbreak	Villalobos *et al.*, 2018
Alstroemeria spp. (Alstroemeriaceae)	LL, FwAb, V, FwMf	sporadic	This report
Aralia sp. (Araliaceae)	LL, Ch	once collected	This report
Catharanthus roseus (Apocynaceae)	16SrI-B: LL, Ch, V	very frequent	Villalobos *et al.*, 2019
Catharanthus roseus (Apocynaceae)	16SrI-P: B	once collected	Villalobos *et al.*, 2019
Citrus paradisi (Rutaceae)	Y, Ch, WB	Sporadic	This report
Citrus sinensis (Rutaceae)	HLB+: M; HLB-: Y	HLB + / - :sporadic	This report
Croton niveus (Euphorbiaceae)	LL, WB	once collected	This report
Cyclanthera pedata (Cucurbitaceae)	LL, WB, Y	sporadic, (1)	Villalobos, 2003
Erythrina costaricensis (Fabaceae)	LL, WB	frequent, (2)	Villalobos *et al.*, 2003
Erythrina glauca (Fabaceae)	Y	frequent, (2)	This report
Erythrina poeppigiana (Fabaceae)	LL, WB	outbreak	Villalobos *et al.*, 2003
Euphorbia heterophylla (Euphorbiaceae)	LL, D	sporadic, (1)	This report
Genipa americana (Rubiaceae)	LL, WB, Y	once collected	Villalobos *et al.*, 2018
Heliocarpus appendiculatus (Malvaceae)	LL, WB	sporadic	This report
Impatiens walleriana (Balsaminaceae)	LL, FwAb	sporadic	This report
Melampodium perfoliatum (Asteraceae)	LL	sporadic, (1)	This report
Phaseolus vulgaris (Fabaceae)	LL, WB, FrAb	outbreak	Moreira *et al.*, 2010
Rictydoctylis carthaginensis (Cucurbitaceae)	LL, Y	very frequent, (1)	Villalobos *et al.*, 2002
Rondeletia buddleioides (Rubiaceae)	LL, Y	once collected (2)	Garita *et al.*, 2008
Saccharum officinarum (Poaceae)	Alajuela: YV, D	outbreak	This report
Saccharum officinarum (Poaceae)	Cartago: RV	sporadic	This report
Sechium edule (Cucurbitaceae)	LL, WB, FrMf	Cartago: outbreak	Villalobos *et al.*, 2002
Sechium edule (Cucurbitaceae)	LL, WB, FrMf	Alajuela (*): once collected	(*) This report
Sechium tacaco (Cucurbitaceae)	LL, WB, FrMf	frequent, (1)	Villalobos *et al.*, 2002
Solanum lycopersicum (Solanaceae)	BB, LL, RL, FwAb	sporadic	This report
Spathodea campanulata (Bignoniaceae)	LL, WB, DB	sporadic, (2)	Garita *et al.*, 2008
Tabebuia rosea (Bignoniaceae)	LL, WB, DB	sporadic, (2)	Garita *et al.*, 2008
Trema micranta (Rubiaceae)	LL, WB	sporadic, (2)	This report
Zea mays (Poaceae)	D, Ch, RL	sporadic	This report

Symptoms: LL = little leaf, WB = witches' broom, FwAb = flower abortion, FrAb = fruit abortion, FrMf = fruit malformation, V= virescence, D = dwarfing, Y = leaves yellows, Ch = chlorosis, DB = dieback, RV = red veins, YV = yellow veins, RL = red leaves, B = bolting, M = mottling. (*) = expanding geographic distribution. Frequency: sporadic = 2 to 10 samples, frequent = 11 to 24 samples, very frequent = more than 25 samples; (1) associated to chayote WB outbreak, (2)= associated to *Erythrina* WB outbreak; HLB+/-: "hunglongbing" positive or negative; Alajuela and Cartago = provinces of Costa Rica.

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Detection of a possible insect vector and alternative host plants for the sesame phyllody phytoplasma in Assam, India

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Abstract

Sesame phyllody is a very serious and destructive disease associated with phytoplasma presence. Roving survey was done to collect symptomatic samples and insect possible vector of the disease. The phytoplasma presence was detected by PCR in some of the insects and other plant species. The amplicons were sequenced and showed 94% to 98% identity with other phytoplasmas. Phylogenetic analysis shows that the phytoplasma infecting *Exitianus indicus* is closely related to aster yellows phytoplasma strains indicating this insect as possible new vector of the disease. Plants in eight diverse species resulted also phytoplasma-infected and the phytoplasma identification is in progress.

Keywords: sesame phyllody, phytoplasma, transmission, PCR, *Exitianus indicus*

Introduction

Phytoplasmas are phytopathogenic prokaryotes without cell wall and inhabit phloem sieve elements in infected plants. Their genome is the smallest among known living microbes and presents one chromosome. The phytoplasmas are transmitted from infected to healthy plants by phloem-feeding insects, mainly leafhoppers belonging to the order of Hemiptera. Sesame (*Sesamum indicum* L.) belongs to the family Pedaliaceae and is one of the most ancient oilseed crop. *Orosius albicinctus*, *Hishimonus phycitis*, *Neoaliturus haematoceps* has been reported as insect vectors of the sesame phyllody phytoplasma in India (16SrI-B group) and abroad (Sertkaya *et al.*, 2007; Nabi *et al.*, 2015; Martini *et al.*, 2018). Sesame phyllody is one of the major constraints throughout the world which causes losses of 5-15%. Therefore, the characterisation of the disease agent and the identification of new host plants are important steps in the development of the management strategies for this disease.

Materials and Methods

During August to November 2017-2018 surveys were done in the major sesame growing districts of Assam (Figure 1), collecting symptomatic samples and insects that were stored at -45ºC. Total DNA was extracted using a reported protocol (Kollar *et al.*, 1990). The DNA of the identified leafhoppers was extracted using a modified procedure from Marzachì *et al.* (1998). For direct and nested PCR, primer pairs P1/P6

and R16F2n/R16R2 were used are reported (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Gundersen and Lee, 1996). The products were resolved on 1.5% agarose gel in 1 X Tris EDTA (TAE) containing ethidium bromide (Sambrook and Russell, 2001). Sequencing was done in Bioserve private Ltd., Hyderabad, India and phylogenetic analysis was done with MEGA 6 software.
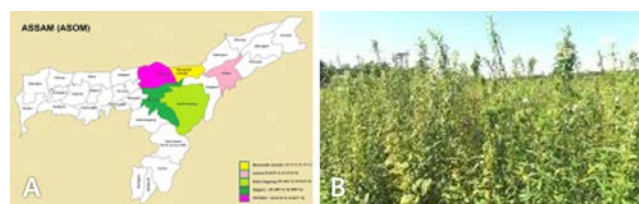
Figure 1. A) surveyed Assam districts; B) overview of a sesame field.

Results and Discussion

Sesame phyllody main symptoms observed where transformation of the flower parts into green leaf-like structures, inflorescence converted into twisted reduced green leaves, with short internodes (witches' broom), development of green flowers (virescence), increase in size of the internodes, shoot like structure of ovary and polysepalar calyx (Figure 2). The leafhoppers collected were identified as *Orosius albinctus* (Dist), *Hishimonus phycitis* (Dist), *Exitianus indicus* (Dist.) and *Cofana unimaculata*

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(Figure 3); among them only *H. phycitis* and *E. indicus* resulted positive in nested PCR analyses (Figure 4).

BLAST analysis of the 16S rDNA partial sequence of the phytoplasma detected in *E. indicus* shows 94-98% identity with other phytoplasmas in the NCBI database. Phylogenetic analysis further confirmed that the phytoplasma detected in *E. indicus* clusters with aster yellows phytoplasmas. On the other hand PCR analysis of eight among the tested plant species e.g *Solanum torvum, Datura stramonium, Linum usitatissimum, Crotalaria juncea, Myosotis* sp., *Gerbera jamesonii, Ageratum* sp. and *Saccharum officinarum* resulted positive for phytoplasma presence (Figure 5). The population dynamics and the detection of phytoplasmas in *E. indicus* suggests that it may play a role as vector for the sesame phyllody phytoplasma transmission in nature together with the reported insect vector *H. phycitis* (Nabi *et al.*, 2015). Identification the phytoplasmas detected in the other species is in progress in order to evaluate their role in the disease dissemination.
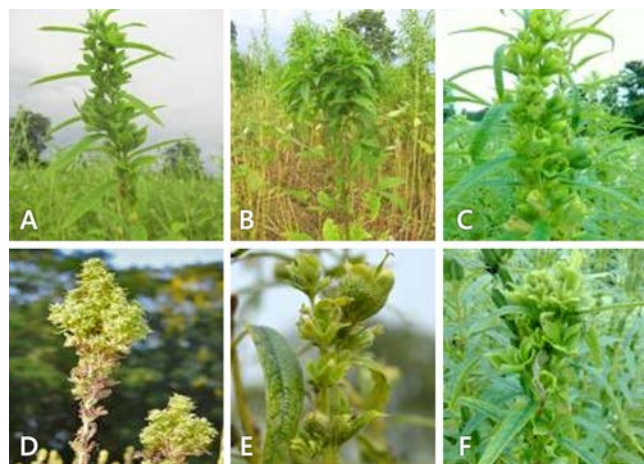
Figure 2. A) Phyllody, B) witches' broom, C) virescence, D) proliferation, E) shoot like structure of ovary and F) polysepalar calyx.
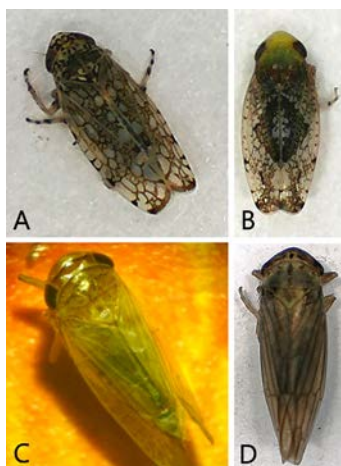
Figure 3. (A) *Orosius albicinctus*, (B) *Hishimonus phycitis*, (C) *Exitianus indicus* and (D) *Cofana unimaculata*.
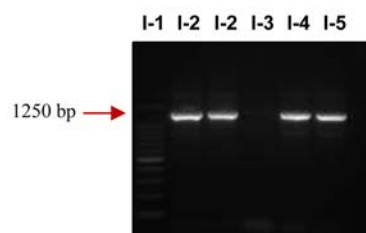
Figure 4. PCR analysis in agarose 1.5% gel of samples I-1: 1 kb marker; I-2: positive controls (sesame phyllody phytoplasma); I-3: water, I-4: *Hishimonus phycitis*, I-5: *Exitianus indicus*.
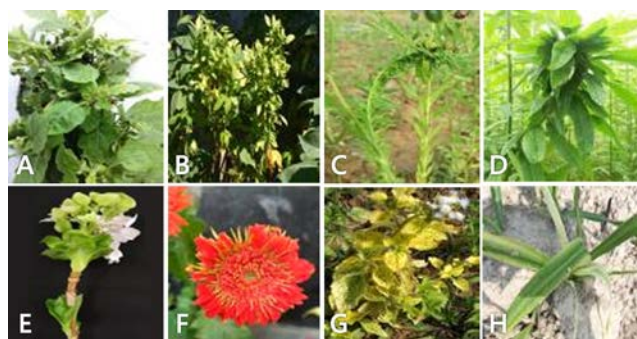
Figure 5. A) *Solanum torvum*, B) *Datura stramonium*, C) *Linum usitatissimum*, D) *Crotalaria juncea*, E) *Myosotis* sp., F) *Gerbera jamesonii* G), *Ageratum* sp. and H) *Saccharum officinarum*.

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Molecular identification of phytoplasmas in Russian vineyards

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Abstract

Phytosanitary surveys of vineyards in the Krasnodar region were carried out for phytoplasma detection. Symptomatic samples were tested by nested PCR. According to the restriction fragment length polymorphism analysis the identified phytoplasma belongs to the "stolbur" subgroup 16SrXII-A.

Keywords: phytoplasma, molecular detection, grapevine, Russian vineyards, *Vitis vinifera*

Introduction

Grapevine is one of the important cultivations in the south of Russia. A total of 91,500 ha of grapevine produced 58 million tons of grapes in 2017 (according to Federal State Statistics Service of the Russian Federation). Phytoplasma presence is associated with severe yield losses in grapevine production (Dermastia *et al.*, 2017; Zambon *et al.*, 2018). This research was aimed to survey selected vineyards in the south of Russia to verify the presence of phytoplasmas in the Krasnodar region.

Materials and Methods

The surveys of Krasnodar region vineyards were conducted in the early autumn of 2017 and 2018. Samples from symptomatic plants were collected for molecular identification of phytoplasmas. DNA was extracted as described in EPPO protocol (2007). Nested PCR was performed with P1/P7 primers (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by R16F2n/R16R2 or U5/U3 primers (Gundersen and Lee, 1996; Lorenz *et al.*, 1995). Amplicons were directly sequenced, aligned and analysed by BLAST. The restriction fragment length polymorphism (RFLP) analysis was carried out *in silico* by the Clone Manager 6N software (Lee *et al.*, 1998).

Results

Both local and imported cultivars were inspected in the survey including Chardonnay, Aligote, Cabernet Sauvignon, Augustin, Traminer, Rkatsiteli, Risling, Livia, Ubileinii, Krasnostop, Muscat, Moldova, Blaufrankish, Kolobok. Specific phytoplasma symptoms were observed such as downward curling of the leaves, yellowing on white varieties and hybrids and reddish on the red ones, black pustules on the green bark and lack of lignification (Figure 1).

Figure 1. Typical symptoms of phytoplasma in infected grapevines in Russia.

Amplicons of specific sizes about 1,250 bp and 630 bp were obtained after the nested amplification with R16F2n/R16R2 and U5/U3 primers respectively (Figure 2).
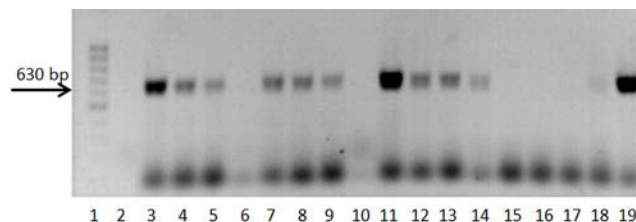
Figure 2. Detection of phytoplasmas by nested PCR with P1-P7 and U5/U3 primers.

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Sequences of these products were assembled to obtain a part of the 16S rRNA gene. BLAST analysis revealed high identity with the GenBank sequences of 'Candidatus Phytoplasma solani'. *In silico* RFLP analyses using 12 restriction enzymes confirmed that the detected phytoplasmas belong to the "stolbur" phytoplasma, subgroup 16SrXII-A.

Discussion

The presence of phytoplasmas in the grapevine samples from the Krasnodar region was confirmed. This is the first large-scale survey of the Russian vineyards for phytoplasma presence. The presence of insect vectors should be further investigated since it is important for vectors and weeds control in the vineyard to prevent the spread of phytoplasma-associated diseases.

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Molecular study of a phytoplasma associated with safflower fasciation in Iran

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Abstract

During the growing seasons of 2017 and 2018, a disease occurred in safflower fields in the south Khorasan province of Iran. Affected plants exhibited extensive fasciation, formation of bushy growth, small leaves, phyllody and shortened internodes. For detection of the putative phytoplasma(s), polymerase chain reaction (PCR) assays were performed using universal phytoplasma primers pairs. Nested PCR analyses revealed that all symptomatic safflowers plants showing fasciation symptoms tested positive for phytoplasma presence, while asymptomatic plants were negative. Nucleotide sequence analysis identified a phytoplasma belonging to the peanut witches' broom group (16SrII). Phylogenetic analysis confirmed the clustering of the safflower fasciation phytoplasma (SaP) with 'Candidatus Phytoplasma aurantifolia'-related strains.

Keywords: 'Candidatus Phytoplasma aurantifolia', fasciation, safflower

Introduction

Safflower (Carthamus tinctorius) is one of humanity's oldest crops commercially cultivated for the oil extracted from the seeds (Zohary et al., 2012). It is infected with several pathogens including fungi, bacteria and phytoplasmas. Over the recent years, a phytoplasma like disease has been observed in several processing safflower crops in the south Khorasan province of Iran. Symptoms included extensive fasciation, formation of bushy growth, phyllody and shortened internodes (Figure 1). The incidence of symptomatic safflower plants in individual fields was less than 0.2%, but symptoms were typical of the diseases associated with phytoplasma presence. Phytoplasmas are obligate bacteria living in the plant phloem tissue (Hogenhout et al., 2008) and in the insect vectors (Weintraub and Beanland, 2006). Phytoplasma taxonomy is complicated and they were only recently cultivated (Contaldo et al., 2016). Phytoplasma taxonomic groups are based on the differences in fragment sizes produced by restriction digests of 16S ribosomal RNA gene sequences (RFLP) or by comparisons of DNA sequences from 16S ribosomal gene (Lee et al., 1998; IRPCM, 2004). The first phytoplasma disease reported in safflower was phyllody in Israel (Klein, 1970) and in 2008 this disease was reported from Iran (Salehi et al., 2008). The objective of this study was to determine the agent associated with the symptoms observed in safflower fields in the south Khorasan province (Iran).

Materials and Methods

Leaf tissues were sampled from 12 symptomatic safflower plants in two locations in Amirabad region (Birjand, South Khorasan) in 2017 and 2018. In addition, seven asymptomatic plants were sampled from these fields to be used as healthy plants. Total DNA was extracted from 0.5 g of leaf tissue using a CTAB-based method (Doyle and Doyle, 1987). A nested PCR assay using primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) followed by R16mF2/R16mR1 (Gundersen and Lee, 1996) and M1/M2 (Gibb et al., 1995) was employed. Mexican lime witches' broom infected sample used as positive control. PCR was carried out in 20 µl reaction mixtures containing 1 µl of undiluted nucleic acid preparation (150 ng), 200 µM of each dNTP, and 0.4 µM of each primer. The following temperature conditions were used: denaturation at 94°C for 2 minutes (5 minutes for the first cycle), annealing for 2 minutes at 58°C, 56°C and 50°C respectively for P1/P7, R16mF2/R16mR1 and M1/M2 primers, extension for 3 minutes (5 minutes in the final cycle) at 72°C. One microliter of diluted (1: 30) PCR product was used as template for nested PCR. The PCR products (6 µl) were electrophoresed through a 1.2% agarose gel, stained in ethidium bromide, and visualized with a UV transilluminator. The amplicons were directly sequenced and obtained sequences were assembled, aligned and compared with nucleotide sequences in the GeneBank database, using BLAST

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(version BLASTN 2.2.18) (NCBI, Bethesda, MD, USA) and deposited in GenBank under the accession numbers MK484137, MK484138 and MK484139. Sequence alignments were performed by using ClustalX. Phylogenetic analyses were done using the close-neighbour-interchange algorithm, on the partial 16S rDNA sequences from safflower samples 129, 130, 135 and from 45 phytoplasma strains using the sequence of *Acholeplasma laidlawii* as outgroup.

Figure 1. Safflower fasciation symptoms (left) compared with healthy shoot (right).
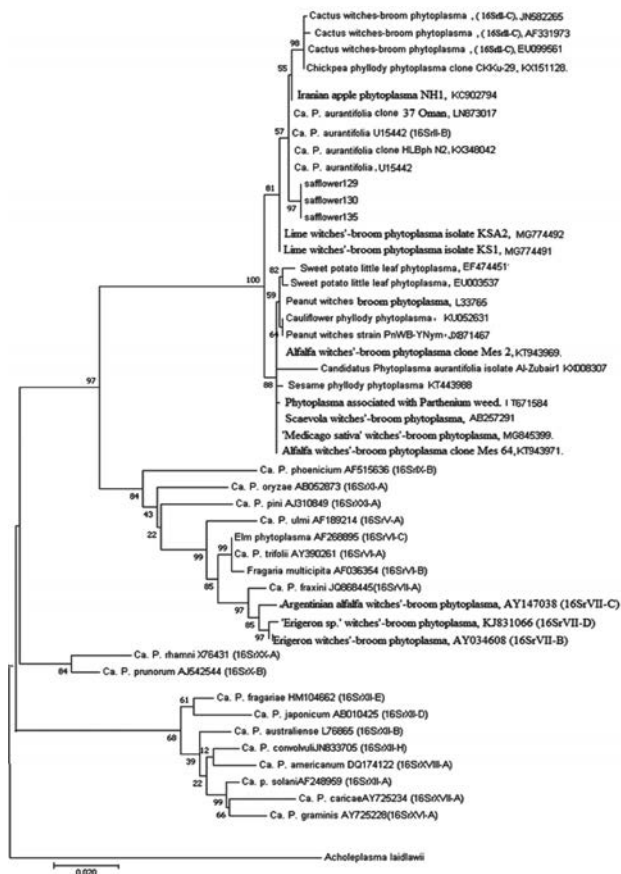
Figure 2. Phylogenetic analysis by the Maximum Likelihood method.

Results and Discussion

Infected safflower plants exhibited extensive fasciation, bushy growth, small leaves, phyllody and elongation of internodes (Figure 1). Phytoplasmas were detected by nested PCR in all the 12 symptomatic safflower plant samples collected while the symptomless samples tested negative. Nucleotide sequence analysis of amplicon from 16S rDNAs confirmed their clustering with 16SrII group phytoplasmas (Lee *et al.*, 1998) in a separate subclade (Figure 2). Phytoplasmas belonging to group 16SrVI have been reported previously associated with safflower phyllody symptom in Iran (Salehi *et al.*, 2009). Some phytoplasma transmitting leafhoppers including *Neoaliturus fenestratus, Macrosteles laevis, Psammotettix striatus and Circulifer haematoceps* have been reported as pest on safflower in Iran (Saeidi *et al.*, 2011) and could be potential vectors for this phytoplasma, the insect transmission to safflower will be carried out in future.

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New additions to the list of phytoplasma plant host species in Brazil

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Abstract

In Brazil, several plant species are affected by phytoplasma diseases, comprising wild and cultivated crops. The majority of the phytoplasmas identified belongs to groups 16SrI and 16SrIII, although there are reports of phytoplasmas affiliated to groups 16SrII, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrXII, 16SrXIII and 16SrXV.

Keywords: yellows diseases, witches' broom, phytoplasmas

Introduction

In Brazil the diseases associated with phytoplasmas have been reported in several plant species belonging to different botanic families (Montano *et al*., 2007, 2011). Amongst the species infected by phytoplasmas there are vegetable and fruit crops, cereals and pulse crops, ornamental plants and shade trees. Following the worldwide distribution of phytoplasmas, the majority of those reported in Brazil belongs to groups 16SrI and 16SrIII, although there are representatives of other 16Sr groups. Interestingly, the presence of a phytoplasma affiliated to group 16SrIII has been recently observed also in citrus plants with "huanglongbing" symptoms (Wulff *et al*., 2019).

Table 1. Plant host species of phytoplasmas in Brazil and phytoplasma classification.

Type of crop	Plant species and phytoplasma classification	Reference
Cereals	*Zea mays* (16SrI-B; 16SrIII-B)	Montano *et al*., 2007, 2011
Fruit crops	*Carica papaya* (16SrXIII-E), *Citrus sinensis* (16SrIII; 16SrIX); *Fragaria* sp. (16SrIII; 16SrXIII); *Passiflora edulis* f. f*lavicarpa* (16SrIII; 16SrIII-V; 16SrVI); *Malus domestica* (16SrIII); *Vitis vinifera* (16SrI-B; 16SrIII)	Montano *et al*., 2007, 2011; Davis *et al*., 2012; Melo *et al*., 2013; Wulff *et al*., 2019
Industrial crops and palms	*Cocos nucifera* (16SrXIII); *Elaeis guineenses* (16SrI), *Eucalyptus urophylla* (16SrII); *Glycine max* (16SrI-B); *Manihot esculenta* (16SrIII-B;16SrIII-L); *Saccharum* sp. (16SrI)	Montano *et al*., 2007, 2011; Flôres *et al*., 2013b; Oliveira *et al*., 2014; Souza *et al*., 2015; Pereira and Bedendo, 2017
Medicinal plants	*Melothria pendula* (16SrIII-U); *Momordica charantia* (16SrIII-J); *Sicana odorifera* (16SrIII)	Montano *et al*., 2007, 2011; Montano and Arocha Rosete, 2019
Ornamental plants	*Begonia* sp. (16SrIII); *Bougainvillea spectabilis* (16SrI-B; 16SrIII-B); *Catharanthus roseus* (16SrII; 16SrIII; 16SrIX; 16SrXV-A); *Celosia argentea* (16SrIII-J); *C. spicata* (16SrIII-J); *Euphorbia pulcherrima* (16SrIII); *Hibiscus rosa-sinensis* (16SrXII; 16SrXV-A); *Polyscias fruticosa* (16SrVII-B); *Thunbergia erecta* (16SrXIII-E)	Montano *et al*., 2007, 2011; Barbosa *et al*., 2012; Eckstein *et al*., 2012; Silva *et al*., 2015; Alves *et al*., 2016; Pereira *et al*., 2016a
Forest and shade trees	*Tabebuia pentaphylla* (16SrII); *Dimorphandra gardneriana* (16SrXIII); *Dimorphandra mollis* (16SrXIII); *Melia azedarach* (16SrIII-B)	Montano *et al*., 2007, 2011, 2015a
Vegetables	*Brassica oleracea* var. *botrytis* (16SrIII-J; 16SrVII-B; 16SrXIII; 16SrXV-A); *B. oleracea* var. *capitata* (16SrI; 16SrIII-B); *B. oleracea* var. *italica* (16SrI; 16SrIII; 16SrXIII); *Ipomoea batatas* (16SrI) *Cucurbita pepo* (16SrIII); *Cucurbita moschata* (16SrIII-J); *Lagenaria siceraria* (16SrIII-Y); *Luffa cylindrica* (16SrIII); *Sechium edule* (16SrIII-J); *Solanum lycopersicon* (16SrIII); *Solanum melongena* (16SrIII-B; 16SrIII-J; 16SrIII-U)	Montano *et al*., 2007, 2011, 2015b; Amaral Mello *et al*., 2011; Canale and Bedendo, 2013; Eckstein *et al*., 2013; Pereira *et al*., 2016b
Weeds and wild plants	*Aegiphila verticillata* (16SrIII-J); *Brachiaria decumbens* (16SrIII-J); *Crotalaria juncea* (16SrI; 16SrIII-B; 16SrV; 16SrVII-C; 16SrIX; 16SrXV); *Erigeron (Conyza) bonariensis* (16SrI; 16SrVII-B; 16SrVII-D); *Helychrisum bracteatum* (16SrIII); *Leonurus sibiricus* *Macroptilium lathyroides* (16SrI); *Sida* sp. (16SrXV-A); *Solidago microglossa* (16SrIII); (16SrIII-B); *Turnera ulmifolia* (16SrXIII); *Vernonia brasiliana* (16SrIII-B; 16SrIII-J;16SrVII-F); *Waltheria indica* (16SrI)	Montano *et al*., 2007, 2011, 2014; Flôres and Bedendo, 2013; Flôres *et al*., 2013a, 2015; Bianco *et al*., 2014; Munhoz *et al*., 2015; Alves *et al*., 2017, 2018; Fugita *et al*., 2017a, 2017b, 2017c

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Phytoplasmas infecting greenhouse cucumber in Iran

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Abstract

During 2014-2018 surveys in greenhouse growing cucumber in central and west of Iran, plants with flower virescence, phyllody and sterility were observed. The disease was observed in Yazd and Chaharmahal and Bakhtiari provinces. Molecular characterization of phytoplasmas associated with this diseases was carried out. Total DNAs extracted from 44 symptomatic and six symptomless plants were subjected to direct and nested PCR using P1/P7, R16mF2/R16mR2 and R16F2n/R16R2 primer pairs. Expected PCR amplicons were obtained only from symptomatic plants. Real and virtual RFLP, phylogenetic and DNA homology analyses of partial 16S ribosomal sequences of the strains detected in the symptomatic plants revealed the presence of phytoplasmas referable to two ribosomal subgroups, 16SrVI-A and 16SrXII-A. By comparing the 16S rDNA sequences available in the GenBank database from phytoplasma strains associated with greenhouse cucumber phyllody in Fars province and leaf, stem and fruit yellowing in Tehran province, it was possible to determine the presence of phytoplasmas clustering with strains in the 16SrII and 16SrVI groups, respectively. Based on the results of the present study and considering the reported presence of phytoplasmas belonging to the same ribosomal subgroups in other crops, these phytoplasmas seem to play an important role in the epidemiology of greenhouse cucumber phytoplasma diseases in Iran.

Keywords: phyllody, virescence, 16SrVI-A, 16SrXII-A, 16SrII

Introduction

Among cucurbitaceous plants grown in greenhouses in Iran, greenhouse cucumber (*Cucumis sativus* L.) with 72.8% of total production is considered the most economically important product (Anonymous, 2017). The development of technology as well as a short growth period of cucurbitaceous plants in greenhouses has provided its development in Iran. Phyllody, virescence, yellowing and sterility are important phytoplasma disease symptoms of greenhouse cucumber. Greenhouse cucumber phyllody was reported for the first time in Jiroft and Kahnooj (Kerman province, Iran) with a disease incidence of up to 80% (Azadvar *et al.*, 2004). In the 2004-2006 surveys in greenhouse cucumber a phyllody disease was proved to be associated with phytoplasmas in Akramia (Yazd province), Pishva (Varamin, Tehran province) and Larestan (Fars province) (Esmailzadeh Hosseini *et al.*, 2006). In Fars province a phytoplasma belonging to the 16SrII group was reported (Dehghan *et al.*, 2014). Recently a 16SrVI phytoplasma was reported from in greenhouse cucumber (*Cucumis sativus*) with yellows symptoms in leaves, stem and fruits in Tehran (Ghayeb Zamharir and Azimi, 2018).

The present work summarizes studies about phytoplasma diseases reported from Iran about greenhouse cucumber.

Materials and Methods

During 2014 to 2018, greenhouse cucumber phyllody (GCP) samples were collected from greenhouse cucumber growing areas of central and west of Iran in Yazd and Chaharmahal and Bakhtiari provinces, and used as sources for molecular studies of the associated phytoplasmas. Total DNAs extracted using the Zhang *et al.* (1998) procedure from 44 symptomatic and six symptomless plants 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. RFLP analysis of nested PCR products with different restriction enzymes was used to identify the phytoplasmas. Twelve samples were directly sequenced using the nested PCR products obtained with P1/P7 and R16mF2/R16mR2. After alignment and assembling, the resulting sequences were trimmed to the R16F2n/R16R2 fragment and submitted to GenBank. A database search of homologous sequences was performed by

Blast analyses at NCBI to determine the closest phytoplasmas. The R16F2n/R16R2 sequence of 16S rRNA gene of phytoplasmas infecting greenhouse cucumber in Iran were aligned using Clustal W with other '*Ca.* Phytoplasma' species and *Acholeplasma laidlawii* as outgroup and a phylogenetic tree was constructed using MEGA7 (Kumar *et al.*, 2016). Bootstrapping was performed 1,000 times to estimate the stability and support for the branches.

Figure 1. Flower virescence, phyllody and sterility in a greenhouse cucumber in Yazd province, Iran.
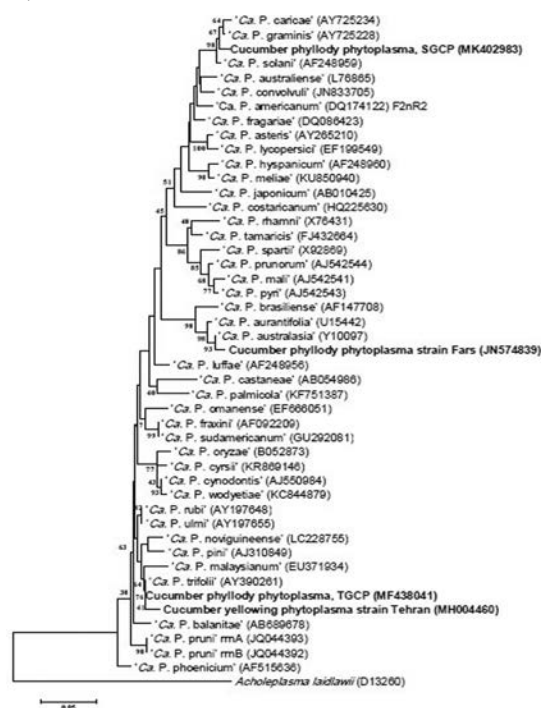
Figure 2. Phylogenetic tree built using neighbor-joining method in Mega 7 for the phytoplasma infecting greenhouse cucumber in Iran (in bold).

Results and Discussion

Greenhouse cucumber phyllody (GCP) disease was observed in all the surveyed areas. The highest infection percentage was 82% in Taft (Yazd province). Infected plants showed flower virescence, phyllody and sterility (Figure 1). Amplicons of the expected size (about 1.8, 1.4 and 1.2 kb) were obtained from all *C. sativus* plants showing flower virescence, phyllody and sterility, but not from the symptomless ones. RFLP analysis of R16F2n/R16R2 amplicons using *Alu*I, *Hae*III, *Taq*I, *Hpa*I, *Hpa*II, *Mse*I, *Rsa*I,

*Kpn*I and *Hha*I enzymes showed two RFLP pattern similar to those of clover proliferation (16SrVI-A) and "stolbur" (16SrXII-A) phytoplasmas, respectively (data not shown). Consensus sequences corresponding to Taft GCP (TGCP) and Shahrekord GCP (SGCP) phytoplasmas in Yazd and Chaharmahal and Bakhtiari provinces were deposited in GenBank under the accession numbers MF438041 and MK402983, respectively. Phylogenetic analysis (Figure 2) and *in silico* restriction of the R16F2n/R16R2 amplified regions using the *i*PhyClassifier program with 17 restriction enzymes confirmed that TGCP and SGCP phytoplasma strains are related to '*Candidatus* Phytoplasma trifolii' (16SrVI-A, GenBank accession number AY390261) and '*Ca.* P. solani' (16SrXII-A, GenBank accession number AF248959), respectively. Based on the results of the present study and considering the reported phytoplasmas belonging to the same ribosomal subgroups in other crops, they could play an important role in the epidemiology of greenhouse cucumber phytoplasma disease in Iran.

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Worldwide

Novel aster yellows phytoplasma subgroup associated with sandalwood spike disease in Kerala, India

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Abstract

The sandal spike disease of sandalwood is known to be associated with aster yellows (AY) phytoplasmas and is a prime threat to sandalwood cultivation in India. The Marayoor Sandalwood Reserve (Kerala state) has an excellent collection of naturalised and matured sandalwood trees and therefore sandalwood forest were surveyed and symptomatic samples were collected from this area. The study confirmed the disease association with AY phytoplasmas and allow to verify the presence of a novel AY phytoplasma strains indicating that phytoplasma strains are differentiating with their plant host species and geographic regions.

Keywords: aster yellows, phytoplasma, sandal spike disease, *Santalum album*, Marayoor

Introduction

Sandalwood spike disease (SSD) is the most destructive disease of sandalwood (*Santalum album* L.) and one of the major causes for the decline in sandalwood production in India. India's production was around 4,000 tonnes per year of heartwood till 1950 which has now decreased to about 400 tonnes per year. This decline in sandalwood production is mainly due to the depletion of sandalwood trees which is attributed to factors like illicit felling, smuggling and SSD (Rao *et al.*, 1999). In this context, this pilot study was undertaken to evaluate the health status of *S. album* in the Marayoor Sandal Reserve (MSR) in Kerala, India which falls under different geo-climatic region and to evaluate the possible presence of diversity in the phytoplasma strains associated with SSD.

Materials and Methods

The survey was conducted in February 2018 in the MSR area (10°16'N 77°09'E; altitude 1,000 to 2,000 m asl). To confirm the presence of phytoplasmas, 2 g of leaf tissue from 9 symptomatic and asymptomatic plants were used for total DNA extraction using the CTAB method (Doyle and Doyle, 1990). The phytoplasma 16S rRNA gene was amplified from 50 ng of DNA samples using primers P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al.*, 1995) followed by nested primers R16F2n and R16R2 (Gundersen *et al.*, 1996). For nested PCR reaction, either 5-, 10- or 20-fold diluted template generated by P1/P7 primers was used. Each PCR reaction was

performed with 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of dNTPs, 1 U of TaKaRa® LA Taq DNA Polymerase (Cat. No. RR002), 2 µg BSA and 1 µM of each primer. PCR products were purified and sequenced directly using bacterial universal primers 343R, 704F, 907R, 1028F and 1492R (Baker *et al.*, 2003) on ABI® 3730xl DNA analyser. The obtained 16S rDNA sequences were curated and analysed using EzTaxon database (Yoon *et al.*, 2017). The PCR products which showed mixed electropherogram signals were amplified again, purified and cloned in pSC-A-amp/kam cloning vector (AGILENT, StrataClone PCR Cloning Kit, Cat. No. 240205) and screened using StrataClone SoloPack *Escherichia coli* competent cells as directed by the manufacturer. The obtained clones were sequenced directly using universal M13 primers. The obtained sequences were curated, trimmed for primers and subjected to further analysis using nBLAST, EzBiocloud, *i*PhyClassifier (Zhao *et al.*, 2009). Computer-simulated RFLP analysis of the 16S rRNA gene was performed to identify the ribosomal group and subgroup of the detected phytoplasmas. Each sequence was digested *in silico* with 17 restriction enzymes and separated on 3% virtual gels (Wei *et al.*, 2007). The virtual RFLP patterns were compared, and a similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula F52Nxy/(Nx+Ny) as described (Wei *et al.*, 2007). Profiles obtained for the phytoplasma associated with the plant studied were compared with published RFLP pattern of phytoplasma representatives of the available ribosomal groups (Lee *et al.*, 1998).
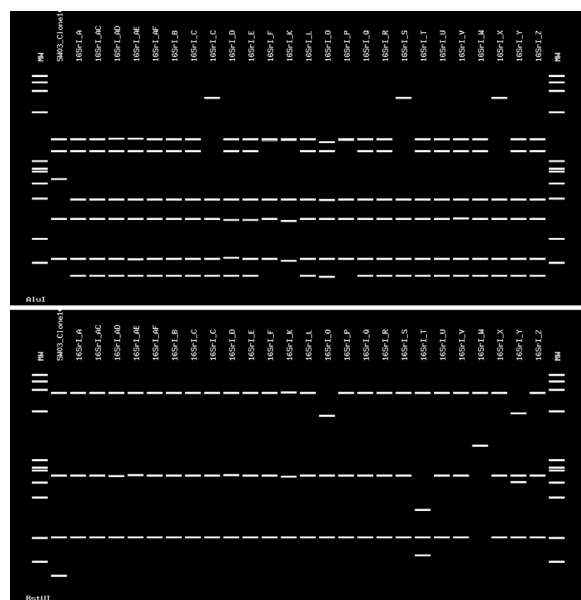
Figure 1. Virtual RFLP gel images showing the unique RFLP profiles of *Alu*I (above) and *Bst*UI (below) digested 16S rRNA clone SW03_10 compared to *i*PhyClassifier available subgroups of the 16SrI group.

Results

The amplification of phytoplasma 16S rRNA gene in all nine symptomatic sandalwood plant samples confirmed the association of phytoplasmas with SSD in MSR region. The 16S rDNA sequence of clone SW03_10 showed 99.28% and 99.76% sequence similarity with strain AYWB (GenBank accession number CP000061) and strain OY-M classified as 'Candidatus Phytoplasma asteris' when analysed using EzTaxon and NCBI-BLAST. The clone SW03_10 scored the virtual RFLP similarity coefficient of 0.96 with published 16SrI subgroups and represents therefore a possible novel subgroup (Figure 1). All other symptomatic sandalwood samples were found to be harbouring phytoplasmas belonging to the 16SrI-B subgroup with RFLP similarity coefficient of one.

Discussion

The 16SrI (AY) group of phytoplasmas in one of the phytoplasmas resulting worldwide distributed. The phytoplasma strains belonging to this group were detected in a wide range of plant species making this the most widespread phytoplasma. Though the association of AY phytoplasmas with SSD was known since a decade, and the presence of 16SrI-B phytoplasmas demonstrated in India (Khan *et al.*, 2008), this study reveals that some diversity of phytoplasma strains are yet to be discovered. This pilot study was aimed to determine the extent of spread of SSD in MSR and diversity of phytoplasma strains associated with SSD. The cloning of 16S rDNA fragments has revealed the presence of a novel phytoplasma strains, which highlights the need for more efforts for verification of new phytoplasma strains

presence in specific plant hosts. The discovery of a possible new subgroup demands more efforts in analysing the alternative plant hosts and specific insect vectors possibly harbouring it. SSD is spreading through alternate plant hosts and unknown polyphagous insect vectors and the efforts made so far could hardly contain the disease for the lack of these basic knowledge. Unlike other crops, loss of a single tree is crucial for the sandalwood growers and therefore it is essential to have an effective strategy for vector management, eventually restricting the SSD phytoplasma spread in neighbouring areas.

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Epidemiology

Network analyses of a global Hemiptera-phytoplasma-plant biological interactions database

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Abstract

A recently published global Hemiptera-phytoplasma-plant database was analyzed using graph theory and network statistics, providing a preliminary meta-analysis of host-phytoplasma associations worldwide. Preliminary results showed that Hemiptera-phytoplasma (HP) and plant-phytoplasma networks have low density and HP associations also show a modular and nested structure, suggesting that different phytoplasmas occupy exclusive ecological compartments of association with their hosts.

Keywords: ecological network, graph theory, leafhopper, planthopper, psyllid, plant pathogen

Introduction

Phytoplasmas belong to a lineage of bacteria (phylum *Tenericutes*, class *Mollicutes*) responsible for major economic loss in cultivated crops and other plants (Strauss, 2009). The great phyletic diversity, widespread present-day geographic distribution, and association of the group with a wide variety of host species, suggest that the group has been evolving in close association with its plant hosts and hemipteran vectors for millions of years (Trivellone *et al.*, 2019). It has long been recognized that microbial associates, including pathogens, play crucial roles in mediating trophic interactions between plants and insects (Janson *et al.*, 2008), and that both indirect and direct interactions may drive ecological and evolutionary processes contributing to dispersal, local adaptation and speciation (Biere and Tack, 2013). However, despite the extensive research devoted to understand the mechanisms that lead to infection and spread of phytoplasmas, no prior attempts have been made to examine patterns of association among phytoplasmas, their plant hosts and the hemipteran insect vectors at a global level. An online database of global Hemiptera-Phytoplasma-Plant (HPP) interactions has been recently published to summarize the information available in the literature (Trivellone, 2019). The preliminary analyses suggested that several phytoplasma strains are widespread and infect a wide variety of hosts, but many groups appear to be more restricted, with a tendency to be associated with particular biogeographic regions and/or plants and hemipteran families or subfamilies. In this study the general

patterns of infection by phytoplasmas on insect (Hemiptera) and plant hosts are analyzed, and preliminary results of network analyses of host-phytoplasma interaction data are presented.

Materials and Methods

Datasets

Analyses were carried out on two datasets extracted from the HPP database (Trivellone, 2019): Hemipteran-Phytoplasma (HP) and Plant-Phytoplasma (PP). The entire HP dataset consists of co-occurrence data for 262 species worldwide, with 246 insect hosts (H, all belonging to the suborder Auchenorrhyncha and family Psyllidae) and 16 phytoplasma groups (P). The list of insect hosts includes species with status of either competent (211) or potential (35) phytoplasma vector as defined by Trivellone (2019). The entire PP dataset is composed of 672 species, with 639 plant hosts (Pl) and 33 phytoplasma groups (P). The list of plant hosts includes species resulted positive for the phytoplasma presence.

Network Statistics

Network representation of biological relationships usually considers organisms as nodes and their direct or indirect interactions as links (ball-and-stick diagram), summarizing the overall trend of a collection of independent studies in a single graph. To reveal patterns of biological associations, the network topology was examined using properties such as density and degree of network centrality. The HP and PP

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Figure 1. Network representation of the interactions between, phytoplasma groups and their potential (light blue) and competent (dark blue) hemipteran vectors (left graph) and phytoplasma groups (yellow nodes) and plant hosts (green) (right graph).
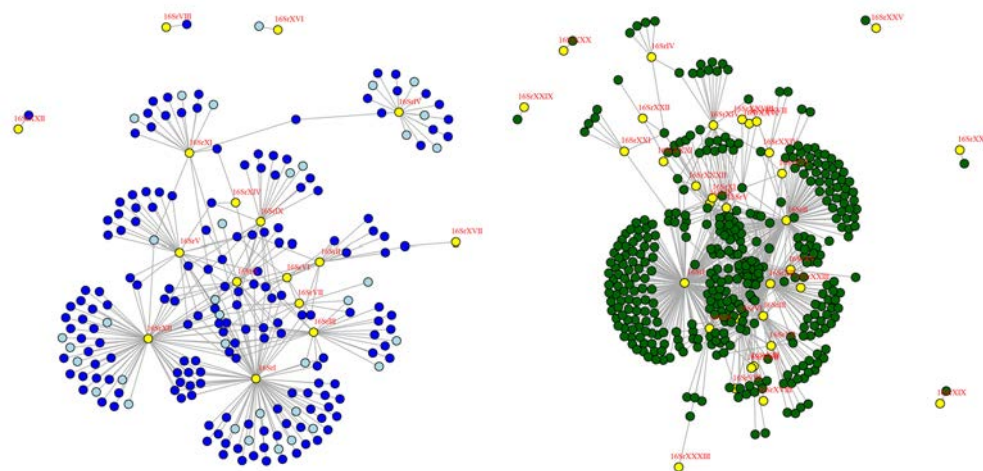
datasets were analyzed and graphed using *igraph* package (Csardi and Nepusz, 2006) in R (R Core Team, 2014). Measures of modular structure and nestedness in the HP network were also calculated. Modularity describes the degree to which a network can be grouped in modules (*i.e.* groups of nodes more densely connected to each other than with other nodes). The standard BRIM algorithm (Barber, 2007), which utilizes the Q index, was used. Q represents how often a particular ordering of phytoplasmas and hosts into modules corresponds to interactions that are primarily inside a module ($Q \approx 1$), primarily outside ($Q \approx -1$) or somewhere in between ($-1 < Q < 1$). Nestedness occurs when specialists from both sets of species interact preferentially with generalists. The NODF algorithm (Almeida-Neto *et al.*, 2008) was applied, and the network was considered nested if the observed NODF value was higher than that predicted by a null model. A Bernoulli random null model was used to measure the statistical significance of modularity and nestedness.

Results

In Figure 1, phytoplasma groups (yellow), competent (dark blue) and potential (light blue) vector species are shown as nodes. The proportion of links from all possible in the network is 0.012. The 16SrI and XII phytoplasma groups are associated with the largest number of insects with 77 and 65 competent and potential vectors, respectively.

The proportion of links from all possible in the PP network (Figure 1) is 0.008. The 16SrI, -II and -XII phytoplasma groups are associated with the largest number of plants with 201, 106 and 116 species, respectively. The entire HP dataset showed significant modularity (Q = 0.57, z-score = 5.11, number of modules = 14) and nested structure (NODF = 0.18, z-score = 45.62). However, 12 out of 14 modules are composed of only one phytoplasma group, it was not possible to calculate modularity and nestedness within internal modules.

Discussion

Understanding the structural organization of biological networks using topological measures gives clues to the

evolutionary processes that may produce the observed host-phytoplasma associations. These preliminary results showed that HP and PP networks have low density, and HP associations also show a modular and nested structure, suggesting that different phytoplasmas occupy exclusive ecological compartments of association with their hosts. Statistical analyses to detected modularity and nestedness of PP dataset are still ongoing.

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Epidemiology

High genetic diversity of '*Candidatus* Phytoplasma solani' infecting pepper in Serbia

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Abstract

Surveys for the presence of symptoms typical for the "stolbur" disease were conducted during 2015 and 2016 in pepper growing areas of Serbia. '*Candidatus* Phytoplasma solani' detection was performed by Stol11 primer amplification. Characterisation was done by multi-locus typing (MLST) of *tuf*, *secY*, *vmp1* and *stamp* genes. The disease was observed in pepper in all the surveyed locations except in south Serbia, whith a low incidence (3-5%). Overall, 13 MLST genotypes were distinguished, and the most common were Rqg31/Rqg50, Rpm35/M5 and STOL.

Keywords: *Capsicum annuum*, distribution, multi-locus (MLST) genotypes, "stolbur" phytoplasma

Introduction

'*Candidatus* Phytoplasma solani' belonging to the 16SrXII-A ribosomal subgroup, known also as "stolbur" phytoplasma, is widely distributed in Serbia in diverse wild plants acting as pathogen reservoirs, and in many cultivated plants in which it is associated with economically important diseases (Mitrovic *et al.*, 2016; Kosovac *et al.*, 2019). Occurrence and economic importance of the "stolbur" phytoplasma infecting pepper plants (*Capsicum annuum* L.) has been known in Serbia since the 1960s (Aleksic *et al.*, 1967). As a result of molecular characterization of a "stolbur" strain from pepper in Serbia, the strain STOL, originating from a diseased pepper plant (maintained in periwinkle) was designated as reference strain for '*C. P. solani*' taxon (Quaglino *et al.*, 2013). Since this includes genetically, ecologically and molecularly diverse phytoplasma strains, it is of importance to obtain additional information on its diversity according with host plant (type host plant) and geographical area (type locality).

Additionally a comprehensive Serbian "stolbur" strain evaluation about distribution, incidence, insect vectors, and genetic and epidemiological characteristics of the phytoplasma by molecular toold is not available. The aims of the present study were to evaluate these aspects in the context of "stolbur" disease of pepper (distribution and incidence) and molecularly evaluate the newly collected topo-types and host type strain(s).

Materials and Methods

Field surveys for the presence of symptoms typical for "stolbur" infection of pepper were performed during August 2015 and 2016 in pepper growing areas of northwestern (districts of Baèka and Srem), eastern (Branièevo and Zajeèar) and southern Serbia (Jablanica). Observed symptoms in affected pepper plants included leaf yellowing, stunting, plant decline, fruit deformation, and complete plant desiccation (Figure 1).

Figure 1. Symptoms of '*Ca.* P. solani'-infection in pepper in Baèka district of northwestern Serbia (left) and Zajeèar district of eastern Serbia (right).

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In all locations the symptoms were severe and the infected plants were easily recognizable, except in the southern Serbia where only moderate leaf yellowing symptoms were noticed. From each locality, at least six symptomatic and three asymptomatic pepper plants were collected resulting in over 60 phytoplasma strains. Identification of '*Ca*. P. solani' was performed using nested PCR amplification with stolbur-specific Stol11 primers (Cvrkovic *et al.*, 2014; Mitrovic *et al.*, 2016). Molecular characterization of the "stolbur" strains inducing disease in pepper was done by multi-locus sequence typing (MLST) of *tuf*, *secY*, *vmp1* and *stamp* genes (Langer and Maixner, 2004; Fialová *et al.*, 2009; Fabre *et al.*, 2011a; Cvrkovic *et al.*, 2014). Obtained nucleotide sequences of *secY* and *stamp* genes were compared to reference strains using MEGA 7, while *tuf* and *vmp1* restriction digestion (RFLP) profiles were compared to published strain profiles.

Results

The "stolbur" disease of pepper was detected in all surveyed locations except in south Serbia. '*Ca*. P. solani' was detected in nearly 95% of the symptomatic peppers, while all asymptomatic plants tested negative. Incidence of the disease was low, ranging from 3% to 5%, and only on a single field it was about 15%. Infected plants were distributed randomly within the pepper fields, indicating incidental infection and non-specific insect vector presence.

Restriction fragment length polymorphism analysis of the amplified *tuf* gene revealed that all symptomatic peppers were infected by the '*Ca*. P. solani' tuf-type b, while the characterization of the other three genes revealed the presence of a high genetic diversity. Three *secY*, four *vmp1* and nine *stamp* genotypes were identified. The *secY* sequence genotypes corresponded to strains of STOL, GGY and BG4560 (Fabre *et al.*, 2011a), while *vmp1* restriction profiles corresponded to V2-TA, V4, V7-A and V14 types (Cvrkovic *et al.*, 2014). The *stamp* sequences grouped within the clusters b-II and b-III (Fabre *et al.*, 2011b) among which the most common genotypes corresponded to strains Rqg50, Rqg31, Rpm35 and STOL (Cvrkovic *et al.*, 2014). Overall, 13 MLST *tuf*/*secY*/*stamp*/*vmp1* genotypes were distinguished. Higher genetic diversity was detected in strains from northwestern Serbia (11 genotypes), followed by strains from eastern Serbia (five genotypes) where two unique genotypes were identified, of which one corresponded to strain BG4560 from neighboring Bulgaria (Fabre *et al.*, 2011a). The most frequent *stamp* sequences identified in northwestern Serbia corresponded to Rqg31/Rqg50 (64%) and Rpm35/M5 (24%) (Cvrkovic *et al.*, 2014; Mitrovic *et al.*, 2016), while the most common strain infecting more than the 70% of symptomatic pepper plants in eastern Serbia was the STOL (Cvrkovic *et al.*, 2014; Kosovac *et al.*, 2019).

Discussion

Based on wide distribution, low incidence and high genetic diversity of '*Ca*. P. solani' in pepper fields in Serbia, multiple vectors and unspecific epidemiology are probably underlying the disease spread and occurrence. MLST analyses of characterized strains and comparison with previously described "stolbur" epidemiological features (Cvrkovic *et al.*, 2014; Mitrovic *et al.*, 2016; Kosovac *et al.*, 2019) indicate the cixiid planthoppers *Reptalus panzeri* and *Hyalesthes obsoletus* from *Convolvulus arvensis* and *Crepis foetida* as probable vectors involved in the phytoplasma transmission. The occurrence of the strain STOL was verified in several locations and its frequent presence in the eastern Serbia points to this area as the most probable origin of the strain.

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Epidemiology

New insights on "bois noir" epidemiology in the Chianti Classico area, Tuscany

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Abstract

"Bois noir" (BN) is the most widespread disease of the grapevine yellows complex in several countries worldwide. BN is associated with the presence of '*Candidatus* Phytoplasma solani', transmitted from herbaceous plants to grapevine by polyphagous insect vectors. In the present study, a preliminary investigation on the epidemiology of BN in the Chianti Classico area was carried out in an organic Sangiovese vineyard. '*Ca.* P. solani' strains identified in symptomatic grapevines and insects were typed through the analysis of the *stamp* gene nucleotide sequences. Obtained results revealed the presence of 4 *stamp* sequence variants in grapevines and the exclusive presence of the sequence variant St10 in *Reptalus quinquecostatus*, the sole insect species found infected by this phytoplasma in the studied vineyard.

Keywords: grapevine yellows, *Reptalus quinquecostatus*, *Vitis vinifera*, nucleotide sequence analysis

Introduction

Grapevine yellows (GY) diseases associated with phytoplasmas constitute a major threat to the worldwide viticulture. "Bois noir" (BN) is one of the most important GY in the European and Mediterranean regions and is associated with '*Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013). Its main insect vector is the planthopper *Hyalesthes obsoletus* Signoret (Maixner, 1994), even though BN incidence is not always correlated to high densities of this insect. Interestingly, recent studies evidenced the capability of other insects to transmit '*Ca.* P. solani' to grapevine (Cvrkovic *et al.*, 2014). Up to now, *Convolvulus arvensis* and *Urtica dioica* have been reported as being the main host plants of '*Ca.* P. solani'. Moreover, other wild and cultivated plants within or near vineyards were found infected by the phytoplasma suggesting their role in BN epidemiology (Mori *et al.*, 2015). The use of molecular markers for '*Ca.* P. solani' strain typing increased the knowledge of BN epidemiology in vineyard agro-ecosystems (Kosovac *et al.*, 2016, 2019). The present study aimed to describe the genetic diversity of '*Ca.* P. solani' strains identified in grapevines and insects. Obtained results allowed to gain new insights into the role of putative insect vectors in BN epidemiology.

Materials and Methods

The study was conducted in an organic Sangiovese vineyard located in Greve in Chianti (Chianti Classico area, Florence province). In September 2018, about 10 leaves were collected from each of 48 symptomatic *Vitis vinifera* cultivar Sangiovese plants. In July and August, yellow sticky traps, placed inside and at the vineyard borders, were used to collect Auchenorrhyncha species, considered potential phytoplasma vectors. Plant DNA was extracted following the protocol described by Li *et al.* (2008). Insect DNA was extracted from captured specimens, maintained in 70% ethanol, as described by Marzachì *et al.* (1998). Detection of phytoplasmas belonging to groups 16SrI and 16SrV, and subgroup 16SrXII-A ('*Ca.* P. solani') was carried out by quantitative PCR according to the protocol by Angelini *et al.* (2007). '*Ca.* P. solani' strains, detected in grapevines and insects, were typed by PCR-based amplification of the *stamp* gene and nucleotide sequence analysis, as described by Pierro *et al.* (2018a).

Results

A total of 347 Auchenorrhyncha specimens were collected, with a prevalence (186 out of 347 specimens) of *Reptalus quinquecostatus* (Table 1). '*Ca.* P. solani' was detected in 45 out of 48 symptomatic grapevines, and in 76 out of 186 specimens of *R. quinquecostatus*, the sole species found phytoplasma infected. Phytoplasma groups 16SrI and 16SrV were not detected neither in grapevines nor in insects. Nested-PCR reactions allowed the amplification of the *stamp* gene in 43

out of 45 '*Ca*. P. solani'-infected grapevines and in 67 out of 76 '*Ca*. P. solani'-infected *R. quinquecostatus* specimens.

Based on sequence identity, four *stamp* sequence variants were identified within the '*Ca*. P. solani' strains infecting grapevines: 3 sequence variants shared 100% sequence identity with St10 (46.6%), St18 (30%) and St5 (20%) sequence variants, while 1 (St59) was identified for the first time in this study and differed from St10 for 2 single nucleotide polymorphisms (nucleotide position 335 and 500) (Figure 1). Sequence analysis revealed that the *stamp* sequence variant St10 was present in all the 67 '*Ca*. P. solani'-infected *R. quinquecostatus* specimens (Figure 1).

Table 1. Insects identified in the surveyed vineyard.

Family	Genus	Species	Collected
Aphrophoridae	*Phylaenus*	*spumarius*	72
Aphrophoridae	*Neophylaenus*	sp.	4
Cicadellidae	*Psammotettix*	sp.	21
Cicadellidae	*Zygina*	*rhamni*	57
Cicadellidae	*Macrosteles*	sp.	1
Cixidae	*Reptalus*	*quinquecostatus*	186
Cixidae	*Cixius*	sp.	1
Dictiopharidae	*Dictyophara*	*europaea*	5
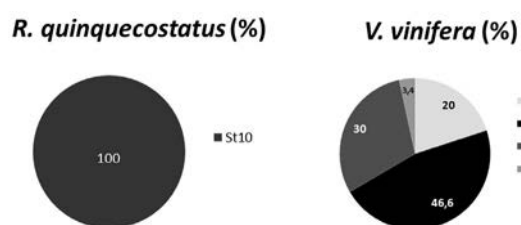
Figure 1. Prevalence of *stamp* sequence variants (%) of '*Ca*. P. solani' strains identified in *V. vinifera* and *R. quinquecostatus* in the studied vineyard.

Discussion

The survey on Auchenorrhyncha surprisingly showed the absence of *H. obsoletus* and the massive occurrence of highly '*Ca*. P. solani'-infected (41%) *R. quinquecostatus* in the examined vineyard. In accordance with previous results obtained in Tuscany (Pierro *et al.*, 2018b), '*Ca*. P. solani' strains infecting grapevines were typed as *stamp* sequence variants St5, St10, and St18, with the prevalence of St10. Interestingly, nucleotide sequence analysis showed the unique presence of the *stamp* sequence variant St10 in *R. quinquecostatus*. Such results could reinforce previous evidences suggesting an important epidemiological role of *R. quinquecostatus* in the transmission of '*Ca*. P. solani' to grapevine (Trivellone *et al.*, 2006; Chuche *et al.*, 2016), at least for phytoplasma strains harboring the St10 *stamp* sequence variant.

Considering the exclusive presence of '*Ca*. P. solani' strains harboring the *stamp* sequence variants St5, St18, and St59 in grapevines, it is reasonable to hypothesize the existence of additional epidemiological pathways for these '*Ca*. P. solani' strains, including other insect vector(s) and/or plant

reservoir(s). Further studies based on '*Ca*. P. solani' transmission trials to grapevine are necessary to clarify the role of *R. quinquecostatus* in "bois noir" epidemiology.

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Epidemiology

A study on the epidemiology and the vector transmission of 'Candidatus Phytoplasma prunorum'

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Abstract

Epidemiology and transmission characteristics of 'Candidatus Phytoplasma prunorum' were studied in Austria and Hungary. The investigations showed high infections rates of up to 70% for wild Prunus spp.. Molecular analysis revealed a large congruence of phytoplasma types in wild and cultivated Prunus as well as in Cacopsylla pruni indicating a joint epidemic cycle. In semi-field transmission experiments an inoculation access period of 4 h was sufficient for phytoplasma transmission.

Keywords: Cacopsylla pruni, wild host, European stone fruit yellows, transmission experiment

Introduction

European stone fruit yellows (ESFY) associated with the presence of 'Candidatus Phytoplasma prunorum' is currently present in all European and Mediterranean regions where susceptible crops are cultivated (Tedeschi et al., 2013). The only known insect species transmitting the pathogen is the plum psyllid Cacopsylla pruni (Scopoli) (Carraro et al., 1998). As basis for appropriate management strategies, the disease epidemiology and characteristics of vector mediated pathogen transmission were examined. Pathogen presence in wild and cultivated Prunus spp. as well as C. pruni was determined and phytoplasma types were characterised by molecular means. Characteristics of insect-mediated phytoplasma transmission were studied by transmission experiments under field and semi-field conditions.

Materials and Methods

Infections of plants and insects in Hungary and Austria

Samples of Prunus armeniaca, P. domestica spp. insititia, P. cerasifera and P. spinosa were collected in July-September 2015-2018 in orchards and their surroundings in Austria and Hungary. C. pruni were caught by beating tray method from March to April in the years 2014-2017 on P. spinosa, P. domestica and P. cerasifera.

Cage transmission trials

For simulation of field conditions in early spring dormant apricot and plum seedlings in pots were used. Ten insects per plant (remigrants collected by the beating tray method on P. spinosa) were caged on the test trees for 7 days (apricot seedlings N=51, plum seedlings N=13). For simulation of vector activity after flowering 10 remigrants per experiments were caged on foliated apricot (N=57), plum (N=7) and blackthorn (N=15) seedlings at 21°C for 7 days. Transmission by remigrant generation insects was investigated by allowing the spring generation insects to feed on foliated apricot seedlings (N=86) for 7 days. In order to study the length of the inoculation access period (IAP) 20 remigrant insects per experiment were kept on apricot seedlings (BBCH stage 03-09) for 4 hours (N=31), 6 hours (N=20) and 3 days (N=12) respectively. After the trials all plants were maintained under an insect proof net and annually tested by PCR.

Monitoring of pathogen spread in the field

Ungrafted apricot seedling rootstocks were planted both in spring 2012 (60 plants) and 2014 (60 plants) in Krems (Lower Austria). In the 2014 planting two times five apricot seedlings were protected from *C. pruni* by an insect proof net. In the following years all plants were tested by PCR and inspected visually.

PCR-analysis and strain characterisation

DNA extraction from plant (leaves and roots) and insect samples was carried out by a CTAB - procedure (Maixner *et al.*, 1995). The presence of phytoplasmas was detected by direct PCR (primer pair f01/r01; Lorenz *et al.*, 1995), nested PCR [primer P1/P7, Deng and Hiruki, 1991; Smart *et al.*, 1996 and R16(X)F1/R1, Lee *et al.*, 1995] followed by RFLP using *Rsa*I and/or quantitative PCR (Christensen *et al.*, 2004). For deeper characterization of phytoplasma strains the genes *aceF* and *imp* were analysed as previously described (Danet *et al.*, 2011).

Results

Investigations of wild and cultivated *Prunus* spp. in Austria and Hungary revealed high infection rates in all the sampling locations. The 61% and 40% of the Austrian *P. spinosa* and *P. domestica* spp. *insititia* root samples and 21% of the *P. spinosa* as well as 13% of the *P. cerasifera* roots collected in Hungary were infected. The pathogen was present in almost all apricot orchards and PCR positive *C. pruni* were widespread (at 11 of 17 sampling sites in Austria and Hungary respectively). In the cage experiments the remigrant *C. pruni* transmitted the phytoplasma to 15.7 % of the *P. armeniaca* and to 7.7% of the *P. domestica* trees at outdoor conditions in March and April. In the foliated test with plants kept at 21°C, transmission rates of 5.3% and 33% were recorded for *P. armeniaca* and *P. spinosa* seedlings, respectively. All plum seedlings remained healthy. Inoculation access of remigrants for 4 hours resulted in 2 infected apricot seedlings out of 31, after an IAP for 6 hours 1 seedling out of 20 and after an IAP of 3 days 1 seedling out of 12 were respectively infected. No pathogen transmission by springtime generation psyllids was observed. In the experiment aiming to monitor pathogen transmission under field conditions 40% and 28% of the ungrafted apricot seedlings planted in 2012 and 2014, respectively were infected in 2017. All trees protected by an insect proof net remained healthy. Molecular analysis of the genes *aceF* and *imp* enabled to distinguish 10 phytoplasma types in apricots. The majority of the phytoplasma types found in apricots were identical to types that were frequent in *P. spinosa*, *P. domestica* spp. *insititia* and in remigrant *C. pruni*.

Discussion

The results of the current study revealed high infection rates of wild *Prunus* spp. The large correspondence of phytoplasma types in wild and cultivated *Prunus* spp. and in *C. pruni* indicated an exchange of phytoplasmas in a joint epidemic cycle. It appears that wild *Prunus* species hosting both insect vectors and the phytoplasma play a crucial role for the disease epidemiology. The cage transmission experiments showed that the risk of phytoplasma transmission is present from the beginning of insect remigration in early spring onwards. In contrast, no indication of pathogen transmission by springtime generation insects was observed. Furthermore the trials indicated that the IAP can be shorter than the previously reported 1-2 days (Carraro *et al.*, 2001). The high infection rates observed for the ungrafted apricot seedlings in the test plantations confirmed a high risk of vector transmission in the field and underlined the need for an effective vector control. However, from previous experiments it can be assumed that only the minority of the available insecticides disrupts insect feeding behaviour in less than four hours at cool spring temperatures and early developmental stages of trees (Paleskic *et al.*, 2017).

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Epidemiology

Looking for wild plant reservoirs and potential insect vectors for '*Candidatus* Phytoplasma solani' in "bois noir"-affected vineyards in Bekaa valley-Lebanon

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Abstract

"Bois noir" is a frequent grapevine yellows in the vineyards of Bekaa valley in Lebanon and is associated with '*Candidatus* Phytoplasma solani'. Genotyping through sequencing of the *tuf* gene and the variable gene *stamp* mainly revealed the presence of '*Ca*. P. solani' strains of genotype tuf-b1/ST14. The high incidence of the disease in two vineyard plots cultivar Chardonnay and the aggregation of "bois noir" cases suggested the establishment in the vineyard itself of reservoir plants and insect vectors. Survey of wild plant and potential planthopper vectors led to the detection the same genotype of '*Ca*. P. solani' in the field bindweed *Convolvulus arvensis* in the two vineyards as well as in several sites in the Bekaa valley and in *Hyalesthes obsoletus* populations collected on this plant species. These data suggest the local propagation of '*Ca*. P. solani' through a classical epidemiological cycle involving bindweed reservoir hosts and *H. obsoletus* planthopper vectors. Surprisingly, a '*Ca*. P. omanense' -related strain was also detected in an old plantation of Syrah as well as in *C. arvensis* and two cixiid planthopper species.

Keywords: grapevine yellows, insect vector, *Hyalesthes obsoletus*, *Convolvulus arvensis*

Introduction

'*Candidatus* Phytoplasma solani', known also as "stolbur" phytoplasma, is a phloem-limited wall-less bacterium that belongs to the phytoplasma group 16SrXII-A. All over Europe, '*Ca*. P. solani' infects a wide range of annual and perennial crops (EFSA, 2014), but it is also occurring in the eastern part of the Mediterranean basin where it associated with the "bois noir" (BN) disease of grapevine (Daire *et al*., 1997; Davis *et al*., 1997; Orenstein *et al*., 2001; Choueiri *et al*., 2002; Ertunc *et al*., 2015). In Europe, the main '*Ca*. P. solani' insect vectors are planthoppers of the family Cixiidae that transmit the phytoplasma from wild plants acting as reservoir, to crops. *Hyalesthes obsoletus* is considered the most important vector of "stolbur" phytoplasmas (Fos *et al*., 1992; Maixner, 1994), while *Reptalus panzeri* acts as BN vector in Balkans (Cvrkovic *et al*., 2014). Deciphering epidemic cycles is essential for controlling BN as it rely on the removal of wild plant reservoirs for '*Ca*. P. solani'. In the Near East, '*Ca*. P. solani' reservoir plants are not yet identified although *H. obsoletus* is suspected to transmit it to grapevine (Sharon *et al*., 2015). Surveys for phytoplasmas have been conducted in Lebanon in grapevine, wild plants present in the vineyards as well as in planthoppers collected on bindweeds. '*Ca*. P. solani' strains detected in grapevines have been genetically characterized and compared to those detected in infected weeds and potential insect vectors.

Materials and Methods

Plant collection and total nucleic acid extraction

Grapevine samples displaying leaf discoloration and rolling, shriveling of grapes and incomplete lignification of canes, were collected in Bekaa valley from 2008 to 2015. Symptomless grapevine plant materials were also sampled as negative control. Yellowing and stunted weeds including *Convolvulus arvensis* (bindweed), *Lactuca serriola*, *Carthamus* sp. were collected in 2014 and 2015. Total nucleic acids were extracted from 1 g of fresh leaf vein using the CTAB method described by Maixner *et al*. (1995).

Insect collection, identification and total nucleic acids extraction

Planthoppers were captured on *C. arvensis* using sweep nets in June 2014 in Ammiq and Kanafar, Bekaa valley. They were identified according to taxonomy handbooks for planthoppers (Holzinger *et al*., 2003), and specimen of *H. obsoletus* and *Reptalus sp*. were finally kept in 70% ethanol at −20°C before nucleic acids extraction using the same CTAB method used for plant samples.

Phytoplasma detection and genotyping

Total nucleic acid extracts were tested by nested PCR using the 16Sr phytoplasma-specific primers R16mF2/R16mR1 followed by R16F2n/R16R2 (Gundersen and Lee, 1996). *Tuf* and *stamp* genes were amplified as previously reported (Balakishiyeva *et al.*, 2018; Fabre *et al.*, 2011). PCR products were analysed by electrophoresis in 1% agarose gel, stained with ethidium bromide and visualized under UV light. All PCR products were directly sequenced on both strands by Beckman Coulter Genomics Company (Takeley, UK) on MegaBACE capillary sequencing instruments. Sequences were assembled, quality edited and consensus sequences were used for phylogenetic analyses as previously described (Balakishiyeva *et al.*, 2018).

Results and Discussion

Almost all phytoplasmas detected in BN grapevine samples were '*Ca.* P. solani'-related strains genotypes tuf-b1 and stamp ST14 corresponding to the Lebanese strain P7 maintained in periwinkle. A '*Ca.* P. omanense'-related strain (Al-Saadi *et al.*, 2008) was detected in one old plot of Syrah (Foissac *et al.*, 2019). Survey from 2008 to 2014 of two plots of Chardonnay indicated that BN symptomatic grapevine plants were aggregating and progressing in some parts of the plots where *C. arvensis* bindweeds were found abundant. Many of them were stunted with yellowish and purplish small leaves. Symptomatic *C. arvensis* were in majority positive for '*Ca.* P. solani' and carrying the same tuf-b1-ST14 genotype. A few of them were positive for a '*Ca.* P. omanense'-related strain. Both phytoplasmas were also detected in *H. obsoletus* collected on *C. arvensis* and '*Ca.* P. omanense' was also detected in a *Reptalus* sp. collected on bindweed. Diseased *L. serriola* were positive for group 16SrIX phytoplasmas as reported in Lebanon (Verdin *et al.*, 2003) and diseased *Carthamus* sp. were positive for a 4 SNP variant of the '*Ca.* P. trifolii'–related strain previously reported in tomato and pepper in Lebanon (Choueiri *et al.*, 2007). These results suggest that the wild plant reservoir for '*Ca.* P. solani' in Lebanon is the bindweed as reported in Western Europe (Langer and Maixner, 2004). Removal of diseased '*Ca.* P. solani' infected *C. arvensis* in the vineyards and their vicinity should help to reduce the BN incidence in the Bekaa valley.

Acknowledgements

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Epidemiology

Epidemiological aspects of phytoplasma diseases in a tropical country

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Abstract

A summary is presented of several epidemiological aspects of the pathosystem involving the urban trees, phytoplasmas, insect vectors and alternative host plants in Bogotá, Colombia. A possible explanation of how and why these phytoplasmas are presently infecting crops in the surrounding areas of this city is presented. The implications of the environmental conditions for phytoplasmas diseases in countries located in the tropical zone are discussed.

Keywords: phytoplasmas, molecular detection, environment, ash yellows, aster yellows

Introduction

Management of phytoplasma diseases require the knowledge of their epidemiology. In Bogotá, Colombia a disease associated with phytoplasmas of the 16SrI and 16SrVII subgroups affect at least eleven species of urban trees, and has also been detected in potato and strawberry crops in the rural areas that surround the city. The present work summarizes the results obtained in almost 20 years of research focused on studying the epidemiology of this pathosystem in the Sabana de Bogotá. Results include information about the pathogens, insect vectors, hosts and alternative plant hosts, environmental and climatic conditions, and about some of their relationships. With the available information, it is possible begin to propose management strategies to reduce the dispersal of the pathogens and to mitigate its negative effects.

Materials and Methods

The detection of phytoplasmas in different plant and insect hosts has been achieved using conventional molecular techniques such as nested PCR with universal primers for phytoplasmas including P1A/P7A, R16F2n/R2, R16mF2/R16mR1 and others, followed by RFLP and sequencing analysis (Duduk *et al.*, 2013). Phytoplasma transmission tests have been performed using *Cuscuta* sp., insect vectors and potato tubers. Microscopy techniques such as DAPI and electron microscopy were also used to confirm the presence of phytoplasmas in some of the tested samples.

Results and Discussion

The first urban tree in Bogotá in which phytoplasmas were detected was *Fraxinus uhdei* where in 2001 phytoplasmas of the 16SrVII group were identified. Morevoer phytoplasmas from these plants were transmitted with *Cuscuta* sp. to *Catharantus roseus* and other plant hosts such as celery and tobacco inducing symptoms associated with phytoplasma presence. DAPI stain allowed the visualization of fluorescence in the phloem that is indicating phytoplasma presence in *F. uhdei* and alternative host plant species. The use of electron microscopy has confirmed this finding. This disease was considered exotic and restricted to this introduced tree species, showing a resemblance to the ash yellows diseases described in North America (Sinclair *at al.*, 1996). Studies on the symptomatology supported by molecular detection, showed the presence of the disease in Bogotà and in other cities of the Andean region of Colombia, indicating that it was widespread in a large area. However, between 2006 and 2007, it was evident that other tree species showed symptoms that could be attributed to phytoplasmas. In the following years, it was shown that phytoplasmas of groups 16SrI and 16SrVII were infecting trees of the following species: *Acacia melanoxylon*, *Croton* spp., *Eugenia neomyrtifolia*, *Liquidambar styraciflua*, *Magnolia grandiflora*, *Pittosporum undulatum*, *Populus nigra* and *Quercus humboldtii* (Franco-Lara and Perilla-Henao, 2014). The presence of both groups of phytoplasmas has also been shown in *Sambucus nigra* (Adoxaceae) and *Salix humboldtiana* (Salicacea) (L. Franco-Lara, unpublished). The prevalence of the disease varies among species being *F. uhdei* the most affected with all its trees symptomatic, whereas *Croton* spp. is the less affected with about 30% with symptoms. In *L. styraciflua*, 16SrV-B, 16SrIX and 16SrXII-A phytoplasma groups were also detected (Franco-Lara *et al.*, 2017).

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In 2010, phytoplasmas of the 16SrI and 16SrVII groups were detected in strawberry crops of the Sabana de Bogotá, and in 2016 in potato crops of the Cundinamarca state, in which the Sabana de Bogotá is located. Phytoplasmas of these groups have also been detected in weeds and grass growing in the green areas of Bogotá. A study analyzed the presence of phytoplasmas in 34 weed species, including *Cenchrus clandestinus*, the predominant grass species of the Sabana de Bogotá. Phytoplasmas of the 16SrVII group were detected in 18 samples from the 150 tested classified as *Amaranthus dubius, Cenchrus clandestinus, Cymbalaria muralis, Fumaria capreolata, Holcus lanatus, Gnaphalium spicatum, Gnaphalium cheiranthifolium, Lepidium bipinnatifidum, Senecio vulgaris, Sonchus oleraceaus* and *Taraxacum officinale.*

For the identification of the insect vectors the presence of Cicadellidae in the *C. clandestinus* grass surrounding infected trees in Bogotá was carried out. Nine species were collected and tested for the phytoplasma presence. Several species were positive for 16SrI and 16SrVII groups. The most abundant species found in the grass were *Amplicephalus funzaensis* and *Exitianus atratus* that resulted positive to phytoplasma presence. Transmission tests were performed with both species and it was shown that they were able to transmit phytoplasmas to broad bean plants (Perilla-Henao *et al.,* 2016). Furthermore, transmission tests have been performed unsuccessfully with *A. funzaensis* to strawberry plants, but with *E. atratus* to two species of potato showed that 16SrI and 16SrVII phytoplasma groups could be transmitted. In another study, the grass Cicadellidae were collected from a rural and an urban location of the Sabana de Bogotà, during one year. In total, 15 species were collected, including *A. funzaensis* and *E. atratus*. DNA barcodes have been generated for 12 of those species, enabling the detection of nymphs in different stages of development. In addition to the known insect vectors, *Haldorus* and *Dalbulus* species were also abundant, infected with phytoplamas and possible new vectors (Silva *et al.,* 2019). *A. funzaensis* and *E. atratus* reproduce on *C. clandestinus,* making this species a key element in the pathosystem. Furthermore *C. clandestinus* can be infected with phytoplasmas of 16SrI and 16SrVII groups, although it is asymptomatic. Recent studies have shown that some of the Cicadellidae collected in the grass could also be sampled from the branches of trees. For example, *A. funzaensis, E. atratus* and *Dalbulus* sp. were collected on *Q. humboldtii* trees. Moreover, *A. funzaensis and E. atratus* were also collected from potato crops of Cundinamarca and the surrounding *C. clandestinus* grass.

Variability analyses have shown that in the studied areas the 16SrI group is represented by several subgroups, while only one subgroup was detected for the 16SrVII group. A study has been conducted to determine if the different temperature, precipitation and humidity conditions, as well as the PM10 (air particulate material) affect the dispersion of phytoplasmas in *Q. humboldtii* trees. The results show that these environmental variables do not impact the group prevalence or distribution of the phytoplasmas in Bogotá. The tropical conditions of Bogotá seem to promote the continuity of phytoplasmas, since there are no overwintering periods for plants and insect vectors. These preliminary results show that although the populations of the insect vectors fluctuate during the year, at any month there is at least one insect vector species present in the grass. In addition, the fact that the trees are long-lasting perennial plants that do not hibernate make the inculum available permanently. Moreover, the known insect vectors seem to be polyphagous and their main host plants are *C. clandestinus,* ubiquitous in this ecosystem.

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Epidemiology

Relationship between climatic and environmental variables and the phytoplasmas detected in *Quercus humboldtii* in Bogotà, Colombia

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Abstract

Phytoplasmas of 16SrI-B and 16SrVII-A subgroups infect the urban *Quercus humboldtii* trees in Bogotà, Colombia. A survey allowed to detect the phytoplasma presence in the 93% of sampled trees, in particular in the 55% of these trees the 16SrVII-A phytoplasmas were detected, in the 37% the 16SrI-B phytoplasmas were present, while the 8% resulted harboring both phytoplasmas. All the studied trees were positive by nested PCR or had unambiguous symptoms. No patterns of distribution of the detected phytoplasma subgroups correlated with ranges of climatic and environmental variables. The insects that are possible phytoplasma vectors presented greater abundance in the trees of the urban parks, with respect to those of separators of the streets.

Keywords: insects vectors, disease severity, prevalence, symptom

Introduction

Phytoplasmas are cell-wall less pathogenic bacteria, obligate parasites of plants and insect transmitted. In the Sabana de Bogotá, Colombia, 'Candidatus Phytoplasma asteris' (16SrI) and 'Ca. P. fraxini' (16SrVII) are the major phytoplasma groups that infect urban trees. The same phytoplasmas infect the urban trees in Bogotà affecting the ecological system services provided, and their aesthetic value. The phytoplasmas are dispersed by sap sucking insect vectors that belong mainly to the families Cicadellidae, Fulgoridae and Psyllidae. Management strategies for the diseases associated with the phytoplasma presence are based on the epidemiological knowledge of the plant - insects vector - phytoplasma relationships due to the fact that the chemical treatments available are not usable for practical and ecological reasons (Lee *et al.*, 2000). *Quercus humboldtii*, a South American endemic species used as urban tree, was studied to verify the relationship between climatic and environmental conditions and the prevalence and spatial distribution of phytoplasmas in this tree species in Bogotá (307 km^2).

Phytoplasma associated symptoms in *Q. humboldtii* include: proliferation of axillary buds, witches' brooms, abnormal elongation of internodes, flaked branches, leaf yellowing, small leaves and changes in the architecture of the crowns. Moreover the richness and abundance of the possible phytoplasmas insect vectors among those reported in Bogotà (Perilla-Henao *et al.*, 2016) for *Q. humboldtii* in

relation to environmental variables and location, in Bogotá, Colombia were studied.

Materials and Methods

Spatial analysis were performed with ArcGIS software comparing the phytoplasma subgroup prevalence in quadrants with annual average temperature, relative humidity, precipitation, PM10 and PM2.5. Bogotá is a tropical city with average temperature constant in the year. The city was divided in 32 quadrants, and 16 random selected trees were sampled per quadrant for a total of 254 trees. Symptoms were recorded, and branches with secondary phloem were sampled for DNA extractions. DNA was tested by nested PCR on the 16S rRNA gene with primers R16mF2 /R16mR1 followed in nested PCR by R16F2n/R16R2 (Gundersen and Lee, 1996). The latter amplicons were analyzed by RFLP with restriction enzymes *Alu*I, *Hha*I, *Mse*I, and *Rsa*I, or by sequencing followed by virtual RFLP analyses to determine subgroup affiliation (Lee *et al.*, 1998; Zhao *et al.*, 2009). With the number of symptoms observed, a disease severity scale was designed (Table 1).

The insect sampling was performed in the same quadrants, in two types of sites: street and parks. Three trees were chosen in each sampling quadrant to capture the insects associated with *Q. humboldtii*, for a total of 96 trees that all showed symptomatology associated with phytoplasmas. The samplings were made with two methodologies: entomologic

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nets and shaking the tree branches to collect the falling insects. The insects were preserved in 15 ml tubes with 96% ethanol and later were taxonomically identified.

Table 1. Severity scale of phytoplasma symptoms in *Q. humboldtii*.

Severity		Number of symptoms
1	non symptomatic	1-2
2	light	3-4
3	moderate	5-6
4	severe	More than 6

Results

The evaluation of symptoms associated with phytoplasmas showed that 80% of the trees presented symptoms, where 43% presented a mild infection, 23% a moderate infection, 14% a severe infection. All the studied trees were positive by nested PCR (93%) or had unambiguous symptoms (7%) suggesting phytoplasma infection. In the 55% of the positive trees phytoplasmas enclosed in the 16SrVII-A subgroup were detected, in the 37% 16SrI-B phytoplasmas were present and the 8% of the sampled trees resulted infected with both phytoplasmas. No patterns of distribution of the subgroups correlated with ranges of temperature (12-26°C), relative humidity (43-90%), precipitation (344-1,036 mm), PM 10 (38-83 µg/m³) or PM 2.5 (10-30 µg/m³). The diversity and abundance of the Hemiptera varied depending on the type of site in which they were collected. In the parks with low human intervention, species richness and abundance were high, while in the streets and road separators there was a low richness and a high abundance of species. In the sites with the highest diversity such as Parque Entre Nubes, Humedal Jaboque and Park Way, the Cicadellidae family was represented by a greater abundance of Deltocephalinae, with 330 individuals and seven morphotypes and Typhlocybinae with 222 individuals and four morphotypes. In a lower proportion Membracidae, Pscoptera (Ectopsocidae and Elipsocidae) and Cixiidae were also found.

Discussion

Of the studied oak trees, 100% presented symptomatology or were infected with phytoplasmas of groups 16SrI or 16SrVII, which had been previously described as predominant in the Sabana de Bogotá (Perilla-Henao and Franco-Lara, 2012, 2014; Perilla-Henao *et al.*, 2012). The prevalence of the detected phytoplasma subgroups resulted not influenced by the climatic or environmental conditions of Bogotá. The results of the insect survey however showed a marked difference in terms of diversity and species richness according to the type of urban location. In places with more human presence, constructions and pollution, a considerable number of insect species was observed suggesting that the insects adapt to these astringent environments. Landscapes with a more conserved site tend to be richer in biodiversity of rare insects compared to the streets. The sites of greatest disturbance generated changes over time, manifesting themselves in ecosystem response processes called resistance and resilience. The loss of resilience is due to the loss of species with a determined function, due to the prolonged disturbances that are generated by creating structural alterations in the environment which causes a change in the biological communities found in this case within the *Q. humboldtii* trees. In conclusion urban areas with particular landscapes represent very diverse habitats for insects and in particular those that could be phytoplasma vectors.

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Towards the evaluation of potential insect vectors of phytoplasmas infecting hazelnut plants in Slovenia

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Abstract

'*Candidatus* Phytoplasma fragariae' and grapevine "flavescence dorée"-related strains have been detected in declining hazelnut trees in several orchards in Slovenia. To define the epidemiological routes the evaluation of possible insect vectors has been carried out, and it is presented in this study.

Keywords: *Corylus avellana*, '*Candidatus* Phytoplasma fragariae', 16SrV phytoplasmas, potential insect vectors

Introduction

Decline and yellows are diseases reported since long time in several European hazelnut (*Corylus avellana* L.) growing areas and were associated since 20 years to 16SrX group phytoplasma presence (Marcone *et al.*, 1996). More recently in stunted, but also in asymptomatic plants a 16SrIII-B phytoplasma was identified in Oregon (USA) (Jomantiene *et al.*, 2000). More recently the presence of '*Candidatus* Phytoplasma fragariae', subgroup 16SrXII-E (Valiūnas *et al.*, 2006) was identified in the United Kingdom (Hodgetts *et al.*, 2015) and 16SrI, 16SrIII, and 16SrV phytoplasmas were reported in Chile (Perez *et al.*, 2017). Phytoplasmas are mainly spread by insects of the families Cicadellidae (leafhoppers) and Psyllidae (psyllids), and the superfamily Fulgoroidea (planthoppers). These feed on the phloem sap of infected plants, and therefore the host range of phytoplasmas is dependent upon the feeding habits of their insect vectors. Phytoplasmas can also be efficiently spread via vegetative propagation, such as through cuttings, grafting and micropropagation practices (Bertaccini *et al.*, 2019). They can also be spread via the formation of root grafts (Ciccotti *et al.*, 2007; Lesnik *et al.*, 2008). Based on the data collected in infected Slovenian plantations in 2018, it seems that the role of the vectors are very important in the phytoplasma epidemiology of hazelnuts. However, the insect vector of '*Ca*. P. fragariae' is not known, and no vectors have been confirmed to transmit FD-related strains from hazelnut to hazelnut. Therefore, the aim of this study was to evaluate the potential vectors in hazelnut plantations to achieve the basic knowledge towards initiating the necessary prophylactic sanitary measures.

Materials and Methods

To determine the presence of phytoplasmas in plant and in insect hosts DNA was extracted from insects, roots and/ or shoots of plants using a procedure based upon the binding of DNA to magnetic beads (Mehle *et al.*, 2013). For detection of phytoplasmas a quantitative PCR approach was employed (Christensen *et al.*, 2004; Hren *et al.*, 2007). Identification and genetic characterization of the detected phytoplasmas were performed on 16S rRNA, ribosomal protein operon, *secY* and *map* genes.

Results and Discussion

Phytoplasmas of three unrelated groups have been detected in declining hazelnut trees in Slovenia: '*Ca*. P. fragariae', and phytoplasmas of the 16SrV and 16SrIX groups. Among these, '*Ca*. P. fragariae' and phytoplasmas of the 16SrV group appear to be the most widespread in the Slovenian hazelnut plantations. '*Ca*. P. fragariae' has been found in Slovenia also in forest trees showing unusually dense proliferation of sprouts from roots and/or trunks. Molecular characterisation of partial 16S rRNA, *secY*, *map* and ribosomal protein genes of hazelnut 16SrV phytoplasma strains shows that they are identical to strains associated with "flavescence dorée" (FD) disease found in Slovenian grapevines since 2005. In Slovenia, FD-related phytoplasma strains have been also detected in *Clematis vitalba*, *Ailanthus altissima*, *Alnus glutinosa* and *Alnus incana*, which all remain asymptomatic (N. Mehle *et al.*, unpublished).

O. ishidae specimens were collected in hazelnuts plantations and testing to verify the phytoplasma presence is in progress to determine their potential role as phytoplasma

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vector among hazelnut trees, or from a potential wild reservoir host plant to hazelnut trees. This is a highly polyphagous species that has been observed in association with many woody plants and deciduous trees, as well as some herbaceous plants (EPPO, 2015). In Slovenia, the first specimens were caught in 2002 in Nova Gorica. Then in July 2004, a substantial population of *O. ishidae* was found in Ljubljana on *Salix* spp. and in Nova Gorica on various fruit trees (Seljak, 2004). Since its first detection, *O. ishidae* has spread across Slovenia (EPPO, 2015).

The insect vector of '*Ca.* P. fragariae' is not known. The leafhopper *Scaphoideus titanus* transmits FD phytoplasmas from grapevine to grapevine, and although it is oligophagous, it lives only on grapevine in Europe (Chuche and Thiery, 2009). It is not known to transmit FD phytoplasma strains among alternative host plants, and although it can feed on a very few other host species, the possibility that it can acquire 16SrV phytoplasma from these hosts cannot be ignored (Chuche and Thiery 2009). Filippin *et al.* (2009) demonstrated that the 16SrV phytoplasmas can be transmitted from *Clematis vitalba* to grapevine by the planthopper *Dictyophara europaea*. *Oncopsis alni* and *Orientus ishidae* have been found to be infected by FD-related strains in Europe (Maixner *et al.*, 2000; Mehle *et al.*, 2010); moreover, transmission of the 16SrV group phytoplasmas to grapevine by *O. ishidae* has been demonstrated (Lessio *et al.*, 2016). It has been reported that *O. ishidae* can also transmit other phytoplasmas to celery plants, including the x-disease phytoplasmas (Rosenberger and Jones, 1978). Casati *et al.* (2017) revealed an abundant population of *O. ishidae* as prevalent at the borders of an FD-infected vineyard and within a wood in which hazel plants were the most present and were shown to be infected by 16SrV strains, although these hazelnut shrubs were asymptomatic.

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Occurrence of '*Candidatus* Phytoplasma ulmi' in native elm trees in Germany

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Abstract

A countrywide survey has been performed to examine the occurrence of the elm yellows phytoplasma in three elm species native to Germany. The DNA of more than 5,000 trees was extracted and the presence of phytoplasmas verified by universal and pathogen-specific quantitative PCR assays. In total, 33% of all plants resulted phytoplasma-positive. The highest presence was found in the European white elm, followed by the wych elm and the field elm. Regional clusters of high incidence were found in east and in south-west Germany, while in the western states the phytoplasma presence was low or absent. Disease symptoms were only occasionally visible as witches' broom for wych elm and yellowing and small leaves for field elm. The survey revealed that '*Candidatus* Phytoplasma ulmi' is widespread in Germany questioning the current quarantine status of this pathogen.

Keywords: elm yellows, European white elm, wych elm, field elm, 16S-23S rRNA spacer region

Introduction

Conti *et al.* (1987) reported the presence of elm yellows (EY) in Italian elm trees in 1987 for the first time in Europe, but symptoms resembling EY have been described earlier (Goidànich, 1951). In Germany, the first report is from southern Germany, where the pathogen was detected in a single wych elm tree (Mäurer *et al.*, 1993). No further report on the occurrence and distribution of '*Candidatus* Phytoplasma ulmi' was published in Germany until 2015, when a survey in the states of Berlin and Brandenburg revealed a high infection rate for European white elm trees (Eisold *et al.*, 2015). In the EPPO region '*Ca.* P. ulmi' is listed as a quarantine pest in Annex I/A1 (EPPO, date 9/201), whereas this status for the EU changed in 2017 and the pathogen moved to the A2 list. However since the knowledge on the distribution of the pathogen in Germany is still poor, it was initiated this project. Beside others, one main objective is to obtain an overview on the pathogen's distribution, which started with a nationwide survey in the spring 2018. The results presented comprise preliminary data on the distribution of '*Ca.* P. ulmi', based on phytoplasma universal- and pathogen-specific quantitative PCR assays.

Materials and Methods

Plant material and locations

Branches from wych elm (*Ulmus glabra*), European white

elm (*Ulmus laevis*) and field elm (*Ulmus minor*) were sampled from April 2018 to November 2018. At each site branches of up to 20 trees were collected randomly without preference for tree condition. Locations for sampling were natural habitats for the species from sea level up to 1,100 m. The geographic location, height and diameter of each tree were recorded.

DNA extraction

DNA was extracted from phloem tissue, according to Ahrens and Seemüller (1992). DNA pellets were resuspended in 200 µl of sterile water and stored at -20°C until PCR analyses.

Quantitative PCR assay

Quantitative PCR analyses were performed with a phytoplasma-universal Taqman assay according to Christensen *et al.* (2004) and a pathogen-specific Taqman assay (hereafter referred to as EY-spacer assay) developed in this study. Primers and probe are located in the 16S-23S rDNA intergenic region and were selected after alignment of 16SrV group member phytoplasma sequences. Endpoint analyses were performed in 10 µl reactions containing 1 µl of DNA extract, 10 pmol of each probe and primers and prima QUANT master mix (Steinbrenner, Germany). After an initial denaturation for three minutes the reactions were cycled 40 times in a two-step PCR protocol with the following

Table 1. Incidence of '*Ca.* P. ulmi' in native German elm species based on qPCR results.

Species	Number of trees analysed	Number of positive samples	Percentage (%)
Ulmus glabra	2,081	675	32.4
Ulmus laevis	1,419	598	42.1
Ulmus minor	1,644	395	24.0

Figure 1. Wych elm showing witches' broom formation (August 2018, Müncheberg, Germany). On the right, a close-up of one shoot with witches' broom.

parameters: 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds, 56°C for 30 seconds for the phytoplasma-universal and the EY-spacer assay, respectively.

Results

In 15 states covering most regions of Germany 2,081 wych elms, 1,419 European white elms and 1,644 field elms were collected. EY-specific disease symptoms were rarely observed. A local accumulation of witches' broom formations was found for wych elms around Müncheberg, Brandenburg (Figure 1). Stunting and chlorosis were observed associated with EY-infected field elms at one location in Rheinland-Palatinate. Due to the widespread infection of wych elm and field elm with Dutch elm disease the health condition of many trees was poor. The highest overall infection rate of 42.1% was recorded for European white elms followed by 32.4% and 24.0% for wych elms and field elms, respectively (Table 1). The species were not uniformly infected throughout the territory. Hot spots were observed in the states of Brandenburg and Saxony and the upper Rhine valley. At about one fourth of the sites the disease incidence ranged between 66.1% and 100%, whereas at 50% of the sites '*Ca.* P ulmi' was not detected. The number of sites free from EY increased slightly with altitude. The infection rate increased significantly for European white elm and wych elm with tree age, but remained stable for field elm. The qPCR results matched almost completely between the universal- and pathogen-specific assays indicating a homogeneous infection of elm trees by '*Ca.* P. ulmi'. In two accessions, 16SrV group phytoplasmas were detected not belonging to the species '*Ca.* P. ulmi'.

Discussion

The survey demonstrated a wide occurrence of '*Ca.* P. ulmi' with high infection rates at 25% of the sites examined.

Despite this high infection rate EY-specific disease symptoms were rarely observed, regardless of the tree age. However, experimental inoculation trials with European white elm seedlings grafted with accessions from infected wych elms induced the witches' broom formation, indicating a host tolerance or a low strain virulence in natural habitats. The pathogen was not uniformly distributed in Germany and hot spots of the disease were identified. The reason for this uneven distribution in Germany is not clear and might only reflect the current state of the spread of this insect-vectored pathogen. The results of the two qPCR assays were matching, except for two accessions from Bavaria and Schleswig-Holstein where different 16SrV group phytoplasmas were identified. The EY-spacer assay showed a lower performance in sensitivity compared to the universal phytoplasma assay which might be due to the lower binding strength of probe and primers. Nevertheless, the results were always clear-cut due to the generally high titre of the pathogen in the samples.

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Are phytoplasmas associated with dieback symptoms of *Fraxinus* in France?

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Abstract

During the ash dieback survey in France it was also performed a study to verify phytoplasma presence in twigs from ash tree displaying dieback symptoms. Two hundred and sixty DNA samples from this national survey were tested for the detection and the identification of phytoplasmas. A phytoplasma related to 'Candidatus Phytoplasma fragariae' was detected in 5% of the tested ash samples. Further investigations are needed to determine the real incidence of phytoplasmas in the French forests.

Keywords: ash dieback, phytoplasma, 'Candidatus Phytoplasma fragariae'

Introduction

Since 2008 in France an active network of ash (*Fraxinus* sp.) dieback survey is based on the observers trained for the recognition of the symptoms and a sensitive and specific molecular method for the detection of *Hymenoscyphus fraxineus* (Ioos *et al*., 2009). Despite those accurate measures, between 2008 and 2014, a relatively large proportion of negative samples (27%) don't allowed the detection and confirmation of *H. fraxineus* presence. This can probably be explained in part by methodological difficulties; however, a risk of confusion with diseases that are associated with similar symptoms must not be completely ruled out, including phytoplasma diseases. In North America, '*Candidatus* Phytoplasma fraxini', also known as ash yellow phytoplasma (ASHY), is associated with symptoms of slow growth, dieback and premature death of *Fraxinus* species. In Europe, little research has been conducted on phytoplasmas in ash trees. Occasionally phytoplasmas such as '*Ca*. P. prunorum' have been found on asymptomatic *Fraxinus excelsior* in the vicinity of heavily infected fruit orchards (Jarausch *et al*, 2001). Strains related to ASHY have been described in Italy (Bruni *et al*., 2005, Zambon *et al*., 2018). In France the objectives of this study were: i) to estimate the frequency of ash infections by phytoplasmas; ii) to identify the main phytoplasmas present in ash; iii) to confirm the absence of '*Ca*. P. fraxini'.

Materials and Methods

Samples

Two hundred and sixty DNA samples were collected from the French network of laboratories which participated to the ash dieback survey. All initial samples from branches or shoots were from ashes that displayed dieback symptoms and were collected between 2011 and 2014 within a broadband focused north west-south east of France.

Phytoplasma detection and identification

DNA samples were amplified by nested PCR targeting the 16S rDNA. Primers pairs P1/P7 and R16F2n/R16R2 were used respectively for PCR and nested PCR amplifications (Deng and Hiruki, 1991; Schneider *et al*., 1995; Gundersen and Lee, 1996) following the author protocols. The amplicons were directly sequenced and the obtained sequences were compared to those available at the NCBI database.

Results

After amplification by nested PCR, the amplicons obtained from 39 samples were sequenced. Sequence analysis of the amplified 16S rRNA confirmed the presence of phytoplasmas in 13 ash samples (5% of the samples tested).

Detected phytoplasmas were identified as '*Ca*. P. fragariae' (subgroup 16SrXII-E) which differs from 3 mutations in 1,100 bp, or share 99.7% identity with the strains detected in strawberry in Lithuania and in France. Twelve of the phytoplasma positive samples come from several departments in the north west quarter of France (Figure 1). The number of positive samples according to the date of collection does not vary during the year (3 or 4 positive per trimester), but the rate of positives is higher in winter (25%) than during the other seasons (less than 8%). While 57% of

the samples were collected in north west quarter of France, 12 of the 13 positive samples came from this area. '*Ca*. P. fragariae' is more frequently present in *H. fraxineus* free samples (9 samples or 9.5%) than in infected samples (2.4%). This difference is significant [p (Chi2) = 0.012].
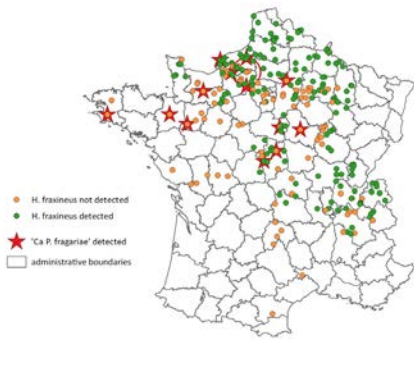
Figure 1. Nature and location of ash samples in which '*Candidatus* Phytoplasma fragariae'-related phytoplasmas has been detected.

Discussion

It was detected, with a frequency of 5%, a phytoplasma in the DNA samples of ash trees showing dieback symptoms. This frequency of detection is low compared to that of *H. fraxineus*, but not negligible considering the pathogen. Indeed, the molecular detection of phytoplasmas in trees is considered difficult because of their low concentration in the phloem and their heterogeneous distribution in the tissues, according to the seasons, and because of possible inhibitors of PCR reactions. In addition, the sampling of plant was focused on the detection of *H. fraxineus*. The detection frequency could be higher by extracting the DNA from the leaves and veins. In comparison, elm-associated phytoplasma was detected at much higher levels (75%) in Croatia in half-symptomless tree samples (Katanic *et al.*, 2016). Phytoplasmas can be aggravating factors for other diseases because they can affect metabolic defence pathways (jasmonic and salicylic acid pathways). The results contradict this possibility since the detection rate of the phytoplasma was higher in the samples in which *H. fraxineus* was not detected than in those where it was detected. All detected phytoplasma strains are closely related to '*Ca*. P. fragariae' (Valiunas *et al.*, 2006). This phytoplasma is a poorly studied plant pathogen whose distribution and range of hosts are not well known. It has previously been associated with diseased *Cornus sanguinea* and *Sambucus nigra* in Italy (Filippin *et al.*, 2008), potato disease in China (Cheng *et al.*, 2012), and hazelnut dieback during an epidemic in the United Kingdom (DEFRA, 2015) and most recently in Slovenia (Mehle *et al.*, 2018). Observations in the UK triggered initiation of a pest risk analysis, which concludes that in the absence of knowledge of the vector of this phytoplasma, risk assessment, and management decisions are subject to high levels of uncertainty (DEFRA, 2015).

The main result of this study is that '*Ca*. P. fraxini' or strains related to this phytoplasma could not be detected in the examined samples of ash. These results call for specific surveys to determine the real incidence of phytoplasmas related to '*Ca*. P. fragariae' on ash and their association with symptoms. However, they should allow future work concerning the role and involvement of the phytoplasmas role in the forest tree diebacks.

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Phytoplasmas detected in insects and spontaneous vegetation near vineyards with yellows diseases in Italy

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Abstract

Surveys in and nearby vineyards in some Italian provinces were carried out to detect alternative host plants and possible insect vectors of phytoplasmas detected in grapevines. Aster yellows is the prevalent phytoplasma detected in both beside the 16SrXII-A and 16SrV-C phytoplasmas. Moreover other phytoplasmas belonging to diverse ribosomal groups were identified indicating a relevant epidemiological role of spontaneous vegetation and possible new insect vectors in the grapevine yellows.

Keywords: phytoplasma detection, insects vectors, spontaneous plants, grapevine yellows, epidemiology

Introduction

No wide knowledge is available about phytoplasmas' impact in the European forests, however several reports are highlighting the importance of these prokaryotes as associated with economic relevant factors having detrimental effects on both production and landscape. In particular a growing number of diverse phytoplasmas are found in the Italian vineyards that are now increasingly detected together with the historical "bois noir" and "flavescence dorée" phytoplasmas (Zambon *et al.*, 2018). The work focused on studying phytoplasma presence in spontaneous vegetation and insects collected in important Italian grapevine-growing areas where the presence of grapevine yellows was reported for several years.

Materials and Methods

The plant samples were collected from four provinces (Bolzano, Forlì, Modena, Treviso) in the North-East Italy from the spontaneous vegetation near symptomatic vineyards in which the phytoplasma presence was known or reported (Zambon *et al.*, 2018). Total nucleic acids were extracted from 1 g of leaf midribs from 11 mainly asymptomatic plant genera *Clematis vitalba, Convolvolus arvensis, Morus* sp., *Partenocissus quinquefolia, Ranunculus* spp., *Robinia pseudoacacia, Rubus* spp., *Skimmia* sp., *Sorghum halepense, Urtica dioica, Zea mais* with liquid nitrogen using a phenol/chloroform protocol (Prince *et al.*, 1993). The insects were captured in two provinces (Modena and Treviso) by yellow sticky traps on wild plants around the grapevine yellows

infected vineyards. The insect were collected during summers 2017 and 2018 and identified under a stereomicroscope using dichotomous keys. Nucleic acids were extracted from single insect or in batches of 2/3 according with the size using a CTAB protocol (Angelini *et al.*, 2001). The plant nucleic acid was diluted in sterile deionized water to a final concentration of 20 ng/µl, while insect DNA was diluted 1:30 in sterile distilled water and in both cases 1 µl of diluted DNA was used in PCR assays. After direct PCR with the phytoplasma universal primer pair P1/P7 (Deng and Hiruki, 1991, Schneider *et al.*, 1995), nested-PCR with R16$_{758}$f (M1)/ m23SR$_{1804}$r (B6) (Gibb *et al.*, 1995, Padovan *et al.*, 1995) or R16F2n/R2 primers (Lee *et al.*, 1995) was performed. Additional nested-PCR assays were carried out using the primers 16R$_{758}$f /16S$_{1232}$r (M1/M2) (Gibb *et al.*, 1995) only on samples negative in the first nested-PCR. Each direct and nested PCR reaction was performed in a total volume of 25 µl containing 2.5 µl of the 10 X buffer, 200 µM of dNTP, 0.625 U of Taq polymerase (abm, Canada), and 0.4 µM of the primer pair. Identification of detected phytoplasmas was done using RFLP analyses on amplified ribosomal DNA fragments with informative restriction enzymes: *Tru1*I, *Taq*I and *Hha*I (Fermentas, Lithuania) and/or direct amplicon sequencing.

Results and Discussion

The samples from spontaneous vegetation resulting positive to the phytoplasma presence are listed in Table 1. Samples of *P. quinquefolia* from two diverse areas and *Skimmia* sp. and *C. vitalba* resulted positive for 16SrXII-A and 16SrV-C

phytoplasmas, respectively. *Clematis* spp., *Rubus* spp., mulberry and sorghum resulted infected with 16SrI phytoplasmas, in one case sorghum was double infected by phytoplasmas in 16SrI and 16SrX groups.

The number of insects captured, in both monitored provinces, was variable and influenced by the type of vineyard management and the presence of spontaneous plants (data not shown); in fact, there was a greater number of insects captured in organic vineyards and in those located near wild plants or woods, where insects perform part of their biological cycle. A total of 211 insects of 8 species (*Scaphoideus titanus* Ball, *Hyalesthes obsoletus* Signoret, *Orientus ishidae* Mats., *Hishimonus hamatus* Kuoh, *Philaenus spumarius* L., *Zygina rhamni* Ferrari, *Psammotettix striatus* Dhlb., *Neoaliturus fenestratus* H-S.) were tested. The analyses allowed to identify the presence of phytoplasmas in all the species except *H. hamatus*, *Z. rhamni* and *P. striatus* (Table 2). In the Modena province phytoplasmas belonging to group 16SrX were detected in *P. spumarius*, and 16SrI and 16SrXII-A in *N. fenestratus*.

Table 1. Results of the detection and identification of phytoplasmas in spontaneous vegetation near vineyards in some Italian provinces.

Plant species	Region (province)	Grapevine	Phytoplasma
Partenocissus quinquefolia	Romagna (Forlì) Alto Adige (Bolzano)	Chardonnay Unknown	16SrXII-A 16SrXII-A
Sorghum halepense	Veneto (Treviso)	Glera	16SrI; 16SrI+16SrX
Clematis vitalba	Veneto (Treviso)	Glera	16SrV-C; 16SrI
Rubus spp.	Veneto (Treviso)	Glera	16SrI
Morus spp.	Emilia (Modena)	Unknown	16SrI
Skimmia spp.	Romagna (Forlì)	Unknown	16SrXII-A

Table 2. Results of the detection and identification of phytoplasmas in insect vectors and putative insect vectors from some Italian provinces.

Insect species	Management (province)	Positive/tested	Phytoplasma (batches)
Scaphoideus titanus	Organic (Treviso)	14/29	16SrV-C (2) 16SrXII-A (4) 16SrVII-A (3) 16SrVI (1) 16SrX-B (2) 16SrI-B (2)
Hyalesthes obsoletus	Conventional (Treviso)	10/27	16SrXII-A (6) 16SrI-B (4)
Orientus ishidae	Conventional (Treviso)	22/69	16SrV-C (4) 16SrVII-A (5) 16SrVI (1) 16SrI-B (3) 16SrXII-A+16SrV-C (2) 16SrXII-A (7)
Philaenus spumarius	Conventional (Treviso)	02/30	16SrX (2)
Neoaliturus fenestratus	Conventional (Modena)	05/29	16SrXII-A (4) 16SrI (1)

In the Treviso vineyards the strain 16SrV-C was detected in *S. titanus* and *O. ishidae*, and 16SrXII-A in *S. titanus*, *O. ishidae* and *H. obsoletus*. The 16SrX-B and 16SrI-B were identified in *S. titanus*, while 16SrI-B was found in *O. ishidae* and *H. obsoletus*. The 16SrVII-A was identified in *S. titanus* and *O. ishidae* (Table 2). A number of plants and one insect species were infected with '*Ca*. P. asteris'-related strains indicating the potential of spreading of this phytoplasma in natural environments. Phytoplasmas enclosed in groups 16SrVI and 16SrVII were detected in *S. titanus* and *O. ishide*; considering that these phytoplasmas were also detected in the same vineyards (Zambon *et al.*, 2018), further studies are necessary to investigate the role of these two insects in the transmission of 16SrVI and 16SrVII phytoplasmas. The recent introduction of several alien species of both possible phytoplasma insect vectors and weeds increased the risk of spreading of new phytoplasmas or new strains that could affect the sanitary condition of the forests surrounding agricultural crops.

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Alder yellows phytoplasmas in *Alnus* species in Serbia

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Abstract

The presence of alder yellows and "flavescence dorée" phytoplasmas (16SrV-C) together with their insect vectors have already been reported in Serbia and the Balkans, as well as have similar phytoplasmas in clematis. The presence of alder yellows phytoplasmas in Serbia have been confirmed in black alder plants showing symptoms of severe leaf yellowing and multiple shoot growth from the basal part of the trunk. The results obtained in this work show that all examined *Alnus glutinosa* trees in Serbia, not exhibiting symptoms but randomly collected among forest trees during a survey, are infected with the alder yellows phytoplasma. However, none of the examined *Alnus viridis* bushes showed to be infected.

Introduction

Phytoplasmas are obligate parasites of plants and insects and are associated with diseases worldwide in hundreds of plant species. In phytoplasmas of the 16SrV ribosomal group, elm yellows (EY) group, nine subgroups, 16SrV-A to 16SrV-I, have been described. In general, the phytoplasmas of the ribosomal group 16SrV share high similarities in their 16S rRNA gene sequences, and four 'Candidatus species' have been described (Bertaccini and Lee, 2018). Phytoplasmas belonging to subgroups 16SrV-C and 16SrV-D are particularly conserved in their 16Sr sequences with only one nucleotide which differentiates between them and form a homogeneous genomic cluster (Lee *et al.*, 2004; Malembic-Maher *et al.*, 2011). However phytoplasmas belonging to the 16SrV-C/D cluster are transmitted by different insect vectors and occupy different ecological niches, regardless to the subgroup affiliation. The "flavescence dorée" (FD) phytoplasma (16SrV-C and 16SrV-D) which is associated with the most severe grapevine yellows in Europe, is transmitted by the ampelophagous leafhopper *Scaphoideus titanus* Ball. On the other hand, alder yellows (AldY) phytoplasma (16SrV-C) infects *Alnus* species and is transmitted by the oligophagous leafhopper *Oncopsis alni* (Schrank) which also occasionally transmits the German Palatinate grapevine yellows (PGY) from alder to grapevine (Maixner and Reinert, 1999). Besides the well-known diseases of grapevine and alder, the 16SrV-C phytoplasmas were also reported in other plant species, with their occasional or potential insect vectors, although their role in the epidemiology and disease

evolution remains unknown. Hypothesis that FD originates from AldY (occasionally transmitted to grapevine by an ampelophagus vector) and that it has happened three times is reflected in the three genetically distinct FD clusters discriminated by the *map* gene sequence analyses and certainly rises concern for emergence of new strains (Arnaud *et al.*, 2007; Maixner, 2009). The presence of FD (16SrV-C) and AldY phytoplasmas together with their vectors has already been reported in Serbia and the Balkans, as well as has similar phytoplasmas in clematis (Duduk *et al.*, 2003; Cvrkovic *et al.*, 2008; Filippin *et al.*, 2009). AldY phytoplasmas in Serbia have been detected in black alder showing symptoms of leaf yellowing and multiple shoot growth from the basal part of trunk (Cvrkovic *et al.*, 2008). Another species of the genus, *Alnus viridis* is present in Serbia, on high altitudes (usually 1,300-1,900 m a.s.l.) and has never been tested for the phytoplasma presence. Hence, the objective of this study was to assess the presence of AldY phytoplasmas in Serbia on *Alnus glutinosa* and *A. viridis* (black and green alder, respectively).

Materials and Methods

Plant material of *A. glutinosa* was collected during summers of 2013, 2015 and 2017. In total, 56 samples were collected form 12 locations in Serbia (Figure 1). Plant material of *A. viridis* was collected during October 2015. In total, eight samples of *A. viridis* were collected form Stara Planina location, its natural habitat (1,700 m a.s.l.), in south-east Serbia. In all cases the leaves were collected from trees/

bushes with no typical phytoplasma infection symptoms. Total nucleic acids were extracted from 0.5 g of fresh leaf midrib tissues using a CTAB protocol (Angelini *et al.*, 2001) and dissolved in TE buffer. Nucleic acids were diluted with sterile distilled water 1: 100 before performing polymerase chain reaction (PCR) assays. Detection of phytoplasmas in collected samples was performed with nested amplification with specific primers, R16(V)F1/R1 on P1/P7 (Deng and Hiruki, 1991; Lee *et al.*, 1994; Schneider *et al.*, 1995). Ribosomal subgroup identification of phytoplasmas belonging to 16SrV was performed with another nested PCR using 16R758f/M23SR1804 primer pair on P1/P7, followed with RFLP analyses using *Taq*I restriction enzyme (Martini *et al.*, 1999).

Results and Discussion

All 56 black alder samples tested, gave amplification of expected size with group 16SrV specific primers, while no amplification was obtained with any of the green alder samples.
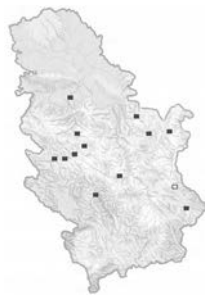
Figure 1. Geographical distribution of samples analysed in this study. Black rectangle, *A. glutinosa*; white rectangle, *A. viridis*.

The RFLP analysis of 16R758f/M23SR1804 amplicons with *Taq*I were identical among all analysed samples referring to the profile of the 16SrV-C subgroup (Martini *et al.*, 1999). All the examined *A. glutinosa* trees, not exhibiting symptoms but randomly collected among forest trees during the survey, resulted to be infected with AldY phytoplasma. The same situation, 100% infection rate of non symptomatic alders, has already been reported for the Spreewald area in Germany (Holz *et al.*, 2016), while in Hungary the infection rate of non symptomatic alders was 86%. In France 84,6% of alders showing yellowing showed to be infected (Ember *et al.*, 2011; Malembic-Maher *et al.*, 2007). Although the presence of AldY phytoplasma infection in *A. glutinosa* in Serbia is known (Cvrkovic *et al.*, 2008), the results of this survey show that it is common/abundant, as it was not possible to find a single full grown non-infected tree. On the other hand, none of the eight *A. viridis* showed to be infected. The same results, although using DAPI, were obtained for the species in mountain regions of central Europe (Lederer and Seemüller, 1991). As suggested by Maixner and Reinert (1999), this could be due to the host preference of the inseect vector *O. alni* or to unfavorable conditions, particularly high altitude, for the vector in the area of natural habitat of *A. viridis*.

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Study of the epidemiology of "flavescence dorée" (FD)-related phytoplasmas and potential vectors in a FD-free area

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Abstract

Epidemiological traits of "flavescence dorée" (FD)-related phytoplasmas were studied in Palatinate, a FD-free viticultural area in southwestern Germany. Grapevines with yellows symptoms, alder trees and leafhoppers on alders were sampled and tested for 16SrV-group phytoplasma presence. Genotyping of positive samples was based on the gene *map*. A high proportion of alder trees was infected by various genotypes including the FD-related genotype M38. About 22% of 330 sampled grapevines were infected with 16SrV-group phytoplasmas, but the map genotyping disclosed only the PGY-type of the 16SrV-C subgroup. The most abundant Deltocephalinae leafhoppers were *Orientus ishidae*, *Allygus mixtus* and *Allygus modestus*. Infection rates by group 16SrV phytoplasmas were 44%, 38% and 41%, respectively, with the predominant map genotype being M38. *Allygus* spp. and *O. ishidae* were able to transmit it to either *Vicia faba*, *Alnus glutinosa*, or both. A survey in vineyards revealed no evidence for the presence of the FD vector *Scaphoideus titanus* in spite of its local occurrence in the adjacent Alsace region. Thus, the Palatinate can still be considered free from FD and its vector.

Keywords: *S. titanus*, 16SrV-phytoplasmas, PGY, Deltocephalinae, map types, vector capacity

Introduction

"Flavescence dorée" (FD) is a quarantine disease of grapevine associated with phytoplasmas of 16SrV-C and -D group (Martini *et al.*, 1999). It is efficiently transmitted from grapevine to grapevine by the Deltocephalinae leafhopper *Scaphoideus titanus* Ball (Schvester *et al.*, 1962). So far, Germany is considered free from FD and from *S. titanus*, but it was detected in 2016 in the Alsace region which is adjacent to the Palatinate area in southwest Germany. A coordinated survey for the presence of this Nearctic leafhopper in both regions was initiated. Besides the monitoring of *S. titanus*, a second focus was to verify the infection of alder (*Alnus glutinosa*) in the vicinity of vineyards with group 16SrV phytoplasmas and to elucidate the potential of leafhopper species collected from alder for transmitting FD-related phytoplasmas from alder to alder or further to grapevine. It has been shown already that genetically closely related phytoplasmas are widespread in alder (Malembic-Maher *et al.*, 2007) and some can be transmitted occasionally to grapevine by the autochthonous Macropsinae species *Oncopsis alni* Schrank (Maixner *et al.*, 2000). So far *O. alni* was only able to transmit the Palatinate grapevine yellows

(PGY, 16SrV-C)-strains from alder to grapevine. But in Germany other Auchenorhyncha species are present on alder such as the autochthonous *Allygus mixtus* and *A. modestus* or the invasive mosaic leafhopper *Orientus ishidae* (Matsumura), for the latter the vectoring ability for FD was demonstrated (Lessio *et al.*, 2016). The aim of this study was to confirm the Palatinate region as free from FD and *S. titanus* and assess the risk posed by infected alders and potential alternative vectors associated with it to the grapevine.

Materials and Methods

Monitoring for immature *S. titanus* was carried out from 2016 to 2018 by checking leaves of suckers in randomly chosen plots of the Palatinate region in southwestern Germany close to Alsace. A geodata based tool (Bischoff, 2016) was used to identify appropriate sampling sites characterized by alder stands close to vineyards. Insects were collected from alders between June and August, while vineyards were examined for symptomatic grapevines in autumn. They were sampled and checked for phytoplasma of ribosomal group 16SrV presence by direct PCR using the group specific primers fAY/rEY (Ahrens *et al.*, 1994). Leafhoppers were collected

from the canopy of trees and grapevines either by sweep net or sticky traps from June until September. Total DNA was extracted from leaf midribs of field-grown grapevine or from branch phloem of alder samples as well as from individual insects as described by Maixner *et al.* (1995). Molecular typing of 16SrV-group phytoplasmas was performed by nested PCR on the *map* gene and subsequent sequencing of the PCR products (Arnaud *et al.*, 2007). Transmission trials were carried out in a climatic chamber at 25°C and a photoperiod of 16 h with nymphs or adults of *A. mixtus, A. modestus, O. alni* and *O. ishidae* collected from alder. Groups of 2-20 individuals were caged on potted faba bean (*Vicia faba*), alder seedlings and *in vitro* propagated grapevines for an inoculation access period of at least 7 days. Insects were then removed and stored at -20°C until DNA extraction. Test plants were sprayed with insecticide and kept in an insect proof greenhouse. Faba beans were tested approximately 6 weeks after the end of the experiments, while alder and grapevine plants were tested about three month after transmission trials and again after hibernation.

Results and Discussion

No *S. titanus* specimens were detected during the 3 years monitoring in the vineyards in south western Germany. From 330 grapevines showing yellows symptoms, 74 (22%) tested positive for 16SrV phytoplasmas while the majority (119 samples) was infected by 'Candidatus Phytoplasma solani' ("bois noir"). The typing of 16SrV-group-positive samples showed 9 different map types, all belonging to the PGY group. Moreover 98% of 488 alder samples was positive with the primers fAY/rEY. Map typing was only performed on a small portion of those samples as described by Arnaud *et al.* (2007). The analysis revealed that map type M38 (FD2 cluster) was found at different sites, but always in mixed infections with PGY map types. Among the most abundant leafhopper species collected on alder about 7% of *O. alni*, 38% of *A. mixtus*, 41% of *A. modestus* and 44% of *O. ishidae* carried 16SrV-group phytoplasmas. Map type M38 was predominant in the Deltocephalinae leafhoppers. It was found in 61% of infected *A. mixtus,* 87% of infected *A. modestus,* and 98% of infected *O. ishidae*. In contrast, all strains detected in *O. alni* were attributed to PGY genotypes. Transmissions trials were successful with *Allygus* spp. and *O. ishidae*, while *O. alni* did not infect any of the tested plants. *Allygus* spp. only transmitted M38 from alder to alder but not to other test plants, while *O. ishidae* was able to infect 72% of *A. glutinosa* and 24% of *Vicia faba* with map type M38. The analysis of transmission trials to grapevine is in progress. The results of the surveillance of *S. titanus* and the sole detection of PGY map genotypes in grapevines infected by group 16SrV phytoplasmas confirmed the absence of FD and its vector from the southwest Palatinate region. However, alder trees close to the vineyards appeared frequently infected with FD-related phytoplasmas such as map type M38 (Arnaud *et al.*, 2007). An important part of the Deltocephalinae *Allygus spp.* and *O. ishidae* collected on alder carried this genotype. Furthermore, transmission trials to faba bean and alder

revealed the principal competence of those species to transmit the M38 genotype. However, their ability to infect grapevine has still to be verified. This question is of great importance for Germany as *S. titanus* is spreading constantly northward. Accidental transmission of FD strains from alder to grapevine, *e.g.* by the still emerging Deltocephalinae *O. ishidae*, could result in *foci* for the further dissemination of FD by *S. titanus*. The ongoing assessment of the vectoring efficiency of *Allygus* spp. and *O. ishidae* to grapevine is therefore of importance. Since the data presented here were specifically collected for the Palatinate region, the work is currently extended to other winegrowing regions of Germany.

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Sporadic outbreaks of "flavescence dorée" in Austrian vineyards and the role of *Phlogottetix cyclops* as a potential vector

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Abstract

Several sporadic outbreaks of "flavescence dorée" in the Austrian vineyards have been recorded since 2009. A long-time monitoring of potential vectors and reservoir host plants of the "flavescence dorée" phytoplasma (FD) revealed that on some monitoring sites *Phlogottetix cyclops* is a common insect species on grapevines as well as on *Clematis vitalba* and *Ulmus laevis* growing near the vineyards. A high percentage of the captured specimens of *P. cyclops* harboured 16SrV-C phytoplasmas. Preliminary results showed that this Asian leafhopper could aquire FD from infected *C. vitalba*, but its ability to transmit the phytoplasma to grapevine still needs to be proven. The possible role of *P. cylops* for occasional transmission of FD from wild *Clematis* plants to grapevines is discussed.

Keywords: grapevine yellows phytoplasma, 16SrV, epidemiology, potential vector, reservoir host plants

Introduction

"Flavescence dorée" (FD) was recorded in the south-eastern part of an Austrian viticulture region in 2009 (Reisenzein and Steffek, 2011). This first outbreak was epidemic, but restricted to a very small area. While this grapevine yellows disease has repeatedly been detected thereafter in different regions and grapevine varieties, its incidence was almost always sporadic. These outbreaks displayed a few single grapevine plants infected by FD and no further epidemic disease spread was observed. The population size of the main vector *Scaphoideus titanus* was initially low in all the demarcated focus zones and a consequent mandatory application of plant protection measures kept it on a low level. All FD phytoplasma strains originating from grapevines were assigned to FD-C (16SrV-C) until 2017. FD-D (16SrV-D) strains were detected in adults of *S. titanus* and in grapevines (cultivar Isabella) for the first time in 2018, always in the south-eastern part of Austria. To understand the epidemiological relevance of these sporadic outbreaks, the presence of potential reservoir host plants and potential insect vectors were studied.

Materials and Methods

The survey for potential reservoir host plant and insect vectors was conducted at 44 locations in Styria, Burgenland and Lower Austria. The sampling was performed from 2012 to 2018. For the vector monitoring, adults were collected with yellow sticky traps (YST) (Rebel Giallo®) which were installed in the leaf wall of the grapevines and in the canopies of wild plants in their surroundings. YST were put up at the end of June until the beginning of October and changed bi-weekly. At several dates in July, August and September the grapevines and other host plant species were sampled by the beating tray method. Symptomatic and asymptomatic plants were collected in late summer and early autumn. A CTAB procedure was used for DNA extraction from plants and insects (Maixner *et al.*, 1995), these samples were tested for the presence of FD phytoplasmas, using a set of quantitative PCR assays (Angelini *et al.*, 2007; Hren *et al.*, 2007). For the strain delineation, RFLP and MLST analyses on 16S rRNA, *secY, map* and *tuf* genes were carried out (Martini *et al.*, 1999; EPPO, 2007, 2016; Arnaud *et al.*, 2007). To evaluate the potential vectoring of FD an acquisition and transmission trial with *Phlogottetix cylops* from infected *Clematis vitalba* plant to healthy grapevines (cultivar Chardonnay) was set up.

Results

The long-time monitoring revealed that different potential insect vectors occur in Austrian vineyards and in vegetation adjacent to the vineyards. *Orientus ishidae* is present, but in a low frequency and only a few individuals harboured FD. *Dictyophara europaea* was very rarely found and the few specimens were not infected. In 2013, *Phlogottetix cyclops*

infected with FD was caught for the first time on *C. vitalba* adjacent to an FD infected vineyard in Glanz, Styria. Since that time it was found that *P. cyclops* was commonly captured in the vineyards and adjacent vegetation beside the main FD vector *S. titanus*. In 2018, *P. cyclops* was caught at 8 monitoring sites. The highest numbers occurred in Burgenland: in Zagersdorf on neglected grapevines overgrown by *C. vitalba* (29 individuals), in Eisenberg on grapevine (12 individuals), in Klingenbach on *C. vitalba* (11 individuals), in Lutzmannsburg on *Ulmus laevis* (7 individuals), in Siegendorf on grapevine (1 individual); lower Austria: in Poysdorf on grapevine (6 individuals), in Prellenkirchen on grapevine (2 individuals) and in Steinebrunn on grapevine (1 individual). These results are in agreement with previously published data (Table 1) (Strauss and Reisenzein, 2018).

Table 1. Number of monitoring sites, total number of *P. cyclops* adults and FD positive *P. cyclops* in Austria in 2012-2017.

	Monitoring		Testing results
	No. sites	No. *P. cyclops*	FD positive/tested
Styria	3	19	10 (19)
Burgenland	7	134	43 (131)
Lower Austria	25	26	2 (6)
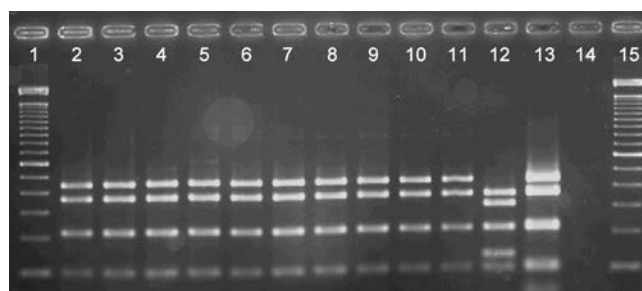
Figure 1. Agarose gel (3%) showing *Taq*I RFLP patterns of phytoplasma rDNA amplified with 16Sr758f/M23Sr primer pair. Lane 1, 100 bp ladder; lane 2 to 10, 16SrV from *P. cyclops*; lane 11, Austrian FD from *V. vinifera* cultivar Scheurebe (16SrV-C subgroup); lane 12, FD from France (16SrV-D subgroup); lane 13, 16SrV-C strain from *A. glutinosa*; lane 15, 100 bp ladder.

P. cylops were caught most frequently (123 individuals) on grapevine, followed by *C. vitalba* (68 individuals) and *U. laevis* (20 individuals). *P. cyclops* specimens were tested for the presence of FD: 46% of the adults captured on *C. vitalba* were FD positive, 29% on *V. vinifera* and 23% on *U. laevis*. The RFLP pattern of all FD strains from *P. cyclops* matched with the pattern of the 16SrV-C Austrian FD strain from *V. vinifera* (Figure 1). The sequence of the *tuf* gene shared 100% identity to sequences of two FD strains from Tuscany (16SrV-C) and with two strains from Serbia (16SrV-C) and were distinct from alder yellows phytoplasmas (Strauss and Reisenzein, 2018). A high genetic similarity of FD strains from *C. vitalba* plants, *P. cyclops* individuals and infected grapevines could be verified from samples of different monitoring sites. Preliminary results of the acquisition and

transmission trials with *P. cylops* indicated the acquisition of FD from infected *C. vitalba* plants by *P. cyclops*, but the transmission could not be proven and needs further investigation.

Discussion

The current epidemiological scenarios in different demarcated areas in Austria, allow the assumption that there could be a transmission pathway from FD infected reservoir host plants to grapevines by other vectors than *S. titanus*. The Asian leafhopper *P. cyclops* was frequently captured in vineyards and particular on the adjacent *C. vitalba* plants. These *C. vitalba* plants were most often infected by 16SrV-C phytoplasmas at the different monitoring sites. *P. cyclops* collected from these plants was very frequently infected with the 16SrV-C strain. The first results of the transmission trials indicate that the acquisition of FD from infected *C. vitalba* plants is possible, but the ability to transmit it to grapevines still has to be proven. Nevertheless, the data support the hypothesis that sporadic outbreaks of FD in the Austrian viticulture could be a consequence of an occasional transmission of FD from wild *C. vitalba* plants to single grapevines by a polyphagous insect species like *P. cyclops*.

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Status of grapevine "flavescence dorée" in Hungary

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Abstract

The Hungarian official survey on "flavescence dorée" (FD) and its vector *Scaphoideus titanus* started in 2004. The leafhopper vector was first reported in 2006, since then it has been widely spread in almost all Hungarian regions. The first detection of FD phytoplasma was in August of 2013 in the south-west part of the country, after that the Hungarian national official survey was getting more extensive. In 2018 in Zala county (the most infected area) more than half of the symptomatic samples were FD positive, in case of *Clematis* plants 50% were infected by FD phytoplasma all around the country. The map FD-2 (16SrV-D) and FD-3 (16SrV-C) strains both are present in Hungary, map FD-2 was found only on grapevines in the south-west area, while the map FD-3 strain was detected in *Clematis vitalba* plants and grapevine samples collected in other regions of the country. During the official survey the grapevine samples from the counties situated east from the Danube river were always negative.

Keywords: grapevine "flavescence dorée", Hungary, FD strains, survey

Introduction

Currently the "flavescence dorée" (FD) is the most devastating and dangerous grapevine disease in Europe. Due to its quarantine status and economic importance the national plant health authorities all around Europe conduct surveys and controls against its expansion. After a first report on the presence of 16SrV phytoplasmas in grapevine (Varga *et al.*, 2000), the Hungarian official survey of "flavescence dorée" (FD) and its vector *Scaphoideus titanus* started in 2004 in the region next to the Slovenian border. This was infact the area where the *S. titanus* was firstly detected (Dér *et al.*, 2007). Later, more and more locations where the disease was detected were found (Ember *et al.*, 2007; Kriston *et al.*, 2013), but still only in western part of Hungary, in counties located west to the Danube river.

Materials and Methods

Sampling

Symptomatic grapevine and *Clematis vitalba* leaves and leafhoppers were collected by plant health inspectors in September and October each year.

DNA extraction

In case of plant samples the CTAB method (Doyle and Doyle, 1987) or the KingFisher with BioNobile kit (Mehle *et al.*, 2013) were used; the DNA from insect samples was extracted by High Pure PCR Template Preparation Kit (Roche).

PCR

Real-time PCR reported in EPPO PM 7/79 (2) Appendix 4 (Hren *et al.*, 2007) and multiplex-nested-PCR (Claire *et al.*, 2003) were carried out, and for strain characterisation PCR products of the *map* gene were digested by two restriction enzymes (Arnaud *et al.*, 2007).

Results

After the first detection of the FD positive grapevine samples in the south-west part of the country, the Hungarian national official survey was getting more extensive as the increase of
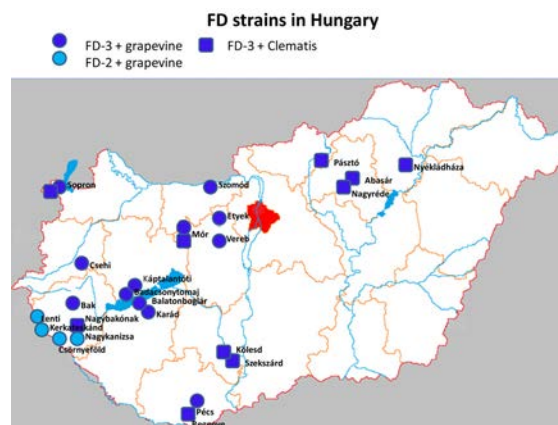
Figure 1. Distribution of FD strains in Hungary.

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Table1. Results of grapevine FD phytoplasma surveys 2007-2018.

Year	Number of samples	"Flavescence dorée" positive	"Bois noir" positive
2007	30	0	10
2008	28	0	18
2009	51	0	26
2010	49	0	17
2011	44	0	32
2012	69	0	18
2013	379	38	113
2014	771	89	283
2015	1,237	207	464
2016	1,188	272	320
2017	2,768	359	923
2018	2,278	475	845

the number of the laboratory tests shows (Table 1, Figure 1). Also *C. vitalba* and *Scaphoideus titanus* leafhopper were sampled and tested. Last year (2018) in Zala county (the most infected area) more than half of the symptomatic samples were FD positive (434/697) and 154 were infected by "bois noir" disease ("stolbur", 16SrXII-A phytoplasmas). In the case of *C. vitalba* plants 50% were infected by FD type phytoplasmas all around the country while all the tested leafhopper vectors were free from this phytoplasma. Both, FD-2 (16SrV-D) and FD-3 (16SrV-C) map strains are present in Hungary, FD-2 was found only on grapevines in the south-west area, FD-3 map strain was identified in strains from *C. vitalba* plants and grapevine samples collected in other parts of the county.

Discussion

After summarizing and analysing the data on surveys, there are different FD outbreak scenarios: i) Zala county where the fast spread of FD was detected and more than 30 vineyards are infected; ii) slow spread or sporadic infections in most of the grapevine growing areas of the western part of Hungary. Finally, in two counties only one or two positive samples were found at diverse locations. So far, during the official survey FD positive grapevine samples from the counties situated east from the Danube river were not found.

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Polymorphism of *rpl*F and *tuf*B genes: expanding the MLST scheme for "flavescence dorée" phytoplasma typing in Croatia

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Abstract

In Croatia, major agents associated with grapevine yellows diseases (GY) are the "bois noir" (BN; '*Candidatus* Phytoplasma solani') and the "flavescence dorée" (FD) phytoplasmas, with the latter emerging and spreading extensively in the last decade. The aim of this study was to perform genotyping of selected FD phytoplasma strains by analysis of the housekeeping genes *rplF* and *tufB* in order to expand the currently used multilocus sequence typing (MLST) scheme. The analysis included samples of grapevine, wild plants from the vicinity of vineyards and insect-vectors from continental Croatia and Istria county. Fragments of *rplF* and *tufB* genes were amplified by PCR and sequenced. The alignment and phylogenetic analysis revealed the presence of 5 and 6 genotypes, respectively. The gene *rplF* showed to be more conserved than the *tufB* gene, but both were proved as good candidates molecular markers in FD phytoplasma genotyping due to their variability. Implementation of data on *rplF* and *tufB* genotypes in the currently used MLST scheme for FD phytoplasma differentiation would give new possibilities for tracking and tracing this quarantine pathogen.

Keywords: grapevine yellows, FD phytoplasma, MLST, *rplF*, *tufB*, phylogenetic analysis.

Introduction

Yet unassigned to a taxon within the '*Candidatus* Phytoplasma' genus, the "flavescence dorée" (FD) phytoplasma encompassing ribosomal subgroups 16SrV-C and –D, is one of the principal agents associated with grapevine yellows (GY) diseases in the Euro-Mediterranean region, together with "bois noir" phytoplasma (BN; '*Ca.* P. solani'). *Scaphoideus titanus* Ball., a grapevine leafhopper of North American origin, is transmitting the disease; however, the role of alternative insect vectors in FD and FD-related phytoplasma epidemiology has been proposed and studied (Filippin *et al.*, 2009; Mehle *et al.*, 2011; Casati *et al.*, 2016). In Croatia, the first detection of FD phytoplasma infecting grapevine was reported in 2009 (Seruga Music *et al.*, 2011) in restricted continental areas of the country. Nevertheless, since 2014 severe FD outbreaks have been recorded in different parts of the country. Recently, an extensive epidemiological study of FD phytoplasmas in Croatia reported the presence of 3 genetic clusters and at least 15 comprehensive genotypes (Plavec *et al.*, 2018). Thus, the objective of this work was to assess the variability of *rplF* (encoding 50S ribosomal protein L6) and *tufB* (encoding the

elongation factor EF-Tu) gene fragments in selected FD strains already analysed by multilocus sequence typing of *secY*, *map* and *uvrB-degV* genes according to Arnaud *et al.* (2007). Furthermore, another objective was to evaluate the use of *rplF* and *tufB* gene fragments as additional markers in the existing MLST scheme for FD phytoplasma typing.

Materials and Methods

Fifty representative FD phytoplasma strains from grapevine, wild herbaceous and woody plants as well as *S. titanus* and *Phlogotettix cyclops* Mulsant & Rey collected in the period 2008-2016 from continental Croatia and Istria county were chosen for this study. All the strains have been previously typed by using MLST scheme described in Arnaud *et al.* (2007) (Plavec *et al.*, 2019). Fragments of *rplF* and *tufB* gene were successfully amplified from all samples, according to previously described protocols (Malembic-Maher *et al.*, 2011). All amplicons were sequenced (Macrogen Europe, Amsterdam, the Netherlands), assembled and edited by using Geneious software, and aligned with ClustalX 2.0. Subsequent phylogenetic analyses were performed by using different methods and parameters in MEGA 7 software and followed by assignment of an appropriate genotype number.

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Results

Alignment and phylogenetic analyses of the *rplF* fragments revealed the presence of 5 genotypes among the analysed strains. Two genotypes were found to be prevalent (comprising 70% of the analyzed samples) and closely related to already reported sequences. One of the genotypes encompassed only sequences from *Alnus glutinosa*, previously described as Palatinate grapevine yellows strain A (PGY-A). Phylogenetic analyses of *tufB* fragments showed even greater polymorphism, with the presence of 6 genotypes. The most represented were genotypes 1 and 6, found in 42.2% and 26.7% of the samples, respectively (Figure 1). Three of these genotypes had sequences identical to those found in GenBank.
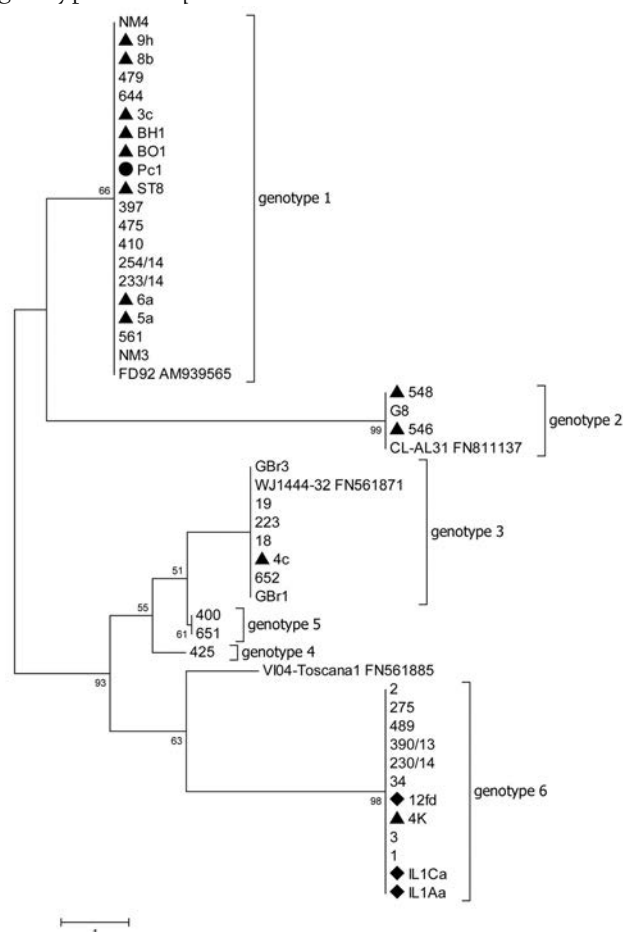
Figure 1. Phylogenetic tree constructed by neighbour-joining analysis (number of differences) of the *tufB* gene sequences from FD phytoplasma strains detected in grapevine, other host plants (black diamonds), *Scaphoideus titanus* (black triangle) and *Phlogotettix cyclops* (black circle) insect species, with all sample identifications according to Plavec *et al.* (2019). Sequences obtained from GenBank are indicated with accession numbers. Affiliation to the *tufB* genotype is shown to the right of the tree.

Discussion

After the first identification of FD phytoplasmas infecting grapevine in Croatia in 2009 and the appearance of severe outbreaks in 2014 and after, extensive efforts have been made in order to track the disease and clarify its epidemiology. The approach using MLST was successfully used; however, this study aimed at expanding the existing clustering scheme. Phylogenetic analyses of *rplF* sequences revealed the presence of 5 genotypes, with two of them corresponding to strains from the map FD2 cluster, one corresponding to map FD1 and map FD2, and one to PGY-A (Plavec *et al.*, 2019). Among the six genotypes found in *tufB* sequence analyses, three genotypes corresponded to strains from map FD2 cluster while one corresponded to map FD1, map FD3 and PGY-A. Altogether, both gene fragments were proved as good additional molecular markers in FD phytoplasma genotyping. Implementation of data on *rplF* and *tufB* genotypes in the currently used MLST scheme for FD phytoplasma differentiation would give even better possibilities for tracking and tracing this quarantine pathogen in the environment.

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Potential role of *Orientus ishidae* in the "flavescence dorée" epidemics in Ticino, Switzerland

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Abstract

The constant, even if low, presence of grapevine "flavescence dorée" (FD) in the vineyards of Ticino, Switzerland, challenged the stakeholders to investigate for other players involved in the FD dynamics. This study was aimed to explore the impact that the exotic leafhopper *Orientus ishidae* may have on FD epidemics in Ticino by monitoring its population density and level of infection with 16SrV group phytoplasmas. In 2018, 283 leafhoppers were collected from three FD infected areas, both from the vineyards and the surrounding landscape. Spatial density maps showed that the captures occurred mostly in the landscape, with a clear clustered distribution among the traps. Molecular analyses revealed a high infection percentage of *O. ishidae* samples in the three investigated plots, both inside and outside the vineyards.

Keywords: "flavescence dorée", leafhopper, *Orientus ishidae*, phytoplasma

Introduction

Grapevine "flavescence dorée" (FD), a serious disease associated to phytoplasmas belonging to the ribosomal subgroups 16SrV-C and D, was detected in Ticino, Switzerland, since 2005. Despite the mandatory strategies of control (uprooting infected grapevines and insecticide treatments against *Scaphoideus titanus*), 12% of the vineyards monitored during 10 years of surveys were yearly infected (Jermini *et al.*, 2014). The prevalence of FD positive grapevines was however low, ranging from 0.4% to 4.4% (Jermini *et al.*, 2017). Recent works reported the possible role of the exotic leafhopper *Orientus ishidae* in the transmission of FD phytoplasmas to grapevine (Lessio *et al.*, 2016). The insect has been reported in Swiss vineyard areas, where it was found infected by FD and FD-like phytoplasmas (Trivellone *et al.*, 2016; Casati *et al.*, 2017). The aim of this work was to monitor the population density and the level of infection of *O. ishidae* in some viticultural areas of Ticino (Switzerland), in order to gain information about the role of the landscape as potential source of FD and related insect vectors.

Materials and Methods

The survey was carried out in three vineyards of Southern Ticino, Switzerland, where FD has been present for at least 10 years, and abundant populations of *O. ishidae* have been detected. One vineyard is cultivated with cultivar Merlot (Montalbano1), two vineyards with Chardonnay (Montalbano2 and Origlio). For each site, 15 to 30 yellow sticky traps, depending on the plot size, were placed horizontally in the grapevine canopy to collect the visiting leafhoppers, while ten to 17 traps, depending on the landscape structure, were placed vertically at 1.2 m from the ground on a gradient going from the forest edge to the inner wood. The traps were replaced weekly from July to October 2018. All individuals of *O. ishidae* were collected from the traps and immediately frozen at -20°C. Insect samples were pooled by trap; each pool consisted of 1-20 individuals, depending on the captures. DNA extraction and quantitative PCR were carried out following protocols previously published (Filippin *et al.*, 2009), in order to identify 16SrV group phytoplasma presence. The capture data were elaborated with the ArcGIS software (ESRI) and spatial density maps were produced for each site.

Results

During the season 2018, 283 *O. ishidae* were collected in the three plots. The captures were highest in Origlio (157 specimens) and lowest in Montalbano1, with only 44 captures (Table 1).

Table 1. Collected *O. ishidae* in the three vineyards: number of individuals collected inside the vineyard and in the surroundings and percentage of infection by phytoplasmas of the 16SrV group (FD and related strains). Insect samples were analysed in pools of 1-20 individuals, depending on the captures on the traps.

Locality	No. of *O. ishidae* in the vineyard	No. of *O. ishidae* in the landscape	*O. ishidae* infected pools in the vineyard (%)	*O. ishidae* infected pools in the landscape (%)
Montalbano1	5	39	80	91
Montalbano2	5	78	60	46
Origlio	3	154	67	50

The flight peaks occurred approximately at the end of July, slightly anticipated in Origlio (week 29), with respect to Montalbano1 (week 30) and Montalbano2 (weeks 30 and 31). Only very few *O. ishidae* individuals were collected inside the vineyards (average number of specimens per trap: 0.33 in Montalbano1, 0.17 in Montalbano2, and 0.10 in Origlio, respectively). In the landscape this number increased by ten to 100 folds (3.54 in Montalbano1, 4.59 in Montalbano 2, and 15.40 in Origlio, respectively). In general, most of the sticky traps outside the vineyards collected at least one *O. ishidae* (ten out of 11 in Montalbano1, 12 out of 17 in Montalbano2 and eight out of ten in Origlio). However, the captures were not uniform among the traps, showing a clear clustered distribution (Figure 1).
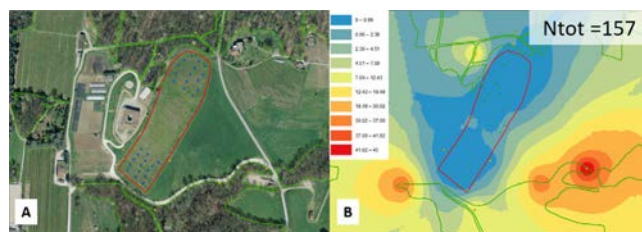
Figure 1. Survey design A) and spatial distribution B) of *O. ishidae* captures in Origlio. In A) red perimeter, vineyard; blue circles, sticky traps inside the vineyard; yellow circles, sticky traps in the landscape. In B) the leafhopper density is rendered with a colour code ranging from blue (0.00-0.96 captures) to red (41.82-45.00 captures).

The PCR analyses highlighted that the pooled samples of *O. ishidae* were infected by phytoplasmas of the 16SrV group in all the sites in considerable percentages, ranging from 50% in Montalbano2 to 53% Origlio, up to 87% in Montalbano1. This infection trend among sites was similar in both the vineyards and the surrounding landscape. In Montalbano1 the infected pools were uniformly distributed in all the traps, whereas in the other two vineyards only six out of 17 (Montalbano2) and five out of ten (Origlio) traps captured phytoplasma-bearing *O. ishidae*.

Discussion

This study was aimed to explore the impact that *O. ishidae* may have on FD epidemics in Ticino. Collected data confirmed that the leafhopper was present in all three investigated vineyards, even if in different amounts. Overall the 96% of the individuals was captured outside the vineyards, confirming the preference of *O. ishidae* for plant host species other than grapevine (Lessio *et al.*, 2016). Surprisingly, high rates of phytoplasma infection were observed both inside and outside the vineyards. The clustered distribution of infected samples outside the vineyards in two sites could make it easier to identify the plant sources of the phytoplasma.

Molecular characterization of phytoplasmas present in the leafhoppers is ongoing. It is indeed fundamental to understand how many strains, which could be transmitted by *O. ishidae* to grapevine, are compatible with the grapevine-to-grapevine transmission by *S. titanus*, and could therefore act as FD source for the epidemics in vineyards.

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Impact of *Orientus ishidae* on "flavescence dorée" emergence in the vineyards of riparian ecosystems

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Abstract

In this study the polyphagous status of *Orientus ishidae* was confirmed by showing that the leafhopper is able to lay eggs, hatch and develop on different deciduous trees of a riparian ecosystem, including the grapevines of neighbouring vineyard plots. It was demonstrated that, in such habitat, the main source plants for the phytoplasma acquisition are the alder trees. Leafhoppers collected on alders were mainly infected by FD phytoplasma genotypes M50 (map-FD1) and M38 (map-FD2) and were able to efficiently transmit it back to alder plants. However the same individuals could not transmit to grapevine after 42 forced transmission trials. These results and the detection of only one M38 infected grapevine stock over a 5 year period of extensive monitoring in neighbouring vineyards, suggest a low transfer frequency of phytoplasmas from alder to grapevine by *O. ishidae*.

Keywords: 16SrV-C and –D phytoplasmas, alternative insect vector, transmission frequency

Introduction

The Asiatic leafhopper *Orientus ishidae* was first detected in Europe in 1998 in Switzerland and Northern Italy and was observed the following years in diverse countries of western and southern Europe. It is described as monovoltine, with overwintering eggs and polyphagous on various deciduous host trees. Recent studies showed that this Deltocephalinae leafhopper could be infected by "flavescence dorée" (FD)-related phytoplasmas of subgroups 16SrV-C and –D, moreover Trivellone *et al.* (2016) and Lessio *et al.* (2016) demonstrated that it was able to experimentally acquire the FD phytoplasma from broad bean and transmit it to grapevine. But this transmission capability must be also assessed in the context of natural source plant(s) for acquisition, in the vineyards and their semi-natural environment in different European regions and ecosystems.

Materials and Methods

Two experimental sites composed of vineyard plots in the vicinity of a riparian ecosystem were selected: Pujols sur Ciron nearby Bordeaux (site P, SW France) and Davayé in Burgundy (site D, NE France). The small isolated plot of Merlot

in P was not treated with insecticides whereas the plots in D, mainly planted with Chardonnay, were submitted to FD mandatory treatments since 2012. For grapevine, extensive survey of symptoms and phytoplasma detection (on pools of 5 plants) were conducted by the plant protection services or INRA between 2015 and 2018 in P, and since 2012 in D. In both sites, the main tree insect species were inventoried. In October 2018, canes were sampled on each tree and phloem was stored for phytoplasma detection. Egg hatching trials were performed as described in Eveillard *et al.* (2016) using 1 to 10 kg of canes collected in January 2017 and 2018 on each tree species. In 2015 and 2016, yellow sticky traps (6 in P and 4 in D) were placed at the edge of the grapevine plots and regularly checked from June to mid-October for the presence of *O. ishidae*. Individuals were unglued and stored for further phytoplasma detection. In July 2015 to 2018, leafhoppers were collected by beating on alder trees and encaged until death by groups of 10 to 40 on *Alnus glutinosa* seedlings or on *Vitis vinifera* (Cabernet Sauvignon and Chardonnay cultivars) obtained from *in vitro* micropropagation. Insects from transmission assays were stored for phytoplasma detection. Plants were incubated in a confined greenhouse for up to 12 weeks at 25°C, regularly observed for symptoms and tested

for phytoplasma presence. For each plant, the same protocol was repeated after overwintering. Total DNA from plant and insect samples was extracted with the CTAB method extended by RNAse treatment and with the TNES protocol (Pecoud *et al.*, 2013) respectively. Phytoplasmas of the 16SrV group were detected by nested-PCR on the *map* gene and genotyping was performed as described in Arnaud *et al.* (2007).

Results

Inventoried species were *Alnus glutinosa, Fraxinus sp., Salix sp., Quercus robur, Coryllus avellana, Tillia mentosa, Crataegus sp., Carpinus betulus, Prunus spinosa, Ulmus minor, Robinia sp.* and *Vitis vinifera* (in the plot only). Their abundance is indicated in Table 1. FD-related phytoplasmas were detected in 92% of alder and in a few hazelnut trees. '*Candidatus* Phytoplasma ulmi' was detected in one hornbeam trees. No detection occurred in the other species. No FD-infected grapevine was detected in P but 3 single stocks were infected by genotypes M38, M54 (map-FD2) and M39 (Palatinate grapevine yellows) in D. Hatchings of *O. ishidae* were obtained from most of the species, with higher populations in P, in the trees bordering the vineyard and in the vineyard itself. Larvae were also present on all species except oak, with higher populations on alder and lime trees. In traps of B, 50/881 (6%) *O. ishidae* were infected while 2/15 were infected in D. They hosted M38, M130 (map-FD2) and M50 (map-FD1) in P and M38 in D. On alders, 856 and 53 *O. ishidae* individuals were collected in P and D - 54% and 49% infected - respectively. Genotypes were M50 and M38 in P and M38, M50, and M38 variants in D. The insects were able to transmit M50 and M38 genotypes to 7 out of 10 young alders but the 42 attempts on grapevine did not succeed.

Table 1. *O. ishidae* presence and FD-related phytoplasma infection on different tree species of riparian ecosystem.

Tree species and abundance[1]			Mean larvae per beating[2]		Hatchings per kg		16SrV detection infected (tested)	
Site	P	D	P	D	P	D	P	D
V. vinifera	0.3	247	3	Nt	7	Nt	0(3)	3(136)
A. glutinosa	40	23	>5	2	2	4	12(12)	12(14)
Fraxinus sp.	25	40	1	0	1	1	0(16)	0(20)
Q. robur	29	4	0	Nt	Nt	Nt	0(19)	0(4)
C. avellana	19	1	1	Nt	2	Nt	2(19)	Nt
Salix sp.	5	11	1	0	0	0	0(5)	0(8)
Crataegus sp.	7	0	>5	Nt	2	Nt	0(9)	Nt
C. betulus	5	0	1	Nt	5	Nt	1(5)	Nt
P. spinosa	3	0	2	Nt	0	Nt	0(3)	Nt
T. mentosa	2	2	>5	0	2	Nt	0(2)	0(2)
U. minor	1	0	1	Nt	7	Nt	0(1)	Nt
Robinia sp.	4	0	1	Nt	17	Nt	0(4)	Nt

[1]For *V. vinifera* only: surface of the grapevine plots in ha.
[2]Mean when tested more than one year. Nt: not tested.

Discussion

In both sites monitored, it was demonstrated that the main source plant for FD phytoplasma acquisition by *O. ishidae* is FD phytoplasma infected alder trees, shown to be the original European reservoir for FD-related phytoplasma diversity (Malembic-Maher *et al.*, 2019). *O. ishidae* is able to transmit FD back to alder with a high frequency but, while experimentally advocated (Lessio *et al.*, 2016), the transfer to grapevine appears to be poorly frequent in natural conditions. Nevertheless, the polyphagous ability of *O. ishidae* could expand the FD ecological niche in the environment of vineyards and other plant species could become secondary hosts and sources of the phytoplasma. Casati *et al.* (2017) showed that hazelnut and willow trees were highly infected by FD near vineyards, with *O. ishidae* populations present in southern Switzerland. Such situations that are highly dependent on the abundance of host plants and autochtonous or introduced Deltocephalinae vectors in the agro-ecosystem, could increase the frequency of phytoplasma transfer to grapevine. Risk assessment of FD emergence should be conducted in different European regions and vineyard ecosystems.

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Integrated management of the vineyard ecosystem for the control of the "flavescence dorée" disease

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Abstract

In Portugal a specific project aims to reduce the risk of "flavescence dorée" phytoplasma (FD) propagation in vineyards in order to increase the capacity to mitigate the severe FD damages. The project is applying more efficient monitoring tools, making use of eco-friendly technologies (smart traps) for automatic detection and identification of the insect vector *Scaphoideus titanus*. It is also evaluating of the role that other host plants and other insects vectors living in the surrounding areas of grapevine plots have in the spread and/or prevalence of FD disease in the Minho region as infection *foci* or insect reservoirs. The last action is the identification and multiplication of the best regional 'rootstock/variety' combinations less FD-susceptible as disease barriers. It is increasingly urgent to have an integrated ecosystem management for pest control rather than the control in individual plots.

Keywords: "flavescence dorée", alternative hosts, monitoring, eco-friendly tools, integrated pest management

Introduction

The wine sector plays an essential role in the national and regional economy of northern Portugal. In the last decades there has been a significant improvement in grape quality and productivity with positive effects in wine quality. The success that the "Vinhos Verdes" have achieved in the international markets, also determines the increasing importance of this activity in the volume of national exports. The "flavescence dorée" phytoplasma (FD) is a quarantine phloem-limited pathogen and a major threat to viticulture for several European regions included in the Directive 2000/29 /EC. The FD was detected in Portugal in 2007 (de Sousa *et al.*, 2003, 2010) in vineyards located in Lima and Cávado rivers valleys, right in the heart of the "Vinhos Verdes" cultivation area, leading the National Phytosanitary Authority (DGAV) to take measures to control the disease. Therefore the destruction of infected plants, mandatory treatments against the insect vector, *Scaphoideus titanus* Ball and the application of hot water treatment to the vegetative propagation material coming from areas affected by FD were implemented. Until now, there is no suitable curative treatment against FD. Facing the disease presence, a significant number of vineyards have to be cleaned up and

replanted, taking some years until becoming productive again. These measures, while necessary, significantly decrease the farmers' incomes. Despite the application of official control measures by grapevine producers for several years, new plants or infected plots are frequently found. In order to increase the effectiveness of the control measures currently in place, it is necessary to consider that other factors than those considered so far, may increase the risk of incidence and prevalence of the disease in the "Vinhos Verdes" region. Additionally, there are other important aspects to take into account in the control of pests: the increased number of plants and their products in the international trade, which endorse the entry of alien pest in the countries; climate changes that modify the vegetative cycle of plants as well the biological cycle of pests and diseases, causing imbalances that make more difficult to control the phytosanitary problems; and the drastic reduction in the number of active substances available in the EU that decreases the farmer's options for insecticide treatments.

Materials and Methods

S. titanus detection is performed through a georeferenced image capture system with wireless transmission capability,

which allows the periodic sending of images and rapid issuance of insect presence alerts. Remote detection allows a more timely control intervention and increase the effectiveness of pesticides. Two smart traps are placed on one farm and two photos per day are sent to the platform for analysis. The survey of plants and insects take place in six vineyard borders. The sampling area (green colour) was set to 25 m to each side of the line between the traps (pink colour) as represented to Cerqueiral farm in Figure 1.

Figure 1. Example of the border area in Cerqueiral farm (green line). Six traps are marked on the map: QC1-6 (pink line).

Six yellow traps were placed in each of the six vineyards (five in the border and usually one of them inside the plot) and replaced every week from May to October. Sampling of plants is performed twice a year (spring and late summer). A circle of about 10 meters in diameter is defined around each trap and samples of the above mentioned potential host plant species are collected for laboratory analysis. The alternative host plants growing in the diseased areas to be surveyed were *Alnus glutinosa*, *Ailanthus altissima* (invasive), *Climatis vitalba*, wild plants of *Vitis* European and American, *Salix* sp. and *Corylus avellana*. The potential insect vectors are *Orientus ishidae*, *Oncopsis alni*, *Dictyophara europaea*, *Metalcalfa pruinosa*, *Phlogotettix cyclops* and *Cacopsylla alni*. Petioles, flowers and phloem scrapings were used for total nucleic acid extraction by CTAB method (Doyle and Doyle, 1990). Phytoplasma detection was performed by nested-PCR with P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by R16F2nR2 (Gundersen and Lee, 1996), sequencing and RFLP analyses (*Taq*I and *Bfa*I enzymes). Other DNA markers will also be used for the molecular characterization of the phytoplasmas detected in the samples. To explore the susceptibility to FD disease, six important regional cultivars and three rootstocks were used. One tolerant (Syrah) and a susceptible cultivar (Cabernet Sauvignon) were included to increase the strength of the results. The inoculation by chip budding will be done. The trial take place in a greenhouse confined with anti-aphid net located at the INIAV facilities.

Results

The results obtained during the 2018 are still partial but are listed below.

S. titanus and *Dictyophara* sp. were present in the borders of five vineyards. Other specimens of putative insect vectors are under evaluation. A great abundance of the cicadellids *Macropsis* sp. was also observed in all the grapevine plots and borders. The alder yellows phytoplasma was detected in leaves and flowers of alder (*A. glutinosa*) in two vineyards (molecular characterization is ongoing). The list of weeds and others potential hosts for phytoplasmas as well the one for insect vectors on the six vineyards are under compilation. Complementary images obtained by drones for characterization of the borders will be used.

Discussion

Despite the treatments in grapevine plots, it was confirmed the presence of *S. titanus* specimens in the borders of the vineyards. Regardless the insignificant presence of this vector inside the plots, these data indicate that the elimination of treatments may re-emerge the disease dangerously in those vineyards. It is considered that it is almost impossible to eradicate the disease from the Minho region due to the predominance of small and continuous plots and because many people have for themselves a few grapevine plants. These factors make difficult to eliminate all infection *foci*. These results reinforce the urgency of complying with the compulsory clean-up of abandoned vineyards as well as making treatments against *S. titanus* in the surroundings of the plots.

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Detection

Genomic similarities among 16SrI phytoplasmas and implications on species boundaries

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Abstract

Phytoplasmas are wall less phytopathgenic bacteria that have great impacts on agriculture worldwide. The current taxonomy for these bacteria is mostly based on their 16S rRNA gene sequences, which may lack sufficient resolution or accuracy. With the increased availability of genome sequences for the 16SrI group within this 'Candidatus' genus, this work quantified their genetic divergence and investigated possible species boundaries. The results indicated that strains belonging to different 16SrI subgroups were sufficiently diverged to be possibly considered as distinct 'Candidatus Phytoplasma' species. However, intragenomic variations could cause conflicting ribosomal subgroup assignments. Thus, genome-based identification of marker genes for multilocus sequence analysis (MLSA) is critical for improving phytoplasma taxonomy and diagnostics.

Keywords: comparative genomics, 16S rRNA, average nucleotide diversity (ANI), taxonomy

Introduction

The advancement in genomics have provided powerful tools for the studies of uncultivated bacteria. Research on phytoplasmas, particularly those belonging to the 16SrI group, have benefited tremendously. Taking advantage of the genome sequence availability for this phytoplasma group, this study compared different quantitative measurements of genomic divergence within this ribosomal group, as well as inferred the putative 'Candidatus species' boundaries.

Materials and Methods

The genome sequences were obtained from GenBank. The 16Sr subgroup assignments were determined using the *i*PhyClassifier program (Zhao et al., 2009). The procedures for homologous gene identification and phylogenetic inference were based on those described in previous studies (Chung et al., 2013; Seruga Music et al., 2019). The proportion of genomic segments mapped (GSM), as well as the average nucleotide identity (ANI), were calculated using fastANI (Jain et al., 2018).

Results

The analysis identified 320 single-copy genes that are shared by all nine genomes analyzed. A total of 25,965 sequence polymorphisms were non-uniformly distributed among these genes, which provided a list of promising candidates for developing molecular markers to discriminate these strains and different lineages within the 16SrI group. The concatenated nucleotide alignment of these genes produced a highly resolved molecular phylogeny with strong support (Figure 1A). The phylogeny is mostly consistent with the 16Sr subgroup assignments, as well as the two measurements of genome similarities (Figure 1B). However, several discrepancies were discovered. First, the wheat blue dwarf (WBD) phytoplasma was described as a 16SrI-B strain (Chen et al. 2014) while the two copies of its 16S rRNA genes were assigned to 16SrI-R and 16SrI-S, respectively. Furthermore, the phylogeny and genome similarities all indicated that this strain is highly divergent from the 16SrI-B strains. The second case involves TW1; while one copy was assigned to 16SrI-B, the other was classified as a possible new subgroup that is most similar to 16SrI-A. Although the phylogeny and ANI results, both support that TW1 is more closely related to other 16SrI-B strains, TW1 shared a much higher proportion of its genome with those 16SrI-A strains.

For the two measurements of genomic similarities, ANI exhibited a clear boundary that distinguishes within- and between-subgroup comparisons (Figure 2). In contrast, the distribution of GSM values did not show such boundary. Although GSM and ANI are only weakly correlated ($r = 0.29$) when all data points were combined, the correlations are high when the analysis was done separately for within- ($r = 0.70$) and between-subgroup ($r = 0.88$) comparisons.

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Figure 1. Relationships among 16SrI phytoplasmas. Subgroup assignments based on 16S rRNA genes are shown. (A) Maximum likelihood phylogeny based on 320 shared single-copy genes. All nodes received >99% bootstrap support. (B) Pairwise similarity scores.
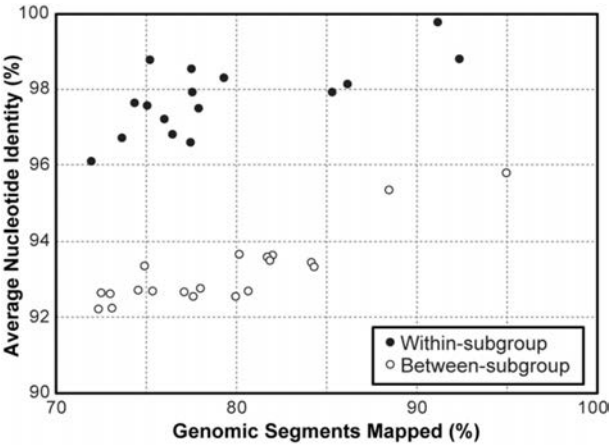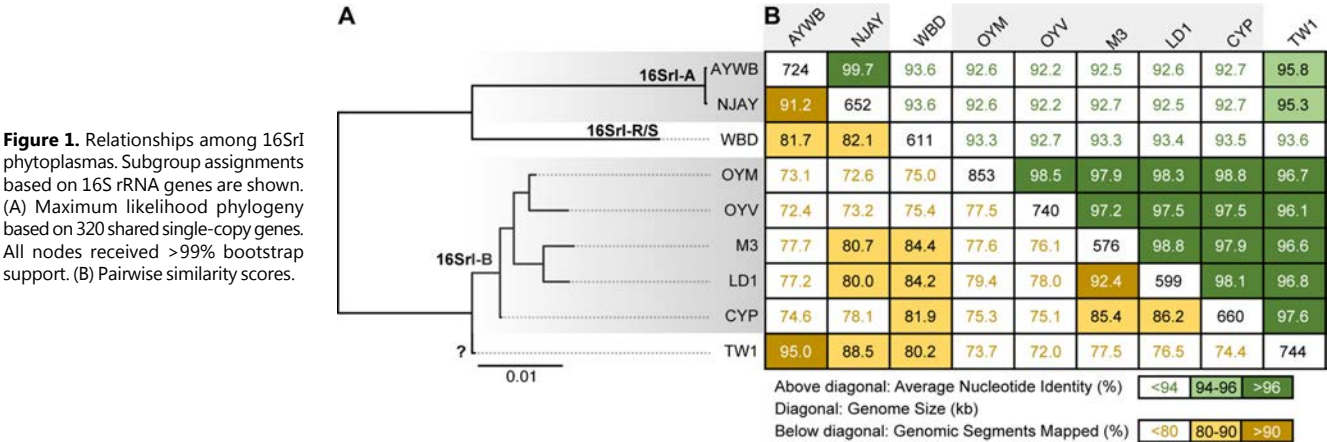
Figure 2. Scatter plot of similarity scores from pairwise phytoplasma genome comparisons.

Discussion

Previous works on bacterial taxonomy suggested an ANI value of 95% as the species boundary (Konstantinidis *et al.*, 2017; Jain *et al.*, 2018). The results indicated that this cutoff also worked well for discriminating 16SrI phytoplasmas belonging to different lineages. However, in addition to ANI, GSM provided another important metric for measuring genetic similarity. Notably, GSM values are expected to be more highly correlated with similarities in gene content, thus has a stronger tie to functions and phenotypes.

The 16Sr subgroup assignments are problematic for some strains. In addition to those two 16SrI strains, others such as '*Ca.* P. australiense' PAa in the 16SrXII-B subgroup also exhibited sufficient intragenomic variation for its two 16S rRNA genes to be assigned to different subgroups (Tran-Nguyen *et al.*, 2008). These findings suggested that the development of additional markers for MLSA is important for accurate classification of phytoplasmas. Based on those genes with high discriminating power identified in this work, PCR primers for this purpose are under development.

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Detection

Development of an anti-Imp serological assay for the detection of "flavescence dorée" phytoplasmas in grapevine, insect vectors and host plants

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Abstract

At present serological assays for the detection of "flavescence dorée" (FD), a quarantine disease of grapevine, are not available. The aim of this study was to produce polyclonal antibodies targeting a surface abundant protein of FD and related phytoplasmas, Imp (immunodominant membrane protein), and to verify its use for the detection of FD phytoplasmas in grapevine, other host plant species, and insect vectors. Two antisera were obtained using recombinant Imp proteins from two FD strains. Preliminary results confirmed the specificity and good ELISA performance in the detection of the target in grapevine, clematis and insect vectors tissues.

Keywords: ELISA, grapevine yellows, phytoplasma, serology, leafhopper

Introduction

Grapevine "flavescence dorée" (FD) is a quarantine disease associated to phytoplasmas and transmitted from grapevine to grapevine by the leafhopper *Scaphoideus titanus* Ball. Control of the spreading of the disease and its vector is mandatory in Europe, and several strategies are suggested, including its early detection in infected plants and insect by means of laboratory analyses. At present the phytoplasma detection is PCR-based since this is a worldwide recognized sensitive, reliable and specific assay, and also due to the absence of alternative reliable diagnostic methods (EPPO, 2016). Serological method development is limited by the low titer of the phytoplasmas in grapevine. Techniques to purify large phytoplasma amounts from herbaceous plants have been exploited to raise polyclonal and monoclonal antibodies (Boudon Padieu *et al.*, 1989, Seddas *et al.*, 1996), however the diagnostic systems were not enough robust for large scale applications. An alternative strategy to obtain a large amount of highly specific antigenic preparations, aimed to raise antisera of improved affinity and sensitivity to the target, and based on recombinant proteins (Liu *et al.*, 2017) was applied. A FD phytoplasma assay based on serology

to perform routine screenings would be particularly useful to ensure a timely detection, or when molecular facilities are not available. Moreover the ELISA test, based on antibodies, is cheaper and easier-to-use than PCR, though less sensitive. The aim of this study was to raise polyclonal antibodies targeting a surface abundant protein of FD and related phytoplasmas, Imp (immunodominant membrane protein), to be used for the detection of this phytoplasma in grapevine, other host plants, and insect vectors.

Materials and Methods

Reference material

FD phytoplasmas used were FD-D and FD-C strains originally collected from Piedmont vineyards. They were obtained from infective *S. titanus* collected alive in the field and allowed to feed on *Vicia faba* in the laboratory. These FD strains were routinely maintained with sequential transmissions from broad beans to broad beans by the experimental vector *Euscelidius variegatus* and by sequential grafting in *Catharanthus roseus*. The *dna*D-*imp*-*pyr*G genomic fragment of FD-D and FD-C (Piedmont) strains were amplified from FD infected grapevine leaves

by nested PCR using primer pair DnaDFDf1/PyrGFDr1 and DnaDFDf2/PyrGFDr2 (patented). A total of 162 amino acids were predicted for full-length FD Imp protein, with residues 44-162 exposed outside the membrane. Two His-tagged partial Imp proteins were synthesized in a *Escherichia coli* system. Each batch was used to immunize two rabbits. The collected antisera, JBA/17 (anti FD-D) and ABA/18 (anti FD-C), were purified by affinity chromatography to prepare IgGs for Western blot (WB) analysis and to prepare two sets of ELISA DAS reagents.

Western blot

Proteins extracted from healthy and FD-C/ FD-D infected periwinkles, broad beans, and *E. variegatus* were used for WB analysis, in order to test the specificity and the functionality of the two antisera.

PCR tests

All samples tested by ELISA were also tested by PCR, using a quantitative protocol (Angelini *et al.*, 2007), and RFLP analysis of the PCR amplified of the 16-23S ribosomal region (Angelini *et al.*, 2001).

Field material

Samples from grapevine (58), *S. titanus* (204), and clematis plants (2) were collected in summer 2018 from known healthy and infected sites, qPCR checked for phytoplasma presence and used for screening the antibody performance.

Results

Expression of the two partial proteins was successful. Titrations of both the antisera were positive in ELISA and in WB against the antigenic recombinant proteins, and confirmed the IgG specificity of both JBA/17 and ABA/18 in infected *C. roseus*, *V. faba*, and *E. variegatus* reference samples. ELISA tests were then carried out for all collected samples. Preliminary data showed an efficient discrimination of the FD-infected and not-infected samples by the two ELISA sets. The JBA/17 showed the best performance, as it was able to detect almost all FD-D-infected grapevines and insects. ABA/18 was able to detect FD-C in infected clematis and insect samples, and in a few grapevine samples.

Discussion

The strategy of producing antisera against the Imp recombinant protein of phytoplasmas has not been used here for the first time. Indeed, Kakizawa *et al.* (2009) produced an antiserum against Imp recombinant protein of OY-W phytoplasma strain; however, this finding was never developed in a kit for routine ELISA detection. In this study, the production of polyclonal antisera against the

recombinant protein targeting FD phytoplasmas and their use in ELISA assays is for the first time reported. Molecular detection of FD phytoplasmas in ELISA-tested field samples is ongoing, with the aim to obtain a full comparison between ELISA and PCR results for the two antisera. Optimization of the protocols for grapevine, host plant species and insect vector sample collection, storage and preparation for serological testing are under study. Once established, the ELISA test for FD phytoplasma will be a valuable tool for routine analyses. Moreover, the developed antisera can be useful for *in situ* studies of FD phytoplasma Imp protein (Trivellone *et al.*, 2019).

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Detection

Development of an endogenous universal internal control for qPCR applications and the importance of different evaluation criteria

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Abstract

Real-time quantitative PCR is the most common technique for detection of phytoplasma, small bacteria associated with several economically important plant diseases worldwide. Infection cycles of phytoplasma-associated diseases involve multitrophic interactions between the bacterium and different hosts, *i.e.* insect vectors and plants, thus diagnostic analyses must be done in different DNA matrices. For routine diagnostics of plant or insect samples by qPCR it is crucial to have an endogenous internal control for the host DNA. While several host specific primer and probe combinations are available as endogenous control for phytoplasma detection, a universal internal control for different eukaryotic host organisms is lacking. Thus a universal internal control that can be used in a broad range of phytoplasma host species was developed and the use of defined strict criteria for the evaluation of qPCR results are elucidated.

Keywords: pathogen detection, real-time PCR, endogenous internal control, evaluation criteria

Introduction

Internal endogenous PCR controls that prove DNA or RNA integrity and the absence of inhibitors are indispensable tools for PCR-based pathogen detection and gene expression studies (Bustin *et al.*, 2009). This is especially valid for diseases associated with phytoplasmas that infect not only plants, but also insects where it would be of advantage to have a universal endogenous control for the different DNA matrices. Phytoplasmas are associated with severe diseases in a wide range of crop plant species (Bertaccini, 2014; Maejima *et al.*, 2014). Nowadays, most tools for phytoplasma detection are based on quantitative real-time PCR (qPCR) techniques. Several qPCR protocols are available for the detection of different phytoplasmas using different host specific primers and probes as an endogenous internal control (Pelletier *et al.*, 2009; Baric, 2012; Monti *et al.*, 2013; Ikten *et al.*, 2016; Linck *et al.*, 2017; Ito and Suzaki, 2017), but no universal endogenous internal control for a broad range of different host species DNA is available. Even though several exogenous internal controls are available on the market, these are not suitable to verify DNA or RNA integrity. Only endogenous internal controls allow to prove the presence and integrity of the nucleic acid and the absence of PCR inhibitors in the respective samples. Endogenous internal controls are not only important as a quality control of the PCR reaction, but they are also an indispensable tool for pathogen quantification (Mittelberger *et al.*, 2017). No endogenous primer and probe combination is freely available, that can be used universally in different eukaryotic host organisms. The aim of this work was the development of a universal internal control, that can be used for the detection of phytoplasmas in diverse host species. Beside the importance of an internal control in qPCR runs, only a diligent evaluation of the generated data guarantees accurate results (D'Haene and Hellemans, 2010). However, critical data evaluation is a weak point in many qPCR studies. Therefore relevant criteria for qPCR evaluation in the context of this study are discussed and emphasized.

Materials and Methods

Based on a highly conserved stretch of the 28S rDNA gene from different eukaryotic species a primer and probe set was designed and tested in a qPCR assay with different eukaryotic DNA samples. The PCR performance was tested with TaqMan and SYBR-Green chemistry and strict evaluation criteria for qPCR results were defined.

For a diligent evaluation and/or quantification of the results all target genes (phytoplasma gene and internal control) were subcloned into the plasmid vector pJET1.2, extracted and measured with a nanodrop 3300 system (ThermoFisher). Plasmid copy content was calculated based on the molecular weight of the plasmid, applying the following formula: plasmid copy content = plasmid concentration (ng/µl)/ molecular weight of the plasmid (ng/copy). With this plasmid solution, multipoint 10 fold standard dilution series for quantification and determination of the detection limit of target genes were prepared and analysed together with each qPCR reaction.

Results

The newly designed primer and probe combination is suitable for the detection and quantification of DNA from a broad range of different eukaryotic species, while it does not amplify bacterial DNA. The multipoint standard dilution series were necessary to determine the limit of detection and the performance of each qPCR reaction. As measures for the qPCR performance, the amplification efficiency and the coefficient of determination (R^2) of the standard curves were used. Only runs with $R^2 > 0.99$ and a PCR efficiency between 95% and 105% were defined as reliable for a relative quantification of the results.

Discussion

A universal endogenous internal control allows to verify sample integrity and the absence of PCR inhibitors. Moreover, 28S rDNA is nuclear encoded and can thus be used for relative quantification of the target gene. This can be helpful when a comparison of pathogen load in different samples of the same host species is requested (Mittelberger *et al.*, 2017). Since the life cycle of phytoplasma comprises different plant and insect hosts it will be helpful to have one common internal control that can be used in a broad range of host species. This is especially useful if many different plant and insect species are screened to analyse their potential as reservoir or transmitting hosts.

Independently of the template and the chemistry used, a careful evaluation of qPCR data is crucial. For example standard curves of the target gene itself and the internal control are important to evaluate the performance of a qPCR reaction. According to Pfaffl (2012) a good standard curve should be linear, have a low variation in the single points, cover the range of quantified DNA amount and display a high correlation coefficient and a slope near -3.33, which corresponds to doubling of DNA in each cycle, and can be expressed as 100% amplification or PCR efficiency. However, involving adequate standard curves and applying strict and comprehensible evaluation criteria are only some of the aspects that need careful consideration when performing qPCRs.

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Detection

Near-infrared spectroscopy analysis – a useful tool to detect apple proliferation diseased trees?

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Abstract

Apple proliferation is associated with the presence of the bacterium 'Candidatus Phytoplasma mali'. An infection with this pathogen leads to changes of the physiological state of the apple tree. In this study, it was applied near-infrared spectroscopy to measure these changes. In preliminary analysis based on a quadratic discriminant analysis the first indications that it might be possible to discriminate diseased from uninfected sample material were achieved.

Introduction

The cell wall-less and phloem restricted bacteria 'Candidatus Phytoplasma mali' is the agent associated with apple proliferation (AP), one of the most important diseases in commercial apple cultivation. An infection with 'Ca. P. mali' induces not only visible symptoms, but also influences the plants hormone levels (Luge et al., 2014; Janik et al., 2016; Dermastia, 2019) and its nutrient household (Lepka et al., 1999; Bertamini et al., 2002). By applying spectroscopic techniques, physiological changes in the plant can be detected. Different spectroscopic techniques have been already applied to study a wide range of plant diseases (Belasque et al., 2008; Graeff et al., 2006; Choi et al., 2004). In this study, near-infrared (NIR) spectroscopy has been applied to discriminate AP diseased from healthy samples.

Materials and Methods

In each of three orchards in South Tyrol (Alto Adige, Northern Italy) 20 healthy and 20 'Ca. P. mali' infected apple trees were chosen. From each healthy tree, 40 leaves were collected. From each infected tree 40 symptomatic leaves (with dentate stipules) as well as 40 asymptomatic leaves were sampled. To improve comparability regarding age and growth stage, the first five leaves from a sprout were taken. Sampling was performed from May till October.

Samples were washed, dried and grounded. The ground material was then scanned with a NIR spectrometer and the wavelength reflectance spectra ranged from 1,100 nm to 2,498 nm with a resolution of 2 nm. The spectra were transformed (reflectance to absorbance, 2nd derivative, Savitzky-Golay-filter) and a principal component analysis (PCA) was performed to their reduce dimensionality. The PCA was full cross validated and based on the first three or five principal components (PCs) quadratic discriminant analysis (QDA) models were calculated (PC QDA). Different models were performed addressing the question if the data allows a discrimination between "infected versus non-infected" and "symptomatic versus asymptomatic". This analysis was performed considering i) the different sample time points (first three PCs were used) and ii) all the samples of one year (first five PCs were used).

Results

The results from the first, preliminary analysis indicate that there are differences between the spectra of infected and non-infected leaves. These observed differences seem to become more apparent during the course of the vegetation period. Differences between symptomatic and asymptomatic leaves on the infected tree were only detectable in June. Analysing the full data set comprising all timepoints and all orchards the model showed a less satisfying accuracy.

Discussion

The preliminary results indicate that NIR analysis can be applied to distinguish between healthy and AP infected plant material. However, the results further indicate that the

explanatory power and accuracy of the results depend on the sampling time point in the year. Later in the vegetation period the results allow a more accurate discrimination between infected and non-infected leaf samples. Symptom manifestation on the tree can be irregular and only partially, *i.e.* not all parts of the canopy show AP specific symptoms. In infected trees, the leaves derived from symptomatic or asymptomatic sprouts were not distinguishable at every sampling time point. Interestingly, in June the NIR spectra analysis allowed a discrimination between symptomatic and asymptomatic leaves. This might be explained by the fact that AP phytoplasma presence in the canopy increases within the year and by their fluctuating colonization (Baric *et al.*, 2011). The results furthermore suggest that in order to explain the differences between infected and uninfected samples comprising all timepoints and all orchards other strategies than PCA combined with QDA might be a recommended option. Thus, variables (PCs or wavelengths) should be selected by algorithm, e.g. successive projections algorithm or genetic algorithm (Pontes *et al.*, 2005), and alternative multivariate statistical models such as partial least square regression discriminant analysis or random forests should be investigated in the future. Furthermore, the impact of season and orchard must be evaluated and the question must be addressed, if the spectral patterns are AP-specific. Abiotic factors, such as water stress or drought, some physiological changes or other disease agents as viruses or fungi might produce similar patterns in the spectra or impact the results. However, the preliminary results of this study are indicating that NIR might be a useful tool to discriminate AP diseased from uninfected material.

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Detection

New phytoplasma subgroups within the 16SrI and 16SrVII groups detected in trees of Bogotá, Colombia

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Abstract

Phytoplasmas of groups 16SrI and 16SrVII are associated with a disease in urban trees in Bogotá, Colombia. The objective of this work was to study the diversity of these phytoplasmas, so 16S rRNA amplicons from the two groups were cloned and sequenced. Using the *i*Phyclassifier tool, sequences of 18 clones were assigned to subgroups 16SrI-B, and to three news subgroups with a restriction pattern similar to that of 16SrI-B and to that of 16SrI-K phytoplasmas. Phytoplasmas of the 16SrVII group were tentatively assigned to a new subgroup with a restriction pattern similar to that of the 16SrVII-A. These results suggest that the phytoplasmas that presently infecting the trees of Bogotà show great genetic variability probably indicating their divergence from original strains from other crops in Colombia such as corn (16SrI-B) and ash (16SrVII-A).

Keywords: ribosomal subgroups, phytoplasma diversity, molecular identification

Introduction

Phytoplasmas are plant pathogenic bacteria of the class *Mollicutes* that lack cell walls, are sensitive to tetracycline and are obligate parasites of the phloem of infected plants and of the tissues of Hemipteran insects. They are transmitted by sap feeder insect species of the Cicadellidae, Cixiidae, Psyllidae, Cercopidae and Delphacidae families, vegetative propagation, grafting and in some cases by seeds. They have small genomes of 600 to 1,600 kb, with low G+C contents (Bertaccini and Duduk, 2009). In the Sabana de Bogotá, Colombia, '*Candidatus* Phytoplasma asteris' (16SrI) and '*Candidatus* Phytoplasma fraxini' (16SrVII) are the major phytoplasma ribosomal groups that infect urban trees. Symptoms such as yellowing and distortions of crown shape and growth patterns are common in *Populus nigra* (Salicaceae), and *Quercus humboldtii* (Fagaceae) infected with phytoplasmas (Franco-Lara and Perilla-Henao, 2014). The objective of this work was to study the genetic variability of the phytoplasmas in the groups 16SrI and 16SrVII infecting the trees of Bogotà using *P. nigra* and *Q. humboldtii* as study models.

Materials and Methods

Samples were collected from 11 *P. nigra* (Pn) and 23 *Q. humboldtii* (Qh) trees. DNA was extracted and used in amplification assays. The 16S rRNA gene was amplified with primers R16mF2/R16mR1 followed in nested PCR by R16F2n/ R16R2 (Gundersen and Lee, 1996). The latter amplicons were analyzed by RFLP with restriction enzymes *Alu*I, *Hha*I, *Mse*I, and *Rsa*I, or by sequencing. Amplicons from selected strains were cloned with the pMiniT™ 2.0 Vectors® (NEB) Kit. DNA of the maize bushy stunt phytoplasma (MBS) (16SrI-B) from Colombia or from the ash yellows phytoplasma (ASHY) (16SrVII-A) from North America were used as positive controls, and water as negative control. PCR assays were conducted in a final volume of 15 μl with 0.05 U/μl of *Taq* Biolase, 1 X reaction buffer, 0.2 mM dNTPs (Bioline®), 0.2 μM primers, and 20–50 ng of template DNA. The thermal profile for amplifications was: initial melting for 10 minutes at 94°C followed by 35 cycles at 94°C for 1 minute, 54°C for 2 minutes and 72°C for 2 minutes with a final extension for 10 minutes. The amplicons were detected in a 1% agarose gel after electrophoresis and RFLP products were separated in a 3% agarose gel. The sequencing reactions were performed by Macrogen, Korea. Forward and reverse sequences were edited with Geneious 9.1.4. and consensus sequences were compared with the BLASTn tool to GenBank database, then analyzed with the *i*PhyClassifier (Zhao *et al*., 2009).

Results

Amplicons of the expected sizes were obtained from 11 Pn, and 14 Qh trees. In both tree species phytoplasmas classified in the 16SrI and 16SrVII groups were detected and no mixed infection was observed (Table 1 and Figure 1). The sequencing analysis confirmed the identity of the phytoplasmas. Since

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the quality and length of the sequences obtained by PCR was not good enough for the *i*Phyclassifier analysis, selected amplicons obtained in nested reactions were cloned and sequenced, as well as the positive ASHY and MBS positive controls. Three clones per sample were sequenced and high quality sequences were obtained for 18 clones. Sequences from the same clone were not identical, with similarities ranging between 99% and 99.3%. Sequences from Pn9, Pn10, Pn11, PnQ12, and Qh22 clustered to phytoplasmas in group 16SrI and sequences Pn3, Pn6, and Qh6 to group 16SrVII. Using the *i*PhyClassifier tool the sequences belonging to the 16SrI group were classified into i) subgroup 16SrI-B (samples Pn9-2, Pn10-1, Pn10-3, Qh22-1); ii) a subgroup named 16SrI-AF with a restriction pattern very similar to that of 16SrI-B (samples Pn9-3, Pn-2, Qh12-1, Qh12-2, Qh12-3); iii) a new subgroup referred here as 16SrI-AH with a restriction pattern very similar to that of subgroup 16SrI-K (sample Pn11-1); and iv) another new subgroup referred here as 16SrI-AG with a restriction pattern very similar to that of 16SrI-AC (sample P9-1). For the 16SrVII sequences the restriction patterns corresponded to a new subgroup 16SrVII-G, very similar to that of 16SrVII-A (samples Pn3-1, Pn3-2, Pn3-3, Pn6-1, Pn6-2, Qh6-2, Qh6-3). All the sequences of the MBS corresponded to the 16SrI-B and all those of ASHY to the 16SrVII-A subgroups.

Table 1. Results of the nested PCR and RFLP analysis, and number of sequences obtained from the cloning of the 16S rRNA gene.

	Number of PCR positives[1]	RFLP		Sequencing	
		16SrI	16SrVII	'*Ca*. P. asteris'	'*Ca*. P. fraxini'
P. nigra	11/11	5	6	2	5
Q. humboldtii	14/16[2]	3	11	4	8
Total	27	8	17	6	13

[1]Numbers of positives of all trees tested in nested PCR analyses.
[2]Samples producing faint bands and not analyzed using RFLP.
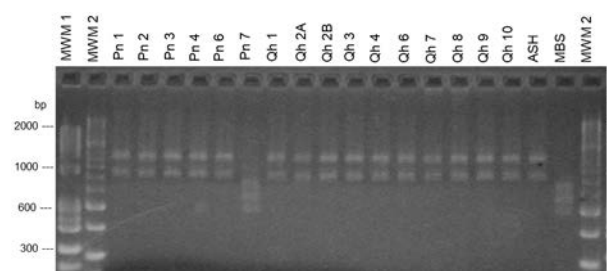
Figure 1. RFLP analysis with *Mse*I of nested amplicons of *P. nigra* (Pn) and *Q. humboldtii* (Qh). ASHY and MBS, positive controls. MWM 1, Hyper ladder 50 bp; MWM 2, Hyper ladder 25 bp (Bioline).

Discussion

The objective of this work was to determine the phytoplasma subgroups that infect *P. nigra* and *Q. humboldtii* in Bogotá. No clones from the same amplicon produced identical sequences, with similarities between 99% and 99.3%, which suggest that the trees are infected with mixtures of very similar phytoplasmas. For example, in sample Pn9, the three clones examined were classified in different subgroups within the 16SrI group *i.e.* 16SrI-B and three subgroups designed 16SrI-AF, 16SrI-AG and 16SrI-AH. A further subgroup 16SrI-AC had been previously reported in Bogotá by verification of GenBank available sequences (Pérez-López *et al.*, 2016). Moreover, all the phytoplasmas assigned to the 16SrVII group infecting both tree species, belonged to the new subgroup 16SrVII-G. These results confirm the high diversity of the phytoplasmas infecting the trees of Bogotà particularly of those classified in group 16SrI and suggest that these strains belong to a diversified population as has been suggested for other phytoplasmas (Davis *et al.*, 2018). Moreover this aspect was already reported in Europe in poplar trees and in North America in ash trees (Griffiths *et al.*, 1999; Seruga *et al.*, 2003).

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Detection

Phytoplasma detection and quantification: make it easy

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Abstract

When high numbers of analyses are required a rapid sample preparation method coupled to RT-qPCR allows to reduce the time and the costs for the phytoplasma detection in plant samples. A comparison between DNA and RNA contribution to qPCR detection revealed the higher input of the latter ensuring the maintenance of the sensitivity and specificity of the assay. The protocol has been validated for the detection and quantification of '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri', '*Ca.* P. prunorum', '*Ca.* P. solani' and "flavescence dorée" phytoplasma by qPCR, RT-qPCR, ddPCR and ddRT-PCR techniques based on TaqMan chemistry.

Introduction

Detection of phytoplasmas is particularly difficult because they are generally present in low concentrations and unevenly distributed in the plant phloem. Conventional detection is usually performed by nested-PCR and quantitative PCR using 16S rRNA general or group specific primers (Christensen *et al.*, 2004; Galetto *et al.*, 2005; Hren *et al.*, 2007). Nested-PCR and PCR-based methods may have some practical limitations for the routine diagnostic. In particular, they are labour-intensive, have the risk of carry-over contamination with possible false positive results.

The protocols for phytoplasmas detection are based on amplification of a DNA target, although previous results on "flavescence dorée" phytoplasma detection showed a higher sensitivity by adding a reverse transcriptase (RT) step to add ribosomal RNA as target of amplification (Margaria *et al.*, 2007; 2009). More recently a RT-qPCR protocol combining RNA and DNA amplification was shown to provide the best sensitivity for the '*Candidatus* Phytoplasma prunorum' detection and quantification (Minguzzi *et al.*, 2016). If the amplification protocol is a crucial step for the pathogen detection, the nucleic acid extraction method adopted is the main time-limiting step to the analysis. Commercial kits for RNA and DNA purification or extraction protocols based on chemical compounds such as cetyltrimethyl ammonium bromide (CTAB) can be used but they are respectively, expensive and time-consuming for large-scale analysis.

Materials and Methods

A quick method based on rapid sap extraction was used, as previously described (Margaria *et al.*, 2007; Minguzzi *et al.*, 2016). One gram of plant tissue was placed in extraction bags and homogenized with 5 ml of grinding buffer [15 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 2% polyvinyl-pyrrolidone (PVP)-40, 0.2% bovine serum albumin (BSA), 0.05% Tween 20 and 1% $Na_2S_2O_5$, pH 9.6] using a ball-bearing tissue grinder. Six mm diameter discs were cut from a nylon membrane (GE Healthcare Life Sciences) using a standard hole puncher. Five µl of sap were spotted onto nylon membrane discs (spot method), placed inside 0.2 ml tubes, and dried under vacuum for 10 minutes. Samples were boiled for 10 minutes after the addition of 100 µl of GES buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA, pH 8) + polyvinylpolypyrrolidone [PVPP] + 0.5% Triton X-100 then centrifuged at 16,000 g for 5 minutes before use.

A CTAB-based method (Angelini *et al.*, 2001; Minguzzi *et al.*, 2016) was also used for total DNA and RNA extraction. Crude extract or total nucleic acids (2 µl) were employed for RT-qPCR amplification in the presence of 10 U of Recombinant Ribonuclease Inhibitor (RNaseOUT) and 2 U of Reverse Transcriptase (M-MLV RT) under the following conditions: 48°C for 30 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Similarly, droplet digital RT-PCR (ddRT-PCR) analyses were performed by a QX200 Droplet Digital PCR System (Bio-Rad)

with the aim to quantify phytoplasma DNA and RNA. Previously described primers and TaqMan probes were used for the detection of 'Ca. P. mali', 'Ca. P. pyri', 'Ca. P. prunorum', 'Ca. P. solani', and the "flavescence dorée" phytoplasma (Ratti et al., 2019).

Results

The results obtained on several samples collected from orchards and vineyards of the Emilia-Romagna region, proved that the sensitivity of the spot method coupled with RT-qPCR is comparable to the CTAB method for all the phytoplasmas assayed. Moreover, an absolute quantification by ddRT-PCR of RNA and DNA in the samples infected by different phytoplasmas, confirmed the relative higher abundance of RNA compared to the corresponding DNA. In particular, the ddRT-PCR analyses indicate that in both CTAB and crude extract preparations the amount of 'Ca. P. mali', 'Ca. P. pyri', 'Ca. P. prunorum', 'Ca. P. solani' and "flavescence dorée" phytoplasma rRNA range between 92% and 99% of the total nucleic acids of the phytoplasmas (Table 1).

Table 1. Quantification by ddRT-PCR of RNA and DNA in plant and insect samples infected by different phytoplasmas.

Phytoplasma	Sample	DNA		RNA	
		Copies/µl	%	Copies/µl	%
"Flavescence dorée"	18/17	411	1.0%	40,269	99.0
	18/17 (spot)	81	0.8%	9,459	99.2%
'Ca. P. solani'	13/17	213	2.7%	7,677	97.3%
	13/17 (spot)	63	7.3%	804	92.7%
'Ca. P. prunorum'	7 (C. pruni)	4,560	0.7%	647,440	99.3%
	127	29,900	7.4%	372,100	92.6%
'Ca. P. pyri'	3,552	600	4.2%	13,800	95.8%
	3,556	265	2.6%	9,985	97.4%
'Ca. P. mali'	813	1,870	0.8%	230,130	99.2%
	36,894	3,325	1.1%	327,420	98.9%

Discussion

Detection and quantification of phytoplasmas can be considerably simplified reducing cost and time of the large-scale or routine analysis by avoiding nucleic acid extraction and adding a reverse transcriptase step to the reaction. RT-qPCR takes advantage of the higher number of copies of 16S rRNA present in active cells compared to the two copies of the 16S rRNA gene.

The reliability of the extraction method has been confirmed by comparing the adopted spot method to the established CTAB protocol used for phytoplasma detection, while high specificity and efficiency are maintained by using appropriate TaqMan® MGB probes. Moreover, the RNA based detection protocols allows to use the same extract for analysis of RNA viruses (Margaria et al., 2009; Minguzzi et al., 2016). Finally, the property of nylon membrane discs to store phytoplasma nucleic acids has been evaluated over a period of 4 months. Discs spotted with samples infected by different phytoplasmas were tested by RT-qPCR immediately or after storing at either 4°C or room temperature. No significant variation on Ct values was observed suggesting that spotted discs could be used to transfer samples over long distance even at room temperature.

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Detection

Proposal to establish a common taxon for 16SrV-C and 16SrV-D phytoplasmas and three genetic clades based on combined 16S rDNA signatures and variability of housekeeping genes

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Abstract

Members of subgroups 16SrV-C and –D form a homogenous genetic cluster with a complex ecology that involves perennial plants such as alders, grapevine and *Clematis* and leafhopper vectors. However, despite the monophyletic origin of the members of these subgroups, a common specific sequence could not be found in their 16S rRNA gene, preventing from describing a common taxon according to the rules for the description of '*Candidatus* Phytoplasma' species. The genetic diversity of 16S rRNA and five housekeeping genes among European and American strains of 16SrV phytoplasmas, including 16SrV-C strains detected in Canadian alders were examined. Members of subgroups 16SrV-C and 16SrV-D share a common combination of three sequence signatures in the 16S rRNA gene that can serve as descriptors for a new common taxon. According to the sequences of *tuf*, *rplV-rpsC*, *rplF-rplR*, *map* and *uvrB-degV* genes, they can be divided into three genetic clusters.

Keywords: "flavescence dorée", alder yellows, Palatinate grapevine yellows, taxonomy

Introduction

According to the convention proposed by Murray and Schleifer (1994) for prokaryotes that can be only incompletely described, phytoplasmas have been described as candidate species within the genus-level taxon '*Candidatus* Phytoplasma' (IRPCM, 2004). The primary rule to describe a '*Candidatus* Phytoplasma' species is to have a 16S rRNA gene sequence similarity below 97.5% with already described '*Candidatus* species'. However, as various phytoplasma ribosomal groups display higher 16S rRNA gene sequence similarity, it had been proposed that in such cases geographically isolated strains, different plant host range or different insect vector, together with evidence of molecular diversity can be used. In addition to the grapevine "flavescence dorée" phytoplasmas, other subgroups of the group 16SrV infect grapevine, alders, *Clematis vitalba*, *Spartium junceum*, *Ulmus* sp. and *Rubus* sp. in Europe (Arnaud *et al.*, 2007). Phytoplasmas infecting *Ulmus* and *Rubus* species have been respectively described as '*Ca.* P. ulmi' (subgroup 16SrV-A) (Lee *et al.*, 2004) and '*Ca.* P. rubi' (subgroup 16SrV-E) (Malembic-Maher *et al.*, 2011). In order to determine the sequence signatures present on the 16S rRNA gene in strains of subgroups 16SrV-C and 16SrV-D (Martini *et al.*, 1999) and that could support the establishment

of '*Candidatus* species', several strains were analysed comparing their 16S rRNA gene sequence. The variability of a set of five house-keeping genes was also analysed.

Materials and Methods

The list of 27 phytoplasma strains used have been previously described (Malembic-Maher *et al.*, 2011). Two additional alder yellows strains were collected on the Mink lake shore in Haliburton (strain A-12-4-2) and on the Erie lake shore in Burnaby (strain A-12-4-12), Ontario, Canada in October and December 2011. Total nucleic acids were extracted according to Maixner *et al.* (1995). The 16S rRNA gene was amplified by semi-nested PCR. A first amplification 16S rRNA gene sequence using the primer pair P1/P7 was followed by a second semi-nested amplification using primer pairs P1-U3 and U5-P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Lorenz *et al.*, 1995). PCR amplified fragments were directly sequenced with the same primers. The *tuf*, *rplV-rpsC*, *rplF-rplR*, *map* and *uvrB-degV* genes were amplified and directly sequenced as previously described (Malembic-Maher *et al.*, 2011). Raw chromatograms were assembled and quality edited to produce a consensus sequence. Multiple alignments were performed using ClustalW and phylogenetic analyses were conducted in MEGA 6 (Tamura *et al.*, 2013).

Table 1. Oligonucleotide signatures in 16S rDNA sequences of 16SrV phytoplasmas.

Position	115	772	1043	1376
FD92	AACAGAAAGGCATCTTTTTGT	GGGG - TAAC-	CTGTCGCTAGTTGC	AACTTCG**A**AA
FDCAM05	AACAGAAAGGCATCTTTTTGT	GGGG - TAAC-	CTGTCGCTAGTTGC	AACTTCGCAA
A12-4-2	AACAGAAAGGCATCTTTTTGT	GGGGG TAACC	CTGTCGCTAGTTGC	AACTTCGCAA
HD1	AACAGAAAGGCATCTTTTTGT	GGGGG TAAC-	CTGTCGCTAGTTGC	AACTTCGCAA
'Candidatus Phytoplasma rubi' (RuS)	AAC**AA**AAAGGCATCTTTTTGT	GGGGG TAACC	CTGTCGCTAGTTGC	AACTT**T**GCAA
'*Ca.* P. ulmi' (EY1)	AACAGAAAGGCATCTTTTTGT	GGGG - **A**AAC -	CTGTCG**Y**TAGTTGC *	AACTTCGCAA
'*Ca.* P. ziziphi' (JWB)	AA**TAA**AAAGGCATCTTTTTGT	GGGG - **C**AAC-	CTGTCGTTAGTT**A**C	AACTTCGCAA
'*Ca.* P. balanitae' (BltWB)	AA**TA**AAAAGGCATCTTTT**AT**	GGGG - TAAC-	CT**T**TCGTTAGTTGC	AACTTCG**T**AA

* '*Ca.* P. ulmi' strains EY1, EI04-2-2 and E04-D438 have CTGCG**T**AGTTGCC while strain E04-D708 has a CTGCG**C**AGTTGCC

Results

The 16S rRNA genes were aligned and informative variable positions are listed in Table 1. Three oligonucleotide sequences starting respectively at 16S rRNA gene positions 115, 772 and 1,043 (underlined in Table 1) could be found in 16SrV-C and V-D subgroup members.

The AACAGAAAGGCATCTTTTTGT sequence in position 115-135 was also found in '*Ca.* P. ulmi', while the TAAC signature in position 776-779 was common between 16SrV-C, 16SrV-D subgroup members, '*Ca.* P. balanitae' (Win *et al.*, 2013) and '*Ca.* P. rubi' (Malembic-Maher *et al.*, 2011). Finally, the CTGTCGCTAGTTGC sequence in position 1043-1056 was found in both 16SrV-C, 16SrV-D subgroup members and '*Ca.* P. rubi'. However, only members of the ribosomal subgroups 16SrV-C and 16SrV-D shared the three sequence signatures in their 16S rRNA gene. Phylogenetic analysis of the concatenated genetic loci *tuf*, *rplV-rpsC*, *rplF-rplR*, *map* and *uvrB-degV* indicates that 16SrV-C phytoplasmas detected in Canadian alders constitute with the American 16SrV-C strain HD1 from *Apocynun canabinnum* a monophyletic discrete genetic cluster that support the establishment of a separate genetic clade. "Flavescence dorée" phytoplasmas of subgroup 16SrV-D appeared clonal and all possess the previously reported specific mutation C>A in position 1383 of the 16S rRNA (Martini *et al.*, 1999). All the other 16SrV-C strains from alders, grapevine, clematis and *Spartium* clustered together on a statistically robust phylogenetic branch.

Discussion

The establishment of a common taxon for the phytoplasmas of subgroups 16SrV-C and 16SrV-D based on the combination of three sequence signatures in the 16S rRNA gene is proposed. These sequence signatures must be conserved all three to assign a phytoplasma strain to the new taxon. The variability of the five house-keeping genes examined supports the description of three different clades for members of the subgroup 16SrV-C and one clade for members of the subgroup 16SrV-D.

Acknowledgements

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Detection

A universal PCR assay amplifying the unique malic enzyme of phytoplasmas

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Abstract

Malic enzyme encoded by the gene *scfA* belongs to the core set of housekeeping genes encoded by phytoplasmas. It enables the conversion of malate to pyruvate, an essential step in the unique pathway of phytoplasmas resulting in the generation of ATP and acetate. Several studies have examined the functionality of this pathway and the phylogenetic origin back to its putative Gram-positive source. The current sequence information on the enzyme relies on available genome sequence data. Comparison of *scfA* gene sequences highlights a low number of conserved sites, even between closely related strains, thereby hampering the design of universal primer pairs for application in PCR. A primer set was designed that enables the amplification of this key gene in phytoplasma metabolism. Successful amplification was performed from strains covering the three main phylogenetic branches of phytoplasma, and, as such, the universal primers scfA115F/865R offer the ability to obtain additional data from closely and distantly related phytoplasmas.

Keywords: malic enzyme, universal primer, PCR

Introduction

Phytoplasmas colonise nutrient-rich environments in insect vectors and plant hosts comprising gut, haemolymph, salivary gland or phloem, respectively. Analyses of complete and draft genome sequences derived from more than a dozen phytoplasma strains highlight that specialising in these particular nutrient-rich niches has resulted in a remarkable loss of metabolic functions in evolution amongst sterol biosynthesis, *de novo* nucleotide synthesis, the phosphortransferase system and the tricarboxylic acid cycle, and in some species, even glycolysis remains uncomplete (Kube *et al.*, 2012). It is remarkable for the class of *Mollicutes* that a malate-sodium symporter was identified. This was highlighted for '*Candidatus* Phytoplasma asteris' strain AY-WB first, and a putative utilisation of this dicarboxylic acid was suggested (Bai *et al.*, 2006). This feature became crucial when the lack of genes encoding the energy-yielding element of glycolysis was recognised for '*Ca*. P. mali' AT (Kube *et al.*, 2008), and further analysis resulted in the suggestion of a phytoplasma specific pathway for utilising malate and enabling the generation of pyruvate, acetyl-CoA, acetyl-phosphate and acetate. It is yielding ATP in the last step (Kube *et al.*, 2012). The malic enzyme (gene *scfA*) is responsible for oxidative decarboxylation of L-malate and

production of pyruvate, CO_2 and NAD(P)H. The coding of *scfA* separates phytoplasmas from other bacteria. Further studies on *scfA* have been carried out successfully, including gene expression (Siewert *et al.*, 2014), their substrates and products (Saigo *et al.*, 2014), as well as protein dependencies and structure (Alvarez *et al.*, 2018). However, the coding of this particular single-copy gene in phytoplasma genomes has uncovered a question: can it be used for further studies apart from its metabolic function with respect to sequence information? To date, the deduced amino acid sequence of the malic enzyme has been used for phylogenetic studies, highlighting its old Gram-positive origin (Siewert *et al.*, 2014; Saigo *et al.*, 2014). Currently, sequence information on *scfA* is limited to genomic (draft) sequences missing for several groups, which is why a PCR assay by applying universal primers to amplify *scfA* was developed.

Materials and Methods

Catharanthus roseus, experimentally infected and without infection (negative control), was used for genomic DNA extraction by applying the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Nucleotide sequences of *scfA* gene were extracted from the genomic sequence (www.ncbi.nlm.nih.gov/genbank) comprising '*Ca*.

P. mali' AT (CU469464), 'Ca. P. solani' 284/09 (FO393427), 'Ca. P. asteris' strains (OY-M, AP006628; AY-WB, CP000061; De Villa CP035949; M3, CP012149), 'Ca. P. ziziphi' (CP025121) and 'Ca. P. australiense' strains (rp-A, AM422018; NZSb11, CP002548). Alignment of the *scfA* gene and visualisation were performed in MEGA X (Kumar *et al.*, 2018) and T-Coffee (tcoffee.crg.cat). MEGA X was also used for calculating the overall mean distance. Universal primers for amplifying the malic enzyme were designed manually, and names are given according to their alignment positions. Potential hybridisation sites were examined via BLASTN against GenBank (blast.ncbi.nlm.nih.gov). PCRs were set up using the oligonucleotides scfA115F and scfA865R (Macrogen Inc., Korea), and the OptiTaq PCR Master Mix (Roboklon, Germany). Reactions were performed according to the following parameters: initial denaturation for 1 minute at 92°C, 35 cycles including 30 seconds at 92°C, 1 minute at 52°C, 45 seconds at 72°C and 1 minute at 72°C for the final extension. PCR amplicons were inspected by electrophoresis in a 1% agarose gel and Sanger sequencing applying the PCR primers (Macrogen Inc., Korea).

Table 1. Primer applied for the partial amplification of the *scfA* gene.

scfA115F	5'- GGAGTNGCNGARCCTTG -3'
scfA865R	5'- CTTTTTTAGCTTCWTSWKRCATAATTTC -3'
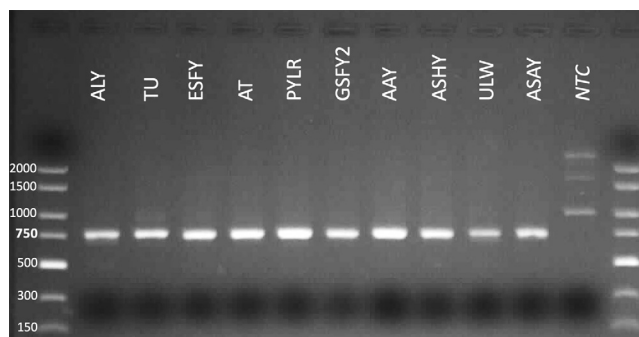
Figure 1. PCR results, lanes: 1 and 13, DNA Perfect Plus Ladder; 2-11, PCR products obtained from *C. roseus* with the phytoplasmas listed in Table 2; 12, healthy *C. roseus*, NTC.

ScfA genes (1,170 nt) extracted from nine genomic sequences show a low number of conserved sites in a multiple alignment with expected high sequence variability, resulting in an overall mean distance of 28%, which also reflects the applied heterogeneous source dataset. Consequently, the primer design was limited to a few locations, and the usage of wobble positions was necessary (Table 1). Amplification, by applying the primer pair scfA115F/865R, was performed successfully, resulting in PCR products of the *in silico* calculated size of 750 bp (Figure 1). Weak PCR products were also obtained from the plant host, albeit limited to the absence of phytoplasma infection and differing in size (above 1,000 bp). Results indicate that this PCR approach is applicable for

templates derived from the three major phylogenetic branches of the 'Candidatus Phytoplasma' taxon. Products were obtained from five 16Sr groups (Table 2). Sanger sequencing results confirmed *scfA* amplification.

Table 2. 16Sr groups and phytoplasmas amplified by the primer pair scfA115F/865R.

16SrI-B	'Candidatus Phytoplasma asteris' (AAY, ASAY)
16SrV-A	'Candidatus Phytoplasma ulmi' (ULW)
16SrV-C	Alder yellows phytoplasma (ALY)
16SrVII-A	'Candidatus Phytoplasma fraxini' (ASHY)
16SrX-A	'Candidatus Phytoplasma mali' (AT)
16SrX-B	'Candidatus Phytoplasma prunorum' (ESFY, GSFY2)
16SrX-C	'Candidatus Phytoplasma pyri' (PYLR)
16SrXII-B	'Candidatus Phytoplasma australiense' (TU)

Discussion

The results highlight the amplification of *scfA* gene in various phytoplasma ribosomal groups by the universal primers despite wobble positions and high sequence variability. Ongoing experiments will provide evidence whether or not this universal primer pair can be applied successfully to all 16Sr groups, whilst continuing analysis of *scfA* gene will help estimate the informative character in general and its application with respect to phytoplasma population studies.

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Detection

Use of *12p* and *36p* genes as molecular markers in support of subgroup identification of two 16SrXIII phytoplasmas associated with strawberry phyllody in Chile

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Abstract

During the year 2017, surveys were carried out in strawberry fields in Biobío and Araucanía regions in Chile, where strawberry plants with symptoms of phyllody, achenes' hypertrophy and leaf reddening had been observed. In the symptomatic plants, two phytoplasmas belonging to the ribosomal subgroups 16SrXIII-F and 16SrXIII-K have been identified. In order to obtain specific molecular tools for the detection of these phytoplasmas, two regions containing the genes encoding the ribosomal proteins 12p and 36p were used, and the phylogenetic trees obtained unambiguously support the distinctness of the two ribosomal subgroups.

Keywords: ribosomal subgroups 16SrXIII-F and 16SrXIII-K, RFLP, phylogenetic trees, South America

Introduction

'Strawberry (*Fragaria* x *ananassa* Duch.) plants have been reported to be infected by phytoplasmas all over the world. In Chile the first case of phytoplasma-associated symptoms in strawberry plants was observed in 2015. During later surveys, symptoms of phyllody, achenes' hypertrophy and leaf reddening and chlorosis were observed in the varieties Camarosa, Albion, Portola, San Andreas and Monterey. Fields with symptomatic plants were discovered in different regions, including Valparaíso, Biobío and Araucanía. The disease was named strawberry phyllody (StrPh), and two phytoplasmas belonging to the 16SrXIII-F and 16SrXIII-K ribosomal subgroups were identified from the symptomatic plants (W. Cui *et al.*, unpublished).

Materials and Methods

During February to April 2017 and December 2017 to March 2018, surveys were carried out in four strawberry orchards in Biobío and Araucanía regions. Seven strawberry plants showing symptoms of phyllody and achenes' hypertrophy were collected and marked as StrPh-CL1 to StrPh-CL7. DNA was extracted according to a modified protocol of Porebski *et al.* (1997). Nested PCR of the 16S rDNA was performed using the universal primer pairs P1/P7 and R16F2n/R2 (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Gundersen and Lee, 1996). Nested PCR of the *tuf* gene was performed using the universal primer cocktail sets designed by Makarova *et al.* (2012).

Direct PCR of the *12p* gene region and nested PCR of the *36p* gene region were performed using the primers designed in this work. DNA from asymptomatic strawberry plants and reactions without DNA template were used as negative controls, and DNA from the phytoplasma strain Vc33 (16SrIII-J, Zamorano and Fiore, 2016) maintained in a periwinkle plant, was used as a positive control. PCR products were visualized in agarose gels with ethidium bromide under UV transilluminator. Amplicons from several genes corresponding to approximately 1.25 kb for 16S rDNA, 440 bp for *tuf*, 820 bp for *12p* and 540 bp for *36p* were recovered and cloned into the vector pGEM-T Easy. The plasmids were transformed into TOP10 chemically competent cells, and the clones were sequenced using the T7/SP6 primers in both directions. Chromatogram showing sequence quality was visualized using BioEdit, which was also used for the alignment of each pair of sequences and the assembly of the full sequence of each clone. Three individual clones of each amplicon from each sample were analyzed. *In silico* RFLP of 16S rDNA was performed by *i*PhyClassifier (Zhao *et al.*, 2009) to identify the phytoplasmas. Phylogenetic trees using 16S rRNA, *tuf, 12p* and *36p* genes fragments were constructed using MEGA7.

Results

Phytoplasmas were detected only in the symptomatic plant samples. With 16S rDNA and *tuf* genes, five strains, StrPh-CL1, StrPh-CL3, StrPh-CL5, StrPh-CL6 and StrPh-CL7, were

identified as 16SrXIII-F phytoplasma, whereas the other two strains, StrPh-CL2 and StrPh-CL4, belong to a newly-proposed subgroup 16SrXIII-K. In the phylogenetic tree constructed using the 16S rRNA sequences, the two 16SrXIII-K strains form a clade separated from the other 16SrXIII subgroups (W Cui *et al.*, unpublished). In the phylogenetic trees constructed using each individual and concatenated sequences of *tuf*, *12p* (Figure 1) and *36p* genes (data not shown), the 16SrXIII-F and 16SrXIII-K clades are unambiguously distinct.
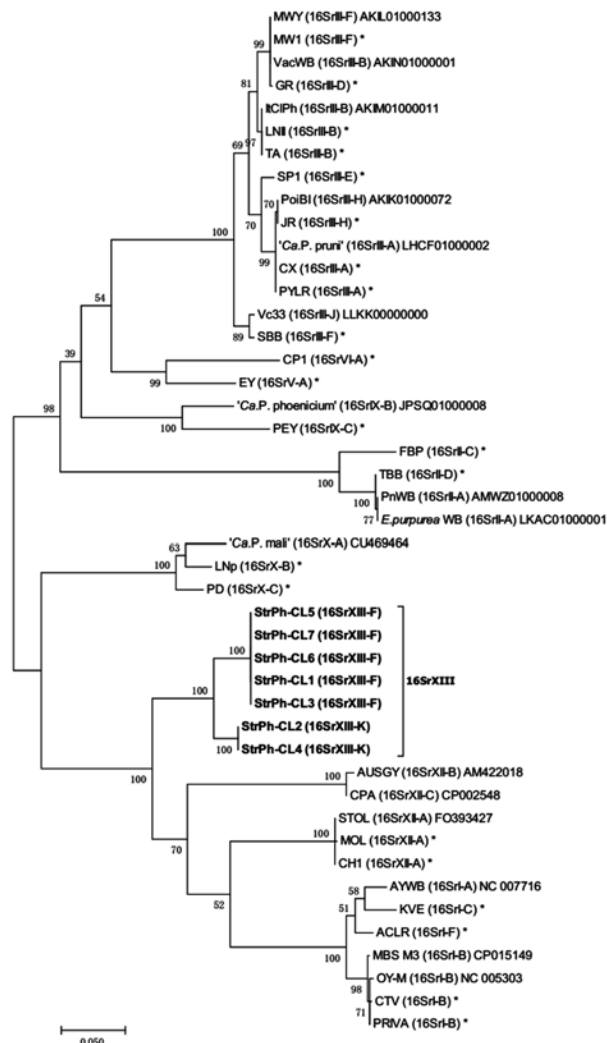
Figure 1. Phylogenetic tree inferred from analysis of *12p* gene region sequences using the Maximum Likelihood method in MEGA7. Asterisks indicate unpublished sequenced produced for this work.

Discussion

The phytoplasmas belonging to the group 16SrXIII were found exclusively in the Americas and six out of 11 subgroups, 16SrXIII-A, 16SrXIII-B, 16SrXIII-F, 16SrXIII-I, 16SrXIII-J and 16SrXIII-K, infect strawberry (Jomantiene *et al.*, 1998;

Fernández *et al.*, 2015; Melo *et al.*, 2018). Among them, subgroups -F, -J and -K were exclusively found in strawberry plants in South America. The phylogenetic tree derived from 16S rDNA showed that they form a clade separate from the rest of the phytoplasma ribosomal groups, suggesting a late divergence of these subgroups after spreading to the continent. In spite of not having sequences in GenBank, the markers *12p* and *36p* were shown to be good tools for subgroup differentiation.

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Detection

Use of mitochondrial divergence in plant-specialized populations of *Hyalesthes obsoletus* for identification of '*Candidatus* Phytoplasma solani' epidemiology

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Abstract

Hyalesthes obsoletus, a major vector of '*Candidatus* Phytoplasma solani' is considered as a polyphagous species associated with diverse wild host-plants. A recent study performed in southeastern Europe documented genetic differentiation among host-plant associations of *H. obsoletus*, while numerous data are pointing to specialized plant-associated epidemiological cycle of '*Ca.* P. solani'. Hence, based on the mitochondrial *COI* gene sequences, a method for routine identification of plant-specialized vector populations and epidemiological routes of pathogen transmission were developed and verified.

Keywords: *COI* gene, epidemiology, genetic segregation, host-plant, insect vector, molecular identification

Introduction

The cixiid planthopper *Hyalesthes obsoletus* (Hemiptera: Cixiidae) is a principal vector of '*Candidatus* Phytoplasma solani' (16SrXII-A ribosomal subgroup), with a major role in pathogen spread, transmission routes and disease epidemiology (Jovic *et al.*, 2019). Generally, *H. obsoletus* is considered as a polyphagous species associated with diverse wild host-plants which, often simultaneously, serve as pathogen reservoirs and consequently are the source of disease transmission. However, recent research performed in southeastern Europe, the distribution centre of the planthopper and the area of many '*Ca.* P. solani'-associated plant diseases, indicated specific host-plant associations of the vector and specific vector-based routes of transmission (Kosovac *et al.*, 2018). Genetic differentiation and host-driven segregation (mitochondrial and nuclear) among three morphologically inseparable host-plant associations of *H. obsoletus* (Figure 1) was evidenced for populations affiliated to: i) *Urtica dioica* and *Convolvulus*, ii) *Vitex agnus-castus*, and iii) *Crepis foetida*.

In different parts of Europe, several studies have also documented the presence of a specialized plant-associated '*Ca.* P. solani' epidemiological cycle and life cycles of *H. obsoletus* (Langer and Maixner, 2004; Johannesen *et al.*, 2008; Imo *et al.*, 2013; Sharon *et al.*, 2015; Kosovac *et al.*, 2016, 2019). The two principal transmission routes of '*Ca.* P. solani' in the continental Europe are based on vector association and pathogen specialization towards *U. dioica* and *C. arvensis*,

while each plant is hosting a specific pathogen strain according to the elongation factor *Tu* gene, tuf-type a and tuf-type b, respectively (Langer and Maixner, 2004). In the Mediterranean coastal zone, along with the aforementioned transmission routes, *V. agnus-castus* is a preferred host-plant of *H. obsoletus* (Sharon *et al.*, 2015) and an independent source of pathogen transmission and of tuf-type b strain (Kosovac *et al.*, 2016). Finally, an additional, independent and recently evidenced, epidemiological cycle of tuf-type b '*Ca.* P. solani' strain in the Balkan region is sourced by *C. foetida* and transmitted by its associated *H. obsoletus* population (Kosovac *et al.*, 2019). The three tuf-type b epidemiological cycles vectored by plant-specialized populations of *H. obsoletus* can be differentiated either by insect vector genetic divergence (Kosovac *et al.*, 2018, 2019) or pathogen divergence using variable, epidemiologically informative marker genes (Cimerman *et al.*, 2009; Fabre *et al.*, 2011) or with a combination of both (Kosovac *et al.*, 2019). The aim of this study was to point to possibilities of using mitochondrial divergence in plant-specialized populations of insect vector for a routine identification of epidemiological routes of '*Ca.* P. solani' strain transmission.

Materials and Methods

The material used in this study is in detail described in Kosovac *et al.* (2018). DNA of selected *H. obsoletus* individuals representing each of the formerly identified AB, EC, ZN, YM and JH mtDNA haplolineages were used for

analysis of nearly full-length of cytochrome oxidase subunit I (*COI*) mitochondrial gene. Obtained sequences were compared and analyzed using MEGA 7. Nucleotide diversity and variability was assessed within and between host-specialized mt*COI* haplogroups. These data were used for designing host-plant-specific multiplex PCR method.

Figure 1. *Hyalesthes obsoletus* on *Convolvulus arvensis* (left) and on *Crepis foetida* (right).

Results

The 1476-bp long mt*COI* gene sequences were obtained for 54 *H. obsoletus* specimens affiliated with one of the three host-plant associations: *U. dioica-C. arvensis*, *V. agnus-castus* or *C. foetida*. Nucleotide analyses confirmed the genetic segregation according to host-plant specialization in all the 18 identified haplotypes originating from a wide geographic area (6 *U. dioica-C. arvensis*, 2 *V. agnus-castus* Greece, 4 *V. agnus-castus* Montenegro and 6 *C. foetida* haplotypes). Mean genetic distance within host-specialized haplogroups was 0.2% for *U. dioica-C. arvensis* and *C. foetida*, and 0.6% for *V. agnus-castus*, while between haplogroups the mean distance was 1.7-2.1%. The sequence comparison of all the haplotypes identified within the *H. obsoletus sensu lato* revealed a genetic variability represented by 57 variable, 11 singleton and 46 parsimony-informative sites. Out of these, 26 sites could be used as informative for the discrimination among *U. dioica-C. arvensis*, *V. agnus-castus* and *C. foetida* host-specialized *H. obsoletus*. Based on mt*COI* sequence variability three forwards primers were designed with binding positions in different regions of the gene, according to variable nucleotide sites specific for each host-specialized haplogroups. In addition, a single reverse primer was designed at the 3'-end of the gene containing nucleotide sequence corresponding to all three host-associated types. All four primers were used as a set, and methodology of differentiation of the three plant-specialized *H. obsoletus* populations was successfully tested on numerous specimens (Kosovac *et al*., 2018).

Discussion

Development of tools for molecular identification and separation of cryptic species or ecological host races is of great importance in insect vector-borne plant diseases of economic relevance. The presented methodology for plant-specialized *H. obsoletus* discrimination enables not only a reliable identification of the vector population (when collected on cultivated plants), but at the same time provides information on the pathogen source plant and disease epidemiology. Although more data are needed regarding '*Ca. P.* solani' differentiation in association with each plant-specialized vector population, available data are supportive of this conclusion (Kosovac *et al*., 2016; 2019).

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Detection

Multilocus sequence analysis for detection of finer genetic variation and phylogenetic interrelatedness in 16SrI group phytoplasma strains infecting different plants in China

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Abstract

The group 16SrI phytoplasmas are associated with severe diseases of many cash and ecological plants throughout the world. To date, the genetic variation and population structure of very closely related phytoplasma strains are still not fully understood in China. In this study, a multilocus sequence analysis (MLSA) scheme was developed using ten housekeeping genes (*rp*, *tuf*, *secA*, *secY*, *ipt*, *dnaK*, *fusA*, *gyrB*, *pyrG* and *rpoB*) fragments compared with 16S rDNA to analyze 18 phytoplasma strains infecting chinaberry, lettuce, mulberry, paulownia and periwinkle from ten provinces in China. The nucleotide site polymorphisms resolved all strains into 15 sequence types (STs), demonstrating extensive genetic diversity among the 16SrI group strain population. All the strains, classified in 16SrI-B and -D subgroup by 16S rDNA analysis, clustered into one clade and clearly differentiated into discrete subclades by phylogenetic analysis of the concatenated gene sequences. The 10 chinaberry witches' broom (CWB) strains that were most closely related to two mulberry dwarf (MD) and hardly distinguished with 16S rDNA, were definitely split into four distinct clusters and 8 STs apparently congruent to their geographical locations. Two lettuce yellows (LY) strains in Sanming, Fujian province, China were more closely related to the onion yellows OY-M strain in Japan than the periwinkle virescence (PeV) and paulownia witches' broom (PaWB) strains in China. The levels of variation in *dnaK* gene were higher than those in 16S rDNA and other genes tested. This MLSA is a promising approach for phytoplasma differentiation as well as for in depth examination of strain diversity and evolution of various 16Sr groups or subgroups.

Keywords: phytoplasma, multilocus sequence analysis (MLSA), sequence type (ST), phylogenetic evolution

Introduction

Five economically and environmentally harmful plant diseases associated with aster yellows (AY) phytoplasma group (16SrI) have been reported widely in China: chinaberry witches' broom, lettuce yellows, mulberry dwarf, paulownia witches' broom and periwinkle virescence. Some of these diseases have also been reported in Australia and other Asian countries such as Korea, Japan, India, and Iran. The 16SrI group of phytoplasmas comprises AY phytoplasma ('*Candidatus* Phytoplasma asteris') and numerous related phytoplasmas, infecting a wide range of host plants globally, and represents the most diverse and widespread phytoplasma group (Lee *et al.*, 2004; Bertaccini and Duduk, 2009). Within this large and intricate group, the genetic variation and population structure of many closely related phytoplasma strains are still not fully understood and characterized. In China, little is known about the phylogenetic

relationships and strain population structure of the five phytoplasmas that are important from the perspective of agriculture and forestry. Furthermore, there is no report on an MLSA assay that is specific for this large group of phytoplasma strains, and research on finer identification, genetic structure analysis, and determination of the phylogenetic relationship of the 16SrI group phytoplasmas is limited. Moreover, inadequate knowledge, especially in China, of the relationship between various strains from different plant hosts and locations has greatly retarded detailed phytoplasma identification and differentiation, as well as efficient pathogen detection, disease diagnosis, epidemiological studies, and disease control.

Materials and Methods

The 16SrI group phytoplasma strains used in the study were collected from different regions of China to represent diverse

environmental niches. The 16S rDNA was amplified using primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995). Other studied genes were: *rp*, including fragments of *rpl22* and *rps3*, *tuf*, *secA*, *secY*, *ipt*, *dnaK*, *fusA*, *gyrB*, *pyrG* and *rpoB*. Sequence analysis was performed using *i*PhyClassifier, EditSeq, DNAMAN version 5.0, MEGA version 7.0 based on 16S rRNA gene and the other gene sequences.
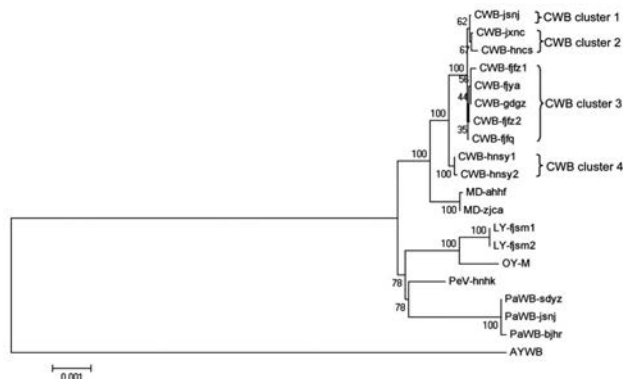
Figure 1. Phylogenetic tree of the 16SrI group phytoplasma strains reconstructed by neighbor-joining analysis of the concatenated gene sequences data set of *rp*, *tuf*, *secA*, *secY*, *ipt*, *dnaK*, *fusA*, *gyrB*, *pyrG* and *rpoB* gene sequences.

Results

Based on MLSA, significant and distinct differentiations were observed that not only correlated with the specificity of the plant hosts, but also with geographic origins of the analyzed strains, which were hardly distinguished by the RFLP analysis of their 16S rRNA gene sequences. The CWB, LY, MD, PaWB, PeV, OY-M, and AYWB strains were clearly differentiated and formed discrete subclades with improved meaningful bootstrap support values. Significantly, 10 CWB strains from diverse regions were further classified into four subclusters, namely CWB cluster 1 (one strain from Jiangsu), CWB cluster 2 (two strains from Jiangxi and Hunan), CWB cluster 3 (including five strains from Fujian and Guangzhou), and CWB cluster 4 (two strains from Hainan). An explanatory chart was drawn to clarify the close relationships among the phytoplasma strains of different CWB genotypes and their geographical distributions (Figure 1), which were hardly dissected by previous single locus studies. The variation was comparatively high in sequence fragments of *dnaK* (3.35%–3.44%), *secY* (2.38%–2.47%), and *ipt* (2.05%–2.17%). In contrast, sequences of 16S rDNA (0.42%) and *tuf* (0.73%–0.74%) were more conserved, as indicated by lower variation in these genes. There were 201 variable *loci* in the 11 analyzed gene sequences. The level of transition in mutant *loci* (57.71%) was definitely higher than that of transversion in mutant *loci* (36.82%). With regard to nucleotide substitution in 201 variable *loci*, the transition between T and C (33.83%) was more frequent compared with that between A and G (23.88%). Transversion between A and C (17.41%) and T and G (12.44%) was common, while transversion between G and C (2.99%) and A and T (3.98%) was comparatively rare. Furthermore, deletion and insertion (6.47%) existed in *rp*, *tuf*, *secY*, and

dnaK sequences. The deletion and insertion of nucleotides in the gene studied resulted in the addition or absence of corresponding amino acids in the deduced amino acid sequence encoded. Based on the analysis of the 11 gene fragments, there were 15 sequence types (STs) in 18 strains. Ten CWB strains were resolved into 8 STs, while three strains, CWB-fjfz2, -fjya, and -gdgz had same ST although they had different geographical origins (Fuzhou and Yongan in Fujian and Guangzhou in Guangdong, respectively). Both LY strains from Sanming, Fujian (LY-fjsm1, -fjsm2) shared the same ST.

Discussion

Intriguingly, ten CWB strains from the Fujian, Guangdong, Jiangsu, Jiangxi, Hunan, and Hainan provinces of south China—a broad area—showed high diversity and were clearly divided into four clusters or eight sequence types. This diversity appeared to be consistent with the difference in the latitude and climate of their specific geographical regions, most possibly attributable to the effect of temperature. Temperature may be the main factor influencing the ecological difference in the diverse environments of the CWB strain sampling sites that were distributed across different latitudes from north to south. The CWB strains from Sanya, Hainan province (island), which has a typically tropical climate, showed obvious differences in the sequence of *ipt*, *pyrG*, *rpoB*, *tuf*, *secA*, *dnaK*, *fusA*, and *gyrB* genes from other CWB strains from mainland China. This finding strongly suggested that geographic separation, low latitude, and constantly higher temperature likely acted as selective pressures and influenced the adaptation and evolution of these cell wall-less prokaryotes. Therefore, the notable divergence between the two CWB strains is possibly caused by geographical isolation of the Hainan province from the mainland, which facilitated population differentiation.

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Detection

Phytoplasma cultivation: lights and shadows

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Abstract

The possibility to grow phytoplasmas in complex media was first demonstrated using micropropagated infected periwinkle shoots and then carried out employing naturally infected field collected plant samples. Phytoplasmas belonging to diverse ribosomal groups were isolated from infected grapevine and coconut palm tissues and in some cases also from asymptomatic tissues, especially from trees or plants growing in severely infected areas. In particular aster yellows and "stolbur" phytoplasmas, group 16SrI and 16SrXII respectively, were consistently grown among other phytoplasmas from the different plant sources employed and the first biochemical and biological characterization of some of these isolates was obtained.

Keywords: complex media, phytoplasma identification, endophytes, plant disease

Introduction

The possibility to grow phytoplasmas in complex media was achieved using micropropagated infected periwinkle shoots (Contaldo *et al.*, 2012) and then employing naturally infected field collected plants (Contaldo *et al.*, 2016; 2019). Moreover the *in vitro* isolation of 'Candidatus Phytoplasma pruni'-related phytoplasmas associated with cassava frog skin disease (CFSD) was recently reported (Alvarez *et al.*, 2017). An overview of the cultivation situation is presented in order to highlight the achieved results and the bottle necks linked to both the isolation methodology and the phytoplasma detection in the solid and liquid media.

Materials and Methods

The phytoplasma growth was obtained from infected micropropagated periwinkle shoots for which the isolation was carried out on commercial media (Piv) with a not disclosed composition (Contaldo *et al.*, 2012). In order to achieve the cultivation from diseased plant materials from the field, media (CBs) supporting the growth from different plants sources and with flexible and modifiable composition were specifically designed (Contaldo *et al.*, 2016). These media were prepared using TSB, which essentially contains tryptone and soy peptone and were used for the isolation and growth of phytoplasmas from infected field collected grapevine samples. One of the key points that was shown to be important within the phytoplasma isolation from these plant sources, was the optimization of the growing conditions, by the use of plate incubation in a specific anaerobic jar in microaerophilic atmosphere (Contaldo *et al.*, 2016). These

media were also successfully employed for phytoplasma isolation from coconut palms infected by Cote d'Ivoire lethal yellowing (CILY) (Contaldo *et al.*, 2019). The use of TSB in liquid and solid media formulations for phytoplasma isolation and culture, however, supports also the growth of bacterial endophytes present in the plant source materials (Figure 1). Therefore to obtain a substantially pure phytoplasma growth, single colonies were purified by filtering and by serial dilution, plated and then used the for molecular phytoplasma detection, performed with diverse PCR assays on the 16Sr RNA gene sequence (Contaldo *et al.*, 2019). Furthermore, to evaluate and determine some of the biochemical properties, isolates of 16SrI-B - 'Ca. P. asteris' from grapevine, 16SrXII-A - 'Ca. P. solani' and 16SrXXII-B - 'Ca. P. palmicola' from coconut palm were employed for the specific tests on degradation of glucose, arginine and urea.
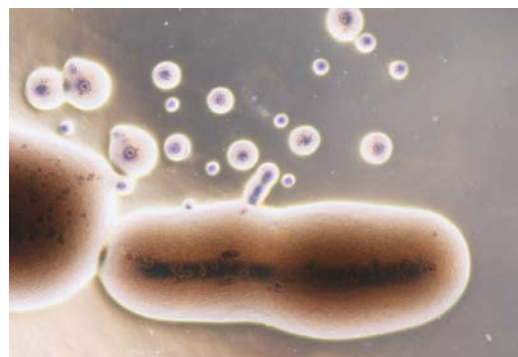
Figure 1. Phytoplasma and bacterial colonies isolated from grapevine, after 2 days from plating under bifocal optical microscope (X 40).

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Table 1. Phytoplasmas isolated in Piv media from micropropagated periwinkle shoots (Bertaccini, 2014).

Phytoplasma	Ribosomal group	Source
CY-TO	16SrI-B	Chrysanthemum yellows, Italy
TBB	16SrII-D	Tomato big bud, Australia
CH-1	16SrXII-A	"Bois noir" grapevine, Italy
STOL	16SrXII-A	"Stolbur" of pepper, Serbia
PWB	16SrVI-A	Potato witches' broom, USA
WBDL	16SrII-B	Witches' broom disease of lime, Oman
PEY	16SrIX-C	*Pichris echoides* yellows, Italy

Table 2. Phytoplasmas isolated in CB media.

Phytoplasma isolate	Ribosomal group	Isolation source
BN-Yan	16SrXII-A	Grapevine
FD-05M	16SrV-C	Grapevine
FD-7bariv	16SrV-D	Grapevine
AY-3bar	16SrI-B	Grapevine
2T-15	16SrXII-A	Coconut palm
20T-15	16SrXXII-B+16SrI	Coconut palm
40T-15	16SrI	Coconut palm
41T-15	16SrI	Coconut palm
11F-15	16SrI	Coconut palm
15F-15	16SrXII-A	Coconut palm
43T-15	16SrV-A	Coconut palm
70L-15	16SrXII-A	Coconut palm
2T-16	16SrI+16SrXII-A	Coconut palm
24T-16	16SrI	Coconut palm
20T-16	16SrI	Coconut palm
L9-16	16SrI	Coconut palm
30T-16	16SrI	Coconut palm
9T-16	16SrI+16SrXII-A	Coconut palm
17T-16	16SrI+16SrXII-B	Coconut palm

Results and Discussion

The DNAs extracted from colonies or liquid aliquots of both, Piv and CB media (Contaldo *et al.*, 2012; 2019) resulted amplifiable on the 16Sr gene in nested-PCR with universal primers, scattered amplifications were also obtained using non ribosomal genes. The difficulty in amplifying non ribosomal genes from these isolates is probably due the presence of different factors ranging from the presence of a mixed bacterial and/or phytoplasma population and/or chemical PCR inhibitors produced in the media. Different phytoplasma strains were isolated and cultivated in both artificial media (Tables 1 and 2, Figure 2). The use of naturally infected plants and of the TSB-based media allowed the isolation and growth of several isolates, reducing also the incubation time. Among the others, the majority of phytoplasmas isolated and grown were aster yellows and "stolbur", probably due to the media composition that seems to be more suitable for these phytoplasmas.

The comparison between the phytoplasma subgroups detected in the plant material and those in the liquid and solid media appears often discordant, with phytoplasmas detected in the culture differing from those founded in the original plants. On the other hand these somewhat contradictory results on phytoplasma identification confirm the presence of a mixed infection in the majority of the samples used in the analyses. The diverse performance of the media tested allow the growth of phytoplasmas belonging to selected ribosomal groups. The biochemical characterization of 16SrI-B, 16SrXII-A, and 16SrXXII-B isolates revealed that they share metabolic features with *Mycoplasma* species group III (glucose positive/arginine positive), which are represented by several mycoplasma species (i.e., *Mycoplasma fermentans*) (Brown *et al.*, 2007). These results confirm that phytoplasmas seems to retain an independent metabolism that allows them to survive outside the host species, very likely with a restricted ability to grow in artificial media and with the presence of endophytic bacteria showing very similar growth requirements.
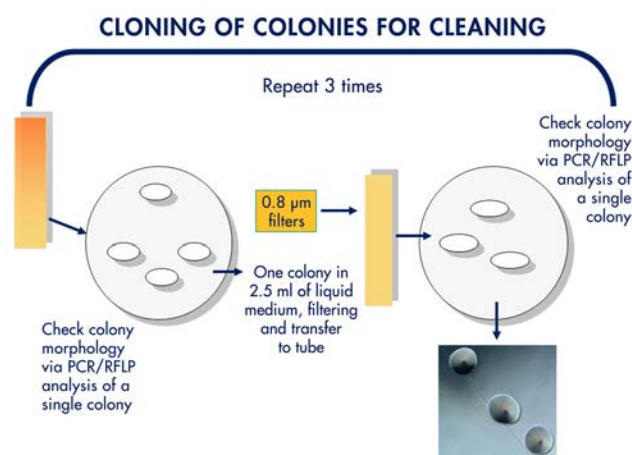
Figure 2. Phytoplasma purification procedure scheme.

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Insect vectors

Searching for the vectors of coconut lethal yellowing: a 50 year unfinished journey

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Abstract

The insect vectors of coconut lethal yellowing have proved elusive. Long running studies in the Caribbean, central America and southern USA have implicated some species and made others more likely. Long running surveys in both east and west Africa have identified many planthoppers and some leafhoppers associated with palms. Occasionally some have shown positive for phytoplasmas. But even after such a long period of study are we little further in identifying the vectors and being able to use this information? This review will give an overview of the work carried out in the various regions where coconut lethal yellowing is found.

Introduction

Lethal yellowing (LY) disease of coconut has been recognised and known by different names for a very long time, perhaps as early as the late 1800's in the Caribbean and at least a hundred years in both East and West Africa (Eden-Green 1997, Howard, 1997). Many kinds of organisms were suspected as being possible vectors but developments in the 1970's made progress possible (Howard, 1997). In a review by Howard (1997) on LY vector studies in the Caribbean he indicated that in the 1970's knowledge that phytoplasmas are associated with plant diseases was a recent discovery and that they were generally transmitted by Auchenorrhyncha was also becoming accepted (Whitcomb and Davis, 1970).

Materials and Methods

This review is based upon papers by Wilson (1987a, 1987b, 1988, 1997 and 2002) and more recent studies (Kwadjo *et al.*, 2018). The survey areas have been based on east and west Africa as well as literature studies on the Caribbean and Central Americas.

Results

From the early 1970's Howard and colleagues were surveying for potential vectors of LY in Florida and others were beginning studies in the Caribbean (Howard, 1997). Their studies focussed on the abundant cixiid planthopper *Haplaxius crudus* (van Duzee), which was shown to be a potential vector for this disease by Howard *et al.* (1983).

A wide variety of Auchenorrhyncha families and species may be found on coconut and some may be abundant (Wilson, 1987a, 1987b), however rather a few seem to be dependent on coconut for their whole life cycle. As noted by Wilson (1997) the Auchenorrhyncha faunas of east and west Africa are largely distinct with only one or two common species that overlap.

Discussion

The search for any vector species involves a considerable commitment to sampling and analysis. The wider use of molecular techniques has enabled more rapid assessment of the potential of insect species to be the vectors of LY. Clearly much still needs to be discovered. Any control measures might be much more problematic. The time taken to grow and test coconut palms for resistance does not easily allow for a rapid solution to this long standing problem.

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Insect vectors

Investigations on pear psyllids as vectors of '*Candidatus* Phytoplasma pyri' in Austria

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Abstract

Investigations of pear psyllids in four pear orchards in lower Austria showed the presence of *Cacopsylla pyri*, *C. pyricola* and *C. pyrisuga* at all locations. Overall, 5.3% of the *C. pyri*, 3.9% of the *C. pyricola* and 8% of the *C. pyrisuga* individuals carried the '*Candidatus* Phytoplasma pyri'. In transmission experiments the three pear psyllid species were able to transmit the phytoplasma to healthy pear seedlings.

Keywords: pear decline, pear sucker, *Cacopsylla pyri*, *Cacopsylla pyricola*, *Cacopsylla pyrisuga*, transmission

Introduction

Pear decline, associated with the presence of '*Candidatus* Phytoplasma pyri', is one of the most destructive diseases of pear in Europe and North America (Seemüller *et al.,* 2011). Several species of the genus *Cacopsylla* are known or suspected to be involved in the pathogen transmission. *C. pyricola* has been shown to transmit the pathogen in USA and Great Britain, *C. pyri* is a confirmed pathogen vector in the continental Europe, two other species namely *C. pyrisuga* and *C. bidens* have been found to carry the phytoplasma but their vectoring ability was not proven (Jarausch *et al.,* 2019). Out of these, three species, namely *C. pyri*, *C. pyricola* and *C. bidens* are polyvoltine and can be found on pear plants all year round, whereas *C. pyrisuga* is an univoltine species overwintering on conifers. The aim of the current study was to determine the presence of pear psyllids in pear orchards in Austria, and to study their infection rates in the course of the year and their ability to transmit the pathogen.

Materials and Methods

Sampling of plant and insects in pear orchards and determination of pear psyllid population dynamics

Psyllid populations were analysed weekly or every 14 days in four pear orchards in Lower Austria from February 2012 until October 2014 by beating tray method using a white plastic tray (30 x 40 cm) and a mouth aspirator. Ten hits per tree were performed (2 branches, 5 hits per branch) (no sampling in December and January). Captured insects were analysed for phytoplasma presence by PCR.

Cage transmission trials

All insects included in the transmission experiments were field collected as stated above. Foliated pear seedlings grown from seeds in laboratory conditions (cultivar Williams and Bosc's Flaschenbirne) cultivated at 21°C served as test plants. On each test plant 10 individuals were caged and allowed to feed for one week. In case of *C. pyricola* 42 trials, in case of *C. pyri* 53 trials, in case of *C. pyrisuga* 24 trials were carried out. After the transmission trials the test plants were maintained under insect proof conditions in a cold greenhouse. Annually root samples were collected for PCR analysis.

PCR-analysis and phytoplasma characterization

DNA extraction from plant and insect samples was carried out by a CTAB - procedure (Maixner *et al.,* 1995). Phytoplasma presence was determined by nested PCR (primers R16F2/R2, Lee *et al.,* 1993 and R16(X)F1/R16(X)R1, Lee *et al.,* 1995) followed by RFLP using *Rsa*I (Seemüller and Schneider, 2004). For a finer phytoplasma characterisation, multi locus sequence analysis was carried out as previously described (Danet *et al.,* 2011).

Results

Analyses in pear orchards in Austria showed the presence of *C. pyri*, *C. pyricola* and *C. pyrisuga* in all orchards. In case

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of *C. pyri* and *C. pryricola* four generations per year were identified. The overall phytoplasma infection rates were for *C. pyri* 5.3%, for *C. pyricola* 3.9% and for *C. pyrisuga* 8%. In the course of the year, the highest infection rates for *C. pyri* and *C. pyricola* were observed in the late winter-early spring and late summer – autumn. Infected *C. pyrisuga* belonged to the remigrant insect generation. The transmission trials resulted in phytoplasma transmission by all three pear sucker species. Multi locus sequence analysis allowed the conclusion that 2 phytoplasma types were transmitted.

Discussion

Investigations allow the conclusion that *C. pyri*, *C. pyricola* and *C. pyrisuga* are regularly present in the pear orchards in Lower Austria. A significant proportion of all the insect species in all tested orchards was phytoplasma infected and thus there is a constant risk for new infections of trees. The higher rates of infected *C. pyri* and *C. pyricola* observed in the late summer and autumn indicate that the infection rates of the insects depend on the phytoplasma concentration in the trees. Moreover, individuals of the overwintering generation developing in autumn on trees with high phytoplasma concentration might have retained the phytoplasma during the winter months and have accounted for the significant infection rates detected in the early spring. Retention of infectivity over the winter has been previously described (Carraro *et al.*, 2001). The results obtained in the current study show that the three investigated *Cacopsylla* species are able to transmit the '*Ca*. P. pyri' and thus confirm the previous hypothesis of transmission by a number of species (Jarausch *et al.*, 2019).

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Insect vectors

Monitoring psyllid vectors of apple proliferation in South Tyrol (Italy): a multi-year survey

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Abstract

Preventing the diffusion of phytoplasma associated diseases is based mainly on indirect control measures against the transmitting insect vectors. In order to survey the occurrence and infectivity of the apple proliferation vectors *Cacopsylla picta* and *C. melanoneura* an intensive monitoring program was realized in the apple growing region of South Tyrol. A constant decrease of population densities for both psyllid species was observed since 2015, partly correlated to the application of insecticides during the blooming phase. Percentages of about 21% of infected adults of *C. picta* indicate that this vector poses a high risk as a vector of the pathogen even at low densities.

Keywords: apple proliferation, vector, *Cacopsylla* spp., infection rate, population dynamics

Introduction

The quarantine pathogen apple proliferation (AP) is one of the economically most important disease in the European apple cultivations. The agent associated with the disease, '*Candidatus* Phytoplasma mali', induces various specific and unspecific symptoms on leaves and fruits, impairing quality and quantity of yield (Bertaccini and Duduk, 2009; Seemüller *et al.*, 2011). In case of AP, two hemiptera have been identified as the main vectors in spreading the disease: *Cacopsylla picta* (Foerster, 1848) (Frisinghelli *et al.*, 2000) and *C. melanoneura* (Foerster, 1848) (Tedeschi *et al.*, 2004). As the disease is not curable, beside the obligate planting of certified pathogen-free stocks and the eradication of the infected plants, its management is primarily based on insecticide applications during the insect vector presence (Weintraub and Beanland, 2006). In South Tyrol (Alto Adige, Italy), severe outbreaks were documented since the 1990. In 2011, an epidemic increase in several orchards of the South Tyrolean apple production area stimulated the investigation of abundances, population dynamics and infectivity of the involved insect vectors. The aim of this long-term monitoring program was to provide additional data about the biology of the insect vectors in order to develop and evaluate suitable and appropriate phytosanitary control strategies.

Materials and Methods

The survey was performed from 2012 until 2018 in apple orchards in the apple proliferation affected area of Burggraviato and Val Venosta (South Tyrol-Alto Adige, Italy). *Cacopsylla* species were collected using beat tray sampling every 7- 10 days from the end of February till late August. Phenology parameters of the apple trees, as well as the number of sampled branches per site were documented. Captured specimen were sexed and identified at the species level. DNA was extracted from selected adults of *C. picta* and *C. melanoneura,* sampled during 2014 to 2017, using the DNeasy Blood and tissue kit (Qiagen, Hilden, Germany) following the procedure for DNA extraction from insects. The PCR assay for specific detection of AP phytoplasma followed the protocol described in Mittelberger *et al.* (2017).

Results

During the survey a total of 3,679 *C. melanoneura* and 567 *C. picta* were captured by beat tray sampling. Highest densities were documented in 2013, recording an overall density of 47.91 *C. melanoneura* per 1,000 branches sampled and 7.52 adults of *C. picta*, respectively (Figure 1). From 2015 onwards, the population densities of these insect vectors decreased drastically (2015: 0.03 *C. picta*/1,000 branches, 2015: 0.76 *C. melanoneura*/1,000 branches).

Flight periods varied between the years for both *C. picta* and *C. melanoneura*. As the climatic conditions differed among the surveyed periods, vector data recorded from 2012 to 2015 were correlated to the phenology status of the apple trees in the orchards (Figure 2).

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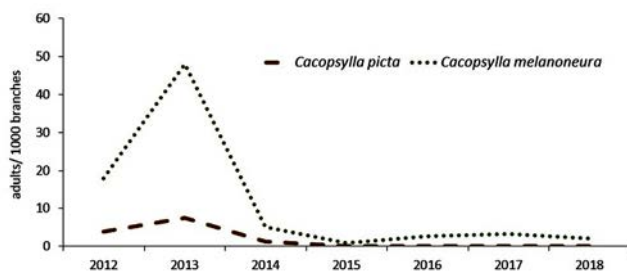
Figure 1. Beat tray captures of AP vectors from 2012 to 2018.
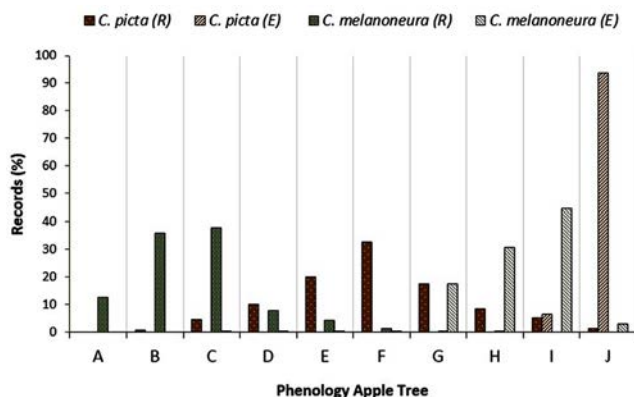
Figure 2. Beat tray captured specimens of *C. melanoneura* and *C. picta* (2014-2018) correlated with the phenology of apple tree registered during sampling (A: dormant status; B-E= developing flowerbuds; F-H: bloom; I-J: fruit setting and development).

Adults of *C. melanoneura* returning from the overwintering sites (remigrants) began to colonise the apple orchards during the dormant status. Highest densities were observed during the first stages of the development of flowering buds (73% of total captures). First individuals of the second generation were found during a tight cluster stage, whereas the highest records were documented during the end of blooming (48%) and the beginning of fruit setting (45%). Remigrants of *C. picta* began to colonise the apple trees with early stages of the development of flowering buds (5%), but the population peak was observed during blooming (59%). Highest densities of the second generation were recorded during the fruit growing (93%). From 2014 to 2017 *C. melanoneura* showed an average infection rate of 1%. In contrast, 21% of the analysed *C. picta* resulted infected with the AP phytoplasma.

Discussion

The field monitoring revealed a constant decrease in abundances for both AP vectors, *C. melanoneura* and *C. picta* since 2015. As the phenology of phytophagous insects are highly correlated to the host plant growth, correlating population dynamics of AP vectors with the growing stage of the apple trees seems appropriate. High densities of *C. melanoneura* were observed at the end of the dormant stadium and in the beginning of flower bud development, whereas the population peak of *C. picta* was highest during the blooming stage. In 2014 the vector control strategy in South Tyrol was extended and an insecticide of the tau-fluvalinate group (non bee-harming) during bloom was applied. The insecticide treatment during this period seemed to have prevented reproduction and efficiently reduced the second generation of *C. picta* and *C. melanoneura*. The laboratory-proven ability of females to transmit the AP phytoplasma to its progeny (Mittelberger *et al.*, 2017), and its high transmission capability shown in laboratory trials are potential reasons for the high percentages of about 21% of infected adults in the field. Despite its status as a sub-resident species in the agroecosystem apple orchard (Fischnaller *et al.*, 2017), *C. picta* can be considered as the main driving force in spreading the apple proliferation disease and thus poses a high potential risk as active vector, even at low population densities.

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Insect vectors

Can polyphagous insect vectors contribute to phytoplasma inventory in diverse ecosystems?

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Abstract

Populations of the polyphagous leafhopper *Euscelis incisus* were screened for natural phytoplasma infection during a period of three years on eight locations in distinct areas of Serbia. Identification and molecular characterization of phytoplasma strains harboured by in-field collected *E. incisus* on diverse habitat types was performed by sequence analysis of 16S rRNA, ribosomal protein genes *rpl22-rps3*, *tuf*, *secY*, *stamp* and *vmp1* genes. Presence of six distinct 'Candidatus Phytoplasma' species was detected in a total of the 8% of the collected *E. incisus* specimens suggesting their tentative role in diverse epidemiological cycles and ecosystems.

Keywords: *Euscelis incisus*, 'Candidatus Phytoplasma' species, multilocus typing, insect vector, epidemiology

Introduction

Phytoplasmas are obligate, wall-less, phloem-limited, prokaryotic organisms belonging to the class *Mollicutes*. Classification of phytoplasmas is primarily based on molecular analyses of highly conserved 16S rRNA gene sequences (Lee *et al.*, 2010). Multilocus typing on other more variable genes (*e.g.* ribosomal protein *rpl22-rps3*, elongation factor *tu-tuf*, and protein translocase - *secY*) are useful for more accurate characterization of the strains and the definition of pathways in epidemiological studies (Arnaud *et al.*, 2007; Lee *et al.*, 2010; Jovic *et al.*, 2011; Cvrkovic *et al.*, 2014; Krstic *et al.*, 2018).

The vector role is crucial for phytoplasma dispersal through ecosystems, while the knowledge about this chain in vector transmitted plant diseases is usually insufficient. Information on natural phytoplasma presence in potential hemipteran vectors thus represents the first step in elucidating their role in spreading through the environment.

Euscelis incisus (Kirschbaum, 1858) is a widespread European species from the subfamily Deltocephalinae. It most commonly inhabits sunny, moderately dry habitats, mostly meadows, pastures and abandoned fields, gardens and urban parks (Nickel, 2003). The wide distribution of *E. incisus*, combined with its ability to inhabit ecologically diverse ecosystems, polyphagous behaviour and continuous presence at sites over the year due to two partly overlapping generations, makes this leafhopper suitable to exchange phytoplasmas in diverse plant species. Natural vector role of this leafhopper was confirmed by the transmission of the 16SrI-C phytoplasma to white clover in Lithuania (Ivanauskas *et al.*, 2014) and 16SrIII-B phytoplasma to *Cirsium arvense* in Serbia along with its epidemiological importance in spreading multiple inflorescence pathogens on creeping thistle (Jakovljevic *et al.*, 2015).

In previous studies on the territory of Serbia, a significant number of *E. incisus* has been observed on pastures around "bois noir" affected vineyards, corn fields affected by maize redness and potato fields affected with "stolbur" (Cvrkovic *et al.*, 2014; Jovic *et al.*, 2009; Mitrovic *et al.*, 2016). Based on these findings, it is assumed that *E. incisus* can be significant as a possible vector in the epidemiology of various phytoplasmas and phytoplasma-associated diseases. The aim of this study was to explore the presence of different phytoplasmas in *E. incisus* in Serbia.

Materials and Methods

The survey was carried out a during period of three years, on eight localities in Serbia where considerably numerous populations of *E. incisus* were recorded. Insects were collected using standard sweep nets and mouth aspirators, stored in 2 ml cryovials (Sarstedt, Numbrecht, Germany) filled with 96% ethanol and kept at 10°C until the return to the laboratory where they were identified with a stereomicroscope (Leica MZ7.5), according to the external morphological features and features of the male genital armature, using the taxonomic keys provided by

Biedermann and Niedringhaus (2004). After identification, the insects were stored in ethanol at −20°C until DNA extraction. DNA was extracted from individual insect specimens following a non-destructive extraction method (Kosovac *et al.*, 2018). Phytoplasma identification and molecular characterization were carried out in: i) the 16S ribosomal RNA gene; ii) the genes coding ribosomal proteins *L22* and *S3*; iii) the *secY* gene region, and iv) the *tuf* gene, the antigenic membrane protein gene *stamp* and the variable membrane protein *vmp1* gene of the "stolbur" phytoplasma. Amplification reactions were performed by direct and nested PCR protocols. All the sequences were aligned with strains retrieved online from the NCBI. The phylogenetic analyses using maximum parsimony were conducted with MEGA 7 software (Kumar *et al.*, 2016).

Results

The insects were collected from the beginning of April until the end of September, during a three year period, from eight distinct areas with diverse habitat types, including vineyard regions, potato fields, meadows, slopes and abandoned pastures. Plant communities were multifarious, with significant presence of species belonging to the Fabaceae, Poaceae, Asteraceae and Plantaginaceae. In total, 8% of collected *E. incisus* specimens were infected with six '*Candidatus* Phytoplasma' species, which included: '*Ca*. P. solani*-, *'Ca*. P. asteris'-, '*Ca*. P. aurantifolia'-, '*Ca*. P. pruni'-, '*Ca*. P. phoenicium'- and '*Ca*. P. oryzae'-related strains. The prevalent was the "stolbur" phytoplasma with 3% of positive specimens from five localities. '*Ca*. P. asteris'-related strains were detected in specimens from three localities, while '*Ca*. P. phoenicium'-related strains were found at two localities. Strains related to '*Ca*. P. aurantifolia', '*Ca*. P. pruni', and '*Ca*. P. oryzae' were detected in naturally infected *E. incisus* from a single locality.

Discussion

E. incisus is a polyphagous species, numerous on plants of spontaneous flora in the immediate vicinity of agroecosystems, which serve as reservoirs of various phytoplasmas. In the south-eastern Europe this species overwinters mostly in the adult stage (Jakovljevic *et al.*, 2015), and it is present in the ecosystems from the end of November until March, with two generations per year, referred to as winter and summer generations. The ability to carry six phytoplasma groups launches this leafhopper as potential bio-indicator of the phytoplasma presence in diverse ecosystems which may have significance in various epidemiological cycles and spreading of these plant pathogens and could be a useful tool in prediction and management of phytoplasma associated diseases. However transmission assays are needed to assess its vector role in spreading the diverse phytoplasmas that resulted to be carried by this insect species.

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Insect vectors

Molecular identification and characterization of phytoplasmas in insect vectors of chickpea phyllody disease in Punjab, Pakistan

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Abstract

This study was conducted to identify the incidence of a chickpea disease consisting in phyllody, virescence and proliferation, its association with phytoplasmas and its insect vector(s). The presence of phytoplasmas in leafhopper species and chickpea plants was verified by nested PCR amplification of 16S rDNA. *Orosius albicinctus, Circulifer haematoceps* and *Neoaliturus fenestratus* resulted phytoplasma positive. The sequence of the detected phytoplasma strain exhibited the maximum nucleotide identity (99.9-100%) with peanut witches' broom phytoplasmas, 16SrII-A subgroup.

Keywords: chickpea, insect vectors, 16SrII-A, phytoplasmas, Pakistan

Introduction

Chickpea (*Cicer arietinum* L.) is a vegetable crop of high relevance in Pakistan. This crop is cultivated in three regions including temperate, tropical and subtropical areas. Among the biotic factors, phytoplasma diseases mainly phyllody is a limitation in farming. The same phyllody disease has been reported to infect chickpea crops in different countries including Australia, Oman and Pakistan (Saqib *et al.*, 2005; Al-Saady *et al.*, 2006; Akhtar *et al.*, 2009) and phytoplasmas enclosed in group 16SrII were detected in the majority of the countries. The aim of this study was to research potential insect vectors of chickpea phytoplasma in Pakistan.

Materials and Methods

Field surveys and sample collection

During 2016, samples were collected from ten chickpea-growing areas of Faisalabad, Rahim Yar Khan and Khanewal districts. Visual examination of 1,000 plants following a W-pattern was done and plants having leaves and exhibiting the phytoplasma symptoms (Figure 1) were collected. The collection of insects was done with the help of aerial net on symptomatic chickpea plants.

Transmission tests

The collected insect captured in infected chickpea fields were maintained on newly emerged chickpea plants in insect proof cages. Then, 30 individuals of every insect species were caged on symptoms showing plants for 48 hours of acquisition periods. Insects (20) from every species were moved in batches of five on overall 20 test plants (three week-old healthy chickpea seedlings). Disease symptom appearance was verified on daily basis. Individual plants (20) were maintained without insect as control.

Figure 1. Phytoplasma infected chickpea plants.

Phytoplasma detection and identification

Extraction of DNA was carried out from insects and plants samples by a CTAB protocol (Doyle and Doyle, 1990) and PCR processed as reported (Ahmad *et al.*, 2017). Direct PCR was carried out with universal primer pair P1/P7 (Deng and Hiruki 1991; Schneider *et al.*, 1995) while in nested PCR primers pair RI6F2n/R2 (Gundersen and Lee, 1996) were

used for phytoplasma detection. Nested-PCR products were digested and the resulting RFLP patterns compared with those available in the pertinent literature. Selected nested PCR amplicons were sequenced from both plants and leafhopper samples and sequences were aligned with Lasergene v. 7.1 software; homology and phylogenetic studies were performed with the MEGA 6 software.

Results

Nested PCR product of the 16S rRNA gene (1.25 kb) allow to confirm the phytoplasma presence in the symptomatic chickpea plants and field-collected insects including Jassid (*Orosius albicinctus, Circulifer haematoceps* and *Neoaliturus fenestratus*) while no phytoplasma detection was achieved in healthy chickpea plant samples, whitefly (*Bemisia tabaci*), aphid (*Myzus persicae*), *Empoasca devastans* and some other unidentified brown plant hoppers. The phytoplasma-positive insect species were used in the transmission trials. After 8 weeks of incubation chickpea plants fed on by the leafhopper species exhibited phytoplasma symptoms and resulted positive in nested PCR while the control plants resulted negative. Several restriction enzymes (*Alu*I, *Hpa*I, *Hinf*I, and *Rsa*I) were used for RFLP of nested PCR products and showed identical patterns from inoculated chickpea plants and leafhoppers consistent to the profile of 16SrII-D strain of sesame phyllody phytoplasma from India (Pamei and Makandar, 2016). The sequence of the chickpea phyllody strain exhibited 99.9-100% of nucleotide identity to peanut witches' broom strain (GenBank accession number L33765) 16SrII-A subgroup.

Discussion

Based on main symptoms, PCR positive plant and insects, results of RFLP and sequence analysis the association of chickpea phyllody disease with phytoplasmas in group 16SrII was confirmed. In the current experiments it was also demonstrated that *O. albicinctus, C. haematoceps* and *N. fenestratus* are the potential reservoir and vectors of the phytoplasma infecting chickpea plants. Of these three species *O. albicinctus* is fairly abundant, and its population exhibit the highest prevalence in phytoplasma diseased chickpea crops. Phytoplasmas of the same group were already reported as associated with chickpea phyllody disease in Pakistan (Akhtar *et al.*, 2009). Because of the widespread existence of insect vectors, the phyllody diseases associated with 16SrII phytoplasmas were reported in different crops in Pakistan, including sesame (Akhtar *et al.*, 2008; Ahmad *et al.*, 2015a), tomato and *Parthenium* (Ahmad *et al.*, 2015a, 2015b). Salehi *et al.* (2015) reported *Orosius albicinctus* as the potential vector of 16SrII-D subgroup phytoplasma associated with squash phyllody in Iran. Moreover, Ikten *et al.* (2014) reported *O. orientalis* as the vector of the same phytoplasma for the sesame phyllody disease in Turkey. *N. fenestratus* was reported to transmit lettuce and wild lettuce phyllody in Iran (Salehi *et al.*, 2007) and this insect was also described as vector for transmission of 16SrII strains associated with bushy stunt of hawkweed oxtongue in Serbia (Mitrovic *et al.*, 2012). The vector status of *C. haematoceps* for transmission

of *Spiroplasma citri* has also reported since almost 50 years in USA, however it was never reported as vector for phytoplasmas. More investigations regarding insect ecology, biology and phytoplasma associations should be achieved to devise and implement the appropriate strategies for the management of this disease in Pakistan.

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Insect vectors

Identification of sesame phyllody transmitting insect vectors in Assam, India

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Abstract

Different leaf hopper species transmitting sesame phyllody phytoplasma were reported. Leaf hoppers were collected from sesame phyllody infected field at different time interval and their population was recorded. Four leaf hopper species were predominantly observed in the field conditions: *Hishimonus phycitis*, *Exitianus indicus*, *Cofana unimaculata* and *Nephotettix nigropictus*. Transmission studies were carried out by giving an acquisition and inoculation feeding period of 7 days each. Among all the leaf hoppers, *H. phycitis* was found to transmit sesame phyllody phytoplasma (83.33%) to healthy sesame plants as confirmed by PCR and nested PCR assays followed by sequencing. The BLAST analysis of 16S rDNA sequences from *H. phycitis* confirmed the phytoplasma presence indicating that is clusters with the aster yellows phytoplasmas.

Keywords: sesame phyllody, phytoplasma, transmission, leaf hoppers, *Hishimonus phycitis*

Introduction

Sesamum indicum L. is one of the oldest and important oilseed crops in the world. Sesame phyllody disease associated with the presence of phytoplasmas is considered as one of its most important diseases, causing yield loss of 5-15% in the world. This disease is transmitted by leaf hoppers especially *Orosius albicinctus* (Dist.) but recently *Hishimonus phycitis* (Dist.) was also found to be a vector (Nabi *et al.*, 2015). The leaf hoppers feed in the phloem of infected plants and acquire phytoplasmas passively which accumulate in acinar cells of salivary gland from which they are transmitted to healthy plants. Therefore, vector screening is a fundamental step in the development of management strategies for this disease. In this study, an effort was made to identify the leaf hoppers present in sesame field and to verify their vector ability.

Materials and Methods

The leaf hopper species feeding on sesame fields were collected during March - November 2017 by net sweep in the morning and evening hours, kept in plastic bags and stored at -20ºC. For the insect transmission seeds from healthy plants were sown in pots and placed in an insect free net house. Three plants were maintained in each pot. The predominant leaf hoppers in the sesame field were used in the transmission test. Five leaf hoppers of each species were given acquisition feeding on phyllody infected plants for seven days in insect rearing cages and then inoculation feeding on the healthy sesame plants for seven days. The leaf-hoppers after the test were killed using insecticide and stored at -20ºC. Inoculated plants were continuously monitored for time required for appearance of symptoms.

Total DNA from all sesame plants was extracted by following the protocol of Kollar *et al.* (1990). The DNA of leaf hoppers used in the transmission studies was extracted using a modified procedure from Marzachì *et al.* (1998). For direct and nested PCR, primers and PCR reaction mixtures were done according to Sertkaya *et al.* (2007). The PCR products were resolved on 1.5% agarose gel in 1X Tris EDTA (TAE) containing ethidium bromide (Sambrook and Russell, 2001). The nested PCR products of insects showing positive results were sent for sequencing from which a contig was prepared in a DNA baser software. Similarity of the sequence was checked with other strains using NCBI BLAST. Phylogenetic analyses were done using MEGA 7 software.

Results

The leaf hopper species identified were *Hishimonus phycitis* (Dist.), *Exitianus indicus* (Dist.), *Cofana unimaculata* (Dist.) and *Nephotettix nigropictus* (Stal.) (Figure 1). The leaf hopper populations counting in each month from March to November

Table 1. Efficiency of different leafhoppers in transmitting the sesame phyllody disease under experimental inoculation conditions.

Leafhoppers	Inoculated plants	Symptomatic plants	Symptoms	Days for symptoms appearance	% transmission
Hishimonus phycitis	12	10	Yellow stunted leaves	28-35	83.33
Exitianus indicus	12	0	No	No	0
Cofana unimaculata	12	0	No	No	0
Nephotettix nigropictus	12	0	No	No	0

2017 indicate that *H. phycitis* and *E. indicus* have the highest population (Figure 2). The results of insect transmission indicate that only *H. phycitis* could transmit the disease to the healthy sesame plants (Table 1 and Figure 3).

Results of the nested PCR assays for *H. phycitis* are shown in Figure 4a. In the BLAST analysis the sequences of the nested PCR products showed high sequence similarities among each other and in the phylogenetic analysis, the phytoplasma detected in of *H. phycitis* clustered together with a phytoplasma strain from *Brassica oleracea* from Italy (GenBank accession number JQ181539) classified as aster yellows (Figure 4b).
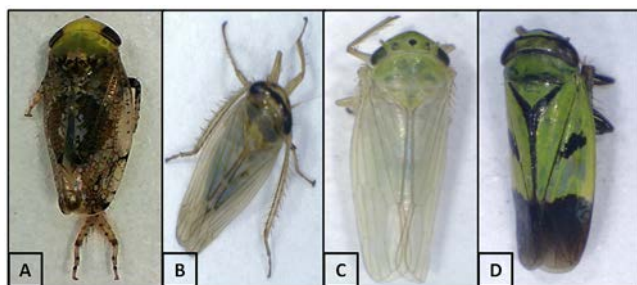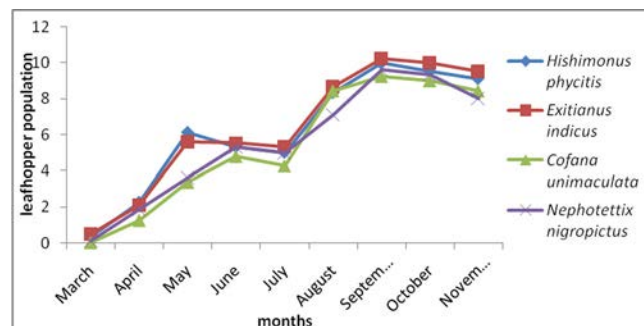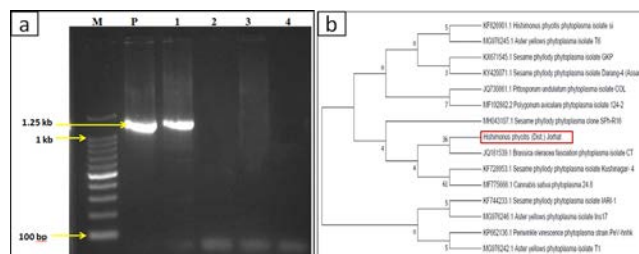
Figure 4. (a) Nested PCR amplification of 16Sr DNA from leafhoppers. M, 100 bp DNA ladder; P, sesame phyllody infected plant; 1, *H. phycitis*; 2, *E. indicus*; 3, *C. unimaculata*; 4. *N. nigropictus*. (b). Phylogenetic relationships of the phytoplasma from *H. phycitis* with known strains (maximum composite likelihood method).

Discussion

In this study the presence of leafhoppers around the year in sesame fields has been analysed. From the data achieved the dates of sowing can be altered to reduce the epidemic of sesame phyllody. From the transmission tests, *H. phycitis* has been recognised as a new vector of sesame phyllody for the North east region of India together with *Orosius albicinctus* which was earlier identified and described as vector by Gogoi *et al.* (2017).

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Figure 1. (A) *Hishimonus phycitis,* (B) *Exitianus indicus,* (C) *Cofana unimaculatus,* (D) *Nephotettix nigropictus.*

Figure 2. Leafhoppers collected in sesame fields, 2017.

Figure 3. Symptomatic sesame plants 35 days after transmission.

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Insect vectors

Experimental and molecular evidence of *Neoaliturus fenestratus* role in the transmission of "stolbur" phytoplasma to lettuce and carrot plants

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Abstract

The vector status of the deltocephalinae leafhopper *Neoaliturus fenestratus* harbouring 'Candidatus Phytoplasma solani' ("stolbur") was tested in two separate transmission trials with seedlings of lettuce and carrot. In both tests, the leafhopper successfully transmitted the "stolbur" phytoplasma to the exposed plants, which expressed partial leaf discoloration and rolling and rapid plant desiccation. Molecular characterization of the *stamp* gene encoding the antigenic membrane protein determined the presence of three "stolbur" phytoplasma strains in field collected *N. fenestratus* and in the insect inoculated plants. The same genotypes of phytoplasma shared by the vector samples and the exposed lettuce and carrot seedlings confirm the ability of *N. fenestratus* to transmit the "stolbur" phytoplasma to the tested plants. These findings indicate a possible role of *N. fenestratus* in the epidemiology of the "stolbur" phytoplasma in vegetable crops.

Keywords: *Neoaliturus fenestratus*, "stolbur" phytoplasma, insect vector, *stamp* gene

Introduction

The leafhopper *Neoaliturus fenestratus* (Herrich-Schäffer, 1834) is a widely distributed species in Europe inhabiting abandoned fields and vineyards and sparsely vegetated meadows, mostly found on different plants from the family Asteraceae (Nickel, 2003). It is reported as vector of the safflower phyllody associated with phytoplasmas in the 16SrI-B subgroup (Raccah and Klein, 1982). Salehi *et al.* (2007) documented its ability to transmit also phytoplasmas belonging to the 16SrIX group *i.e.* lettuce phyllody and wild lettuce phyllody. Furthermore, *N. fenestratus* was also confirmed as a vector of the *Picris hieracioides* bushy stunt, a phytoplasma in 16SrII-E subgroup (Mitrovic *et al.*, 2012) and chicory phyllody phytoplasma of the 16SrIX-C subgroup (Ermacora *et al.*, 2013).

The host plants of the "stolbur" phytoplasma ('Candidatus Phytopalsma solani') include diverse herbaceous and woody species, crops and native flora. *N. fenestratus* has been reported in association with many plant hosts of "stolbur", in particular in the wild compartments and detected to carry "stolbur" phytoplasma in vineyards across Europe (Batlle *et al.*, 2000; Orenstein *et al.*, 2003; Riedle-Bauer *et al.*, 2006).

Due to polyphagous feeding preferences and its vector status for diverse phytoplasmas, *N. fenestratus* represented a good candidate to investigate for the capacity to transmit the "stolbur" phytoplasma to lettuce and carrot, one of the main vegetable crops of great economic importance in Serbia.

Materials and Methods

In late August 2014, adults of *N. fenestratus* were sampled in locality Jasenovik in South Serbia in abandoned vineyards with the predominant weeds represented by *Picris hieracioides* and *Crepis foetida* (Asteraceae), previously confirmed to harbour the "stolbur" phytoplasma. Two separate transmission trials were set up, with lettuce (*Lactuca sativa*) and carrots (*Daucus carrota*) seedlings. A total of 163 *N. fenestratus* were separated in groups of six to 21 specimens and released on seedlings which were daily changed until there were no surviving adults. All exposed plants were kept in a growth chamber for the following two months and monitored for symptoms development, after which the leaves and roots were sampled and together with leafhopper specimens analysed for phytoplasma presence.

Initial molecular identification was based on a nested PCR analysis with 'Ca. P. solani'- specific stol11F2/R1 and

R16 F3/R2 primers; the phytoplasmas detected in the positive samples were then characterized by amplifying and sequencing the *stamp* gene encoding the antigenic membrane protein by nested PCR with the primers pair stampF/R0 followed by StampF1/R1 (Mitrovic *et al.*, 2016).

Results

In total 95 *N. fenestratus* specimens were included in the first trial and offered fresh carrot seedlings in six series (Table 1). Overall, 21 plants were exposed, of which six were positive for "stolbur" phytoplasma presence (29%). BLAST analysis determined identity of the *stamp* genotypes in carrots comparing them with the reference strains either Rqg31, STOL or BG4560 (Table 1). The same three genotypes of "stolbur" phytoplasma have also been identified from the analysed leafhoppers.

In the second trial, 22 lettuce seedlings were exposed in five series to 68 *N. fenestratus*. The "stolbur" phytoplasma was detected in six plants (27%), with five *stamp* sequences identical in the strain STOL and one to Rqg31 (Table 1). On the other hand, the three genotypes identified as STOL, Rqg31 and BG4560 were detected in the insect vector specimens. Two months after the exposure, all carrot and lettuce seedlings tested positive for "stolbur" phytoplasma expressed partial leaf discoloration, leaf rolling and rapid plant desiccation.

Table 1. Results of transmission trials with *N. fenestratus*.

	Trial 1/carrot	Trial 2/lettuce
No of series in trial/ No of exposed plants per series	6/1-6	5/3-6
Total No of exposed plants/ No of plants tested positive (%)	21/6 (29%)	22/6 (27%)
Stamp genotypes detected/ No of positive plants	Rqg31/2 STOL/3 BG4560/1	Rqg31/1 STOL/5
Total No of leafhoppers in trial/ No of leafhoppers per exposed plant	95/7-21	68/6-21
No of leafhoppers tested positive (%)	55 (58%)	24 (35%)
Stamp genotypes detected/ No of positive leafhoppers	Rqg31/20 STOL/30 BG4560/5	Rqg31/4 STOL/16 BG4560/4
Stamp genotypes detected/ No of positive leafhoppers	Rqg31/20 STOL/30 BG4560/5	Rqg31/4 STOL/16 BG4560/4

Discussion

In spite of its wide distribution in Serbia *N. fenestratus* has not been investigated as "stolbur" vector. The species mostly inhabits abandoned vineyards and grasslands, commonly formed by plant species harbouring the "stolbur" phytoplasma. The '*Ca*. P. solani' genotypes identified in the field sampled leafhopper specimens (STOL of the cluster b-III, and Rqg31 and BG4560 of the cluster b-II) have been previously reported in association with diverse native plants and crops in Serbia (grapevine, maize, potato). *Hyalesthes obsoletus* and *Reptalus panzeri* are the main vectors involved in "stolbur" transmission in crops and vineyards in Serbia, with several confirmed and as many assumed epidemiological cycles (Mitrovic *et al.*, 2016). Due to its polyphagous host preferences, wide distribution and vector status, *N. fenestratus* should be considered as a leafhopper species of interest for further investigation on its vector role in the epidemiology of "stolbur" phytoplasma and disease dissemination within the native compartment and subsequently into vineyards and other crop systems, including vegetables.

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Insect vectors

Genetic variability of *Scaphoideus titanus* populations and a new host plant of 16SrV group phytoplasmas in Bosnia and Herzegovina

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Abstract

During surveys for "flavescence dorée" phytoplasma and *Scaphoideus titanus* presence in vineyards of the Republic of Srpska in 2018, the life cycle of the leafhopper and a genetic study of two populations present in northwest and southeast vineyards were examined. *S. titanus* nymphs and adults were monitored using yellow sticky traps. The first nymphs were found in the last week of May; adults occurred from the end of June to the end of October. A 623 bp fragment of the mitochondrial cytochrome oxidase II gene showed a high dominance of the previously reported haplotype i. Nested PCR analyses on grapevine, *Clematis*, *Ailanthus* and *Alnus* species collected inside and outside the vineyards demonstrated the presence of a 16SrV group phytoplasma in one sample of *A. altissima*.

Keywords: vector, mitochondrial haplotype, nested PCR, 16SrV phytoplasma group

Introduction

Scaphoideus titanus Ball (Hemiptera: Cicadellidae: Deltocephalinae), the main vector of the quarantine- regulated "flavescence dorée" phytoplasma (FD), is a Nearctic leafhopper native to North America which was accidentally introduced to Europe where it was first reported from vineyards in France, after, it was also reported from other European countries (Chuche and Thiery, 2014). Surveys and laboratory analyses performed since 2005 have not detected the presence of FD phytoplasma in grapevine in Bosnia and Herzegovina (BiH) although the presence of *S. titanus* was found in all the grapevine growing areas of the country. The results of the first survey for *S. titanus* conducted in 2017, indicated the presence of the leafhopper in grapevine production regions in BiH (Radulovic *et al.*, 2018). Therefore, in 2018 a new extended survey was done in order to examine *S. titanus* population dynamics and the life cycles in the two climatically different grapevine growing regions (Mediterranean and moderate continental). Moreover, genetic variability studies of the two populations were also performed. Finally, the latest results of the survey of the FD phytoplasma in grapevine and alternate host plants are reported.

Materials and Methods

Field surveys for insects and mitochondrial COII genotyping

The research was carried out during 2018 in two vineyards located in northwest BiH, (Prnjaor, 445242 N 174507 E), and southeast BiH, (Trebinje, 424042N, 183239E). In both vineyards an abundant population of *S. titanus* was observed during the preceding summer (Radulovic *et al.*, 2018). Woody grapevine material was collected during the winter pruning period in February-March. Canes were inspected for the presence of *S. titanus* eggs using a dissecting microscope. Visual inspection (5 leaves per transect row) started in the second week of May and continued until the middle of June when the presence of larval instars was first observed. Additionally, the sampling of *S. titanus* nymphs and adults was made using yellow sticky traps (Bug-Scan, Biobest, 25 x 40 cm). Four traps were placed inside the grapevine canopy, approximately 30-50 cm above the ground and checked every two weeks from May to October. All the captured *S. titanus* individuals were identified and counted. The population dynamics were based on nymph and adult captures on the sticky traps. During July, the branch beating method was used to collect adults for the genetic variability study, they were immediately placed in 96% EtOH, in which they were stored until DNA extraction. Total DNA was extracted from 9 individual insects (4 from Trebinje and 5 from Prnjavor) using DNeasy Blood and Tissue Kit (Qiagen, Germany). The mtDNA fragment containing a portion of tRNA^{LEU}-COII genes was amplified by PCR with M13/pUC primers (Papura *et al.*, 2014). The 623 bp PCR products were sent for sequencing to Macrogen Europe.

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Clematis vitalba-sourced ''flavescence dorée" phytoplasmas and *Wolbachia* in naturally infected populations of *Dictyophara europaea*

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Abstract

Screening for "flavescence dorée"-related phytoplasmas (FD) sourced by *Clematis vitalba* as reservoir plant and *Wolbachia* infection in *Dictyophara europaea* populations was carried out in south eastern Europe and north Italy. Both FD and *Wolbachia* were characterized by multilocus sequence typing (MLST). All FD infected populations from Serbia and Italy share the identical *Wolbachia* strain *w*Eur. Populations from Montenegro and Serbia differed by very high FD infection rate in the absence of *Wolbachia* (De*w*Eur-) and low FD infection (ca. 3%) in the presence of *Wolbachia* (De*w*Eur+), respectively. These populations did not differ in vectoring abilities and shared the most reported FD MLST genotype. Additionally, *C. vitalba* as FD source plants exhibited similarly high infection rate at both locations. All molecular diversity parameters of *D. europaea* populations were found to be lower in Serbia and Italy where De*w*Eur+ populations are present. No evidence of direct fitness effects on *D. europaea* caused by *Wolbachia* infection was registered. Bacterial competition for limited resources in De*w*Eur+ populations and an absence of competition in De*w*Eur- populations, suggests that bacterial interactions have shaped the observed discrepancy in FD infection rates in the current source-plant pathosystem.

Keywords: *Dictyophara europaea*, 16SrV-C phytoplasma, *Clematis vitalba*, *Wolbachia*, interaction

Introduction

Dictyophara europaea (L.) (Dictyopharidae) is a widespread polyphagous planthopper of economic importance regularly found in association with the climbing shrub *Clematis vitalba* (Filippin *et al.*, 2009; Krstic *et al.*, 2016). *C. vitalba* frequently occupies the vineyard ecosystems and acts as a reservoir plant of the "flavescence dorée" phytoplasma (FD), subgroup FD-C (Filippin *et al.*, 2009). Epidemiological studies of *D. europaea* as an alternative vector of FD in south eastern Europe, revealed populations naturally infected with *Wolbachia*, the most widespread endosymbiont present in arthropods, known for various reproductive manipulations of the host (Werren, 1997). Detection of *Wolbachia* presence in natural populations of *D. europaea* and multifold differences in FD infection levels between geographically distant populations was the starting point for this study (Krstic *et al.*, 2018). Therefore, differences in the genetic properties of diverse populations of *D. europaea*, *C. vitalba* infection rates and harbored phytoplasma genotypes were examined as a possible cause of the observed discrepancy in FD infection rate, as well as *Wolbachia* presence status

and effects, to understand possible interactions between the bacteria in the current source plant pathosystem.

Materials and Methods

For the genetic population studies *D. europaea* specimens were collected from locations in Serbia, Montenegro, North Macedonia and Greece, moreover previously described DNA material from Italy was used (Filippin *et al.*, 2009). For the phytoplasma transmission trials, insects rearing procedures and comparative analysis of fitness characteristics, *D. europaea* adults were collected from two locations, the village of Nudo in western Montenegro, where populations of *D. europaea* were found to harbour more than 60% FD (Krstic *et al.*, 2018). The second location was near Aleksandrovac in central Serbia. This latte insect population was found to harbour FD at low percentages (ca. 3%; Filippin *et al.*, 2009). *C. vitalba* plants were randomly sampled within each of the two locations. Transmission trials were conducted to grapevine seedlings using naturally FD infected planthoppers from Montenegro, collected on *C. vitalba*. Population genetics analyses of *D. europaea* were based on

the mitochondrial *COI* and the nuclear *ITS2* genes as markers. Phylogenetic relationships of haplotypes were visualized through network reconstruction by applying median-joining and statistical parsimony methods. Haplotype and nucleotide diversity estimates as well as association between mt*COI* variations and different hierarchical levels of tentative causes of variability (*Wolbachia*, FD infection and geography) were assessed by analysis of molecular variance (AMOVA). Identification and characterization of FD phytoplasmas in insect and plant material was performed using FD9 nested PCR and 16S rRNA, *rpl22-rps3*, *secY* and *map* gene typing, respectively. Identification of *Wolbachia* presence was carried out using *ftsZ* gene and subsequent characterization using *wsp* gene and standard *Wolbachia* MLST genes (*ftsZ*, *coxA*, *fbpA*, *hcpA*, and *gatB*). *Wolbachia* effects on fitness components of *D. europaea* (fecundity, longevity and body weight) were tested in outdoor control conditions using reared populations of *D. europaea* naturally infected with *Wolbachia* (De*w*Eur+) and compared to the uninfected population (De*w*Eur-).

Results

Among all *D. europaea* populations collected in south eastern Europe, FD was found only in specimens on locations where the insects were associated with *C. vitalba*. Combined results for Serbia and Italy from 2002 to 2015, revealed a low FD-infection rate of approximately 3%. By contrast, in Montenegro, significantly infected populations were found in the monitoring from 2008 harboring FD at high levels (higher than 60%). Additionally, specimens used in the transmission trials were infected at a rate of 61.7% and all the grapevine plants inoculated with *D. europaea* exhibited symptoms of phytoplasma infection and tested positive for FD. Sampled source plant *C. vitalba* showed a similarly high infection rate of approximately 30%, both in Serbia and Montenegro. Five FD-C MLST genotypes were identified among the strains detected in *D. europaea* and *C. vitalba*. Three genotypes were found only in strains from Montenegro and one in Serbia, while the most widespread and frequent was the G1 genotype found in both locations and in both host plants. Screening for *Wolbachia* presence revealed its presence in six out of eleven populations sampled from Serbia and in all populations from Italy. In contrast, *Wolbachia* was not detected in any of the populations originating from Montenegro. The *Wolbachia* colonizing *D. europaea* belongs to the supergroup B, and it is identified as Deur_B_wEur (*w*Eur). Sequencing of mt*COI* gene revealed the presence of 26 haplotypes across south eastern Europe and Italy. The Montenegro population exhibited all diversity parameters higher than De*w*Eur+ populations from Serbia and Italy. AMOVA results revealed the presence of a significant difference when the populations were grouped according to geography. *Wolbachia* and FD presence in the populations accounted for a low percentage of variation, although statistically significant. No evidence of direct fitness effects on *D. europaea* was registered with regard to *Wolbachia* presence.

Discussion

This study shows that *D. europaea* specimens from FD-infected populations De*w*Eur+ and De*w*Eur- do not differ in FD vectoring abilities. Only subtle differences in phytoplasma genotypes infecting each population were recorded, while in general insects and source-plant strains were found to be infected with the same most frequent and widely distributed FD genotype G1, indicating the exclusion of phytoplasma as a cause of differentiation between these two populations of *D. europaea*. The MLST characterization of FD present in *C. vitalba* and *D. europaea* revealed the existence of new and previously described genotypes from diverse hosts in Europe. This indicates an overlapping or intercrossing of epidemiological routes and transmission pathways of FD. The haplotype networks showed a high frequency of haplotypes associated with *Wolbachia* in the respective populations, suggesting the presence of mitochondrial sweep patterns. All parameters of molecular diversity were found to be lower in Serbia and Italy where De*w*Eur+ populations are present. Geographically isolated Serbian and Italian populations exhibit the same FD infection rates and share identical *Wolbachia* strain *w*Eur. Bacterial competition for limited resources in De*w*Eur+ populations and an absence of competition in De*w*Eur- populations, suggests that bacterial interactions have shaped the observed discrepancy in FD infection rates among the populations tested. These findings highlight the need for further research on endosymbionts and their interactions with phytoplasmas and insect vectors.

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Interaction

Phytoplasma inoculum titre and inoculation timing influence symptom development in newly infected plants

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Abstract

Previous study unveiled that a single phytoplasma infection may induce mutually distinct symptoms in a single host plant. Four sequentially-developed symptoms were identified in potato purple top (PPT) phytoplasma-infected tomato plants. The symptoms included big bud (BB), cauliflower-like inflorescence (CLI), disrupted sympodial growth patter (DSGP), and witches' broom growth (WB). In the present study tomato seedling were graft-inoculated at different developmental stages and with PPT phytoplasma inocula of different titres. The results indicated that both phytoplasma inoculum titre and inoculation timing could influence the symptom development in infected plants. Findings from the study support the notion that the type of symptoms in any given shoot is determined by the developmental stage of the shoot apex when it became infected by the phytoplasma.

Keywords: phytoplasma, inoculum titre, inoculation timing

Introduction

Phytoplasmas are insect-transmitted, cell wall-less bacteria that parasitize plant phloem sieve elements. Nearly a thousand diverse plant species are known to be susceptible to phytoplasma infections and develop diseases with abnormal plant growth patterns (Weintraub and Beanland, 2006). The Columbia Basin potato purple top (PPT) phytoplasma, also known as beet leafhopper transmitted virescence agent (BLTVA), was identified from diseased potato plants in Pacific Northwest, USA (Munyaneza, 2005). In a previous study four mutually distinct symptoms that were sequentially induced by potato purple top (PPT) phytoplasma infection in tomato plant were described. The four identified symptoms are big bud (BB), cauliflower-like inflorescence (CLI), disrupted sympodial growth pattern (DSGP), and witches' broom (WB). In that study the PPT phytoplasma infection was established by graft inoculation. Newly developed branches that exhibit typical WB symptom were used as inocula (scions), and four-leaf stage tomato seedlings were used as host (rootstocks) (Wei et al., 2013). In the present study, new scion/rootstock combinations for graft inoculation were employed and investigated whether the inoculum titre and inoculation timing play a role in the symptom expression in plants.

Materials and Methods

In this study PPT phytoplasma infection was established by a single graft-inoculation. Symptomatic young branches (YB)

or single leaves (SL) were used as PPT phytoplasma inocula (scions). The cultivar Money Maker tomato seedlings of four-leaf (4L) and ten-leaf (10L) stages were used as the host (rootstocks). Titres of YB and SL inocula were about 19.25±0.46 ng/µl and 0.87±0.07 ng/µl, respectively as determined by a separate study (W. Wei et al., unpublished). The infectious scions were grafted onto the primary stem of healthy tomato seedlings. The PPT phytoplasma infections in tomato plants were established with four different combinations of inoculum titre and inoculation timing: i) high-titre inoculum/early inoculation (YB/4L), ii) high-titre inoculum/late inoculation (YB/10L), iii) low-titre inoculum/early inoculation (SL/4L), and iv) low-titre inoculum/late inoculation (SL/10L). The grafted plants were maintained in a greenhouse.

Results

Consistently with the results of a previous study (Wei et al., 2013), the YB/4L-inoculation plants produced four mutually distinct symptoms. BB was observed starting from the first inflorescence (28 day post inoculation, dpi). At 45 dpi the newly developed inflorescence stopped producing BB symptoms, and branched into smaller segments, forming CLI. Starting from fourth or fifth inflorescence, more leaves were produced in each sympodial unit instead of three leaves and one inflorescence. At around 60 dpi, the typical WB symptom was observed. In the present study low-titre inocula (scions) and ten leaf stage rootstock plants (late inoculation timing) were tested.
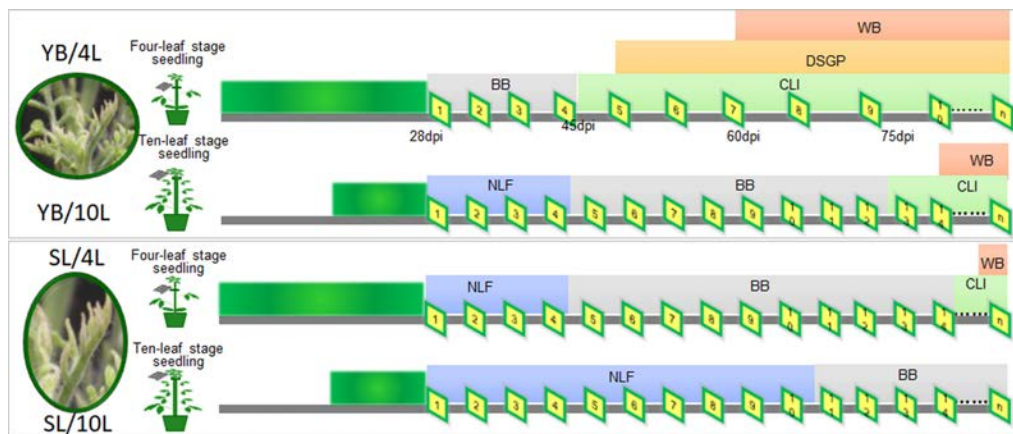
Figure 1. PPT phytoplasma induced symptom expression in infected plants with different graft inoculations. YB/4L: newly developed symptomatic young branches (YB) as a grafting scion and four leaf-stage (4L) seedling as a rootstock; YB/10L: newly developed symptomatic young branches (YB) as a grafting scion and ten leaf-stage (10L) seedling as a rootstock; SL/4L: a symptomatic single leaf (SL) as a grafting scion and four leaf-stage (4L) seedling as a rootstock; SL/10L: a symptomatic single leaf (SL) as a grafting scion and ten leaf-stage (10L) seedling as a rootstock. Green bars represent the vegetative growth before the formation of the first inflorescence. Each yellow-green diamond represents an inflorescence. Rectangles labeled with letters indicate the time length for each symptom presence. NLF: normal looking flower; BB: big bud; CLI: cauliflower-like inflorescence; WB: witches' broom; DSGP: disrupted sympodial growth pattern.

Compared with YB/4L inoculation, in YB/10L-inoculation plants, the first several inflorescences only formed normal looking flowers. The BB symptom appeared around the fifth inflorescence, and lasted until 80 dpi, before CLI and WB were sequentially observed. No visible DSGP was observed. SL/4L-inoculation plants exhibited a similar symptom expression profile to that of YB/10L-inoculation plants. In SL/10L-inoculation plants, BB appeared until 70 dpi, and no CLI, WB or DSGP symptoms were observed (Figure 1).

Discussion

Phytoplasma infection can reshape the growth and developmental pattern of the host plant by overriding genetically preprogramed plant meristem fate and change the identity of the meristem cells (Wei *et al.*, 2013). During the infection, while the phytoplasma cells are restricted in the phloem sieve cells, virulence factors (effector molecules) can move to the meristems (Hoshi *et al.*, 2009) and exert their pathological effect. In a previous study, four symptoms were identified in tomato plants infected with the PPT phytoplasma and it was hypothesized that each symptom corresponds to a distinct phase in the derailment of meristem fate. Based on this hypothesis, it is predicted that the earlier a plant gets infected by a phytoplasma, the more symptom types the host plant may develop on different shoots. The specific type of symptom exhibited by any given shoot must be determined by the developmental stage of the shoot apex when the plant becomes infected by the phytoplasma. Graft inoculation makes it possible not only to establish infection in host plant but also to control inoculum titre and inoculation timing. The graft inoculation on small tomato seedlings offers an additional benefit that the sequentially developed symptoms induced by phytoplasmas can be traced reliably and no early symptoms would be missed. The present study tested this hypothesis. A total of

four titre/timing inoculation combinations were investigated. Compared with YB/4L-inoculation plants, in the YB/10L- and SL/4L-inoculation plants it was observed not only a delayed symptom appearance but also a corresponding shift symptoms to later branches/shoots. BB symptom appeared late, and lasted long; CLI and WB were only observed at the very late infection stage; no apparent DSGP symptom was observed. In SL/10L-inoculation plants, only BB symptom developed at a much late time and no CLI, DSGP or WB symptoms was observed during the infection. These results indicated that, while both phytoplasma inoculum titre and inoculation timing (plant development stage) can influence the outcome of symptom development, conceivably both impact the critical time point when the phytoplasma infection affects the developing shoot apex. These findings will help understand phytoplasma induced symptom expression and interaction of phytoplasmas with plants.

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Interaction

"Flavescence dorée" phytoplasma has multiple *ftsH* genes showing host-dependent expression in both natural and experimental pathosystems

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Abstract

"Flavescence dorée" (FD) is an European severe epidemic disease of grapevine associated with the presence of a phytoplasma. Differences in virulence among strains of '*Candidatus* Phytoplasma mali' have recently been associated to variations in FtsH/HflB and membrane AAA+ ATPases. The present study aims to predict and characterize the *ftsH* gene repertoire of FD phytoplasma, the orientation of these proteins in the phytoplasma membrane and to measure their expression profile.

Keywords: membrane topology, AAA+ ATP-dependent zinc-binding membrane protease, leafhopper, grapevine

Introduction

"Flavescence dorée" (FD) is an epidemic disease of grapevine in European vineyards associated with the presence of the FD phytoplasma (Boudon-Padieu, 2002). In the class *Mollicutes*, the *ftsH* genes are present as a single copy, however, four *ftsH* genes were found in '*Candidatus* Phytoplasma mali' (Kube *et al*., 2008; Seemüller *et al*., 2013). Moreover differences in virulence among strains of '*Ca*. P. mali' were correlated with differences of one of these genes coding HflB/FtsH membrane proteases (Seemüller *et al*., 2011; 2013). This study aims to predict and characterize the *ftsH* gene repertoire of the FD phytoplasma, the orientation of these proteins in the membrane, and to measure their expression profile.

Materials and Methods

Phytoplasma strains, plants and insects

S. titanus and grapevine plants cultivar Cabernet Sauvignon (CS), broad bean and *Euscelidius variegatus* were infected with FD strain 92 as described in Eveillard *et al*., 2016. Plants were collected 7 weeks' post-inoculation.

Nucleic acids extractions and phytoplasma quantification

Plant DNA extraction was done as described in Eveillard *et al*. (2016). RNA extraction was done using the Plant Total RNA kit (Sigma-Aldrich) with some modifications. Insect

DNA and RNA extraction were done using the TRIzol Reagent. All RNA samples were treated with RQ1 DNase (Promega). The phytoplasma quantification was done by quantitative PCR (qPCR) using the protocol described in Eveillard *et al*. (2016).

cDNA synthesis and real-time reverse transcription-PCR

For each sample, 1 µg of RNA was used for cDNA synthesis employing the Superscript Reverse Transcriptase III and random primers (Invitrogen). The final pellet was resuspended in 20 µl of RNase free water. Real-time RT-PCR was run using 1 µl of cDNA template in 1X Light Cycler 480 SYBR Green Master Mix (Roche) with 0,1 to 1 µM of each primer in a final volume of 20 µl. Reaction conditions were: 15 minutes at 95°C and 36 or 40 cycles of 20 seconds at 95°C, 30 seconds at 56°C, 60°C, 62°C or 64°C and 30 seconds at 68°C. Specific primers were designed to amplify each *ftsH* gene. Three biological replicates (3 CS, 3 broad bean, 3 *S. titanus* and 3 *E. variegatus*) were used. Normalization was done with reference genes *gyrA* and *dnaB*.

Molecular phylogenetic analysis

The 8 FD phytoplasma *ftsH* genes were deposited in the European Nucleotide Archive under the accession numbers LT999755 (FtsH1) to LT999762 (FtsH8). The evolutionary history for phylogenetic tree production was inferred by using the maximum likelihood method based on the JTT matrix-based model (Jones *et al*., 1992).

Data analysis

Mean Normalized Expression (MNE) (Simon, 2003) or Relative Gene Expression (RGE) (Pfaffl, 2001) were calculated. Statistical analyses were done with R 3.5.2 software (R Core Team, 2018).

Results

With comparison with the FtsH proteins of *Bacillus subtilis* and *Escherichia coli*, eight FtsH were identified in the predicted FD phytoplasma proteome (Carle *et al.*, 2011). The selected sequences contain FtsH specific sites, *i.e.* the two transmembrane domains, the ATP-binding sites, the pore signature, the ATP-hydrolysis site, the zinc-coordinating motif, the 3rd Zn ligand glutamic acid residue, the conserved catalytic aspartic acid and the conserved leucine-zipper residues. Phylogenetic analysis of all predicted proteins in the complete phytoplasma genomes available revealed that only FD phytoplasma, '*Ca*. P. mali' and '*Ca*. P. ziziphi' encode multiple copies of FtsH, whereas the other phytoplasmas encode a single FtsH and multiple copies of AAA+ membrane ATPases lacking both zinc-binding site and protease domain. Maximum likelihood phylogenetic analysis of bacterial FtsH indicated that all phytoplasma FtsH had a common ancestor, FtsH6 in the case of the FD phytoplasma. The 8 FD *ftsH* genes expression was determined in the natural hosts CS and *S. titanus*, and in the experimental hosts broad bean and *E. variegatus*. MNE showed that all but one were expressed in every hosts. Only the *ftsH*8 gene was nearly not expressed in the insects. The expression profiles were similar between the two plants and the two insects, respectively. The RGE showed that *ftsH* genes 3, 4 and 8 were more expressed in CS as compared to *S. titanus*. On the contrary, *ftsH* genes 1, 2, and 6 were more expressed in *S. titanus* as compared to CS. The results were slightly different in the experimental pathosystem. Using Phobius, the C-tail carrying the putative ATPase and proteolytic activities was predicted to be intracellular with the exception of FtsH6 and 7 which active sites were predicted to be extracellular, meaning in direct contact with hosts.

Discussion

Up to now, no virulence factor has been identified in the FD phytoplasma genome. No specificity of expression was observed in the natural pathosystem CS/*S. titanus* as compared to the experimental pathosystem broad bean/*E. variegatus* which could be explained by the fact that the FD phytoplasma multiply in both and can be transmitted by both insects. A difference of the expression profile was found between plants and insects, indicating that some FtsH may have an increased role in plants or insects depending on the *ftsH* gene copy. Six out of the eight FtsH are predicted to be intracellular. The FtsH with cytoplasmic catalytic sites may have a role similar to those identified for the eubacterial universal FtsH, known to catalyse the degradation of

denatured, misassembled or damaged proteins and enable cellular regulation of protein stability. Two out of the eight FtsH are predicted to be extracellular. In comparison, approximately half of the '*Ca*. P. mali' FtsH were predicted to have extracellular active sites, suggesting an involvement of these proteins in pathogen-host interactions (Seemüller *et al.*, 2013). One may hypothesize that the extracellular FtsH proteases and the transporter units could act together to allow the exchange of peptides between the host environment and the FD phytoplasma cell. Preliminary data confirm that the soluble part of three FtsH in *Escherichia coli* exhibited an ATPase activity *in vitro*.

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Interaction

Characterization of candidate effectors of "flavescence dorée" phytoplasma: expression and subcellular localization

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Abstract

"Flavescence dorée" is a severe disease of grapevine that expands across Europe. The associated agent is a phytoplasma belonging to ribosomal group 16SrV, and transmitted in the field by the insect vector *Scaphoideus titanus*. Genome sequence analysis of the "flavescence dorée" phytoplasma allowed to define a restricted set of 12 putatively secreted proteins of unknown function. In order to provide a first characterization of these candidate effectors, their expression level was determined both in host plants and insect vectors. The results indicated variable expression levels among candidates, with differential transcript accumulation between plant and insect hosts. The subcellular localizations of the candidate effectors were determined in both plant and insect cells by fusion with a fluorescent protein and compared. All but one candidate displayed a similar pattern in plant and insect cells. In total, this study provides a first glimpse of "flavescence dorée" phytoplasma candidate effectors, and builds a foundation for a deeper understanding of the virulence mechanisms deployed by this phytoplasma.

Keywords: "flavescence dorée", phytoplasma, effector, virulence, subcellular localization

Introduction

Phytoplasma, like other phytopathogenic organisms, have been shown to secrete proteins termed effectors that alter the physiology of the host. Up to now, three phytoplasma effectors have been characterized in detail. Tengu has been shown to modify plant architecture, and was suggested to act through an inhibition of auxin-related pathways (Hoshi *et al.*, 2009). SAP11 was demonstrated to bind and destabilize specific plant transcription factors, leading to a decrease in jasmonate production and to enhanced vector reproduction (Sugio *et al.*, 2011). SAP54 also induces developmental alterations of flowers through interaction with plant transcription factors (Maclean *et al.*, 2014), and increases insect vector attraction to the plants (Orlovskis and Hogenhout, 2016). With the focus on the "flavescence dorée" phytoplasma, the identification and characterization of the effectors that are secreted by this phytoplasma were studied.

Materials and Methods

Determination of expression levels

Plants (broad beans and grapevine cultivar Cabernet-Sauvignon) and insects (*Scaphoideus titanus* and *Euscelidius variegatus*) were grown under confined greenhouse conditions. Infection of plants and insects with strain FD92 was performed as described in Eveillard *et al.* (2016). Insects and plant samples were collected 4 and 7 weeks post-transmission respectively. RNA extraction was performed using either the Plant Total RNA kit (plant samples) or the TRIzol Reagent (insect samples). Transcript accumulation of candidate effectors was determined using qRT-PCR and Light Cycler 480 SYBR Green Master Mix (Roche). Three biological replicates of each sample type were run 3 times in duplicates to quantify the expression levels. Gene expression was normalized with two phytoplasma reference genes (g*yrA* and *groEL*).

Subcellular localization in plant and insect cells

Effector coding sequences were cloned in plasmids pB7YWG2 or pB7WGY2, or in custom binary vectors. Constructs were transformed in *Agrobacterium tumefaciens* strain GV3101. *Nicotiana benthamiana* plants were transiently transformed by infiltration with agrobacteria suspensions and observed 48h-72h later with a confocal microscope (Leica SP2 or Zeiss LSM 880). For insect cells, effector coding sequences were cloned into plasmids pAGW and pAWG (Drosophila Genomics Resource Center). *Drosophila* S2 cells were transfected using GeneCellin (Eurobio); 48 h after

transfection, the cells were fixed with paraformaldehyde and imaged using a confocal microscope (Zeiss LSM 880).

Results

A bioinformatic analysis of the genome sequence of strain FD92 (Carle *et al.*, 2011) allowed to predict genes encoding putatively secreted proteins and to establish a list of 12 selected candidate effectors (C. Garcion *et al.*, unpublished). The presence of Tengu, SAP11 or SAP54 effectors was not detected. No function could be assigned to the candidate effectors based on primary sequence. Since the phytoplasmas colonize and multiply in two different hosts (plants and insects), each candidate could target either plant or insect components (or both). The expression level and subcellular localization of candidates in the two types of hosts (plants and insects) were therefore compared. Expression levels of candidate effectors showed similar profiles between grapevine and broad bean, and between *S. titanus* and *E. variegatus*: four and three candidates showed a strong expression in grapevine and in *S. titanus* respectively. Comparison of expression levels between host types (plant or insect) showed that three candidates were expressed more than 10 times in one host type relative to the other, and that 7 candidates out of 12 were more expressed in insects than in plants, with expression ratios ranging from 2.6 to 48.

Subcellular localizations of candidate effectors were determined by fusion with a fluorescent protein and expression of the constructs in plant or insect cells. Both N- and C-terminal fusion orientations were tested. In plants cells, four candidates were found to display a nucleo-cytoplasmic distribution pattern. Two candidates showed a fluorescent signal that was associated with vesicle-like structures, part of which co-localized with a Golgi apparatus marker. Two other candidates were found to be located in punctuate structures of variable size and of unknown identity. Various fluorescent markers demonstrated that these structures are different from plastids, mitochondria, peroxysomes or Golgi organelles. Another candidate showed a nucleolar localization. Finally, three candidates showed very low levels of expression and could not be imaged.

All candidate fusions showed a similar pattern of distribution between insect and plant cells, except one that was located in the nucleolus in plant cells, but in the cytosol and nucleus in insect cells. This discrepancy might indicate a specific recognition of plant components. The three candidates with low expression levels in plants showed a nucleo-cytoplasmic pattern in insect cells.

Discussion

The expression study of candidate effectors did not reveal a strict association of some candidates with a specific host type (plant or insect), indicating that there is no tight control of transcription depending on the host. However, there does exist differences of transcript accumulation between host types. A simple expectation would be that a stronger expression in one host type indicates a stronger requirement in this host, and would point out to the host targeted by the effector. Nevertheless, reality may be more complex. Indeed, the candidate showing a differential subcellular localization between plants and insect cells, suggesting a specific recognition of plant components, is the one that is most strongly expressed in insects compared to plants. The subcellular localization studies showed a striking similarity between the patterns observed in plants and in insect cells, even though insect cells used were *Drosophila* cells instead of insect vector cells due to technical constraints. This may illustrate a strong conservation of targets despite the very old divergence between plants and insects, or alternatively an adaptative selection for the candidate effectors to interact with both hosts. Finally, the putative association of some candidates with vesicles that could belong to trafficking system of host cells is intriguing. We speculate that it may be linked to the intracellular journey of phytoplasmas in the cells of the intestinal epithelium or salivary glands of the insect vector.

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Interaction

Role of membrane protein Imp of "flavescence dorée" phytoplasma in interaction with insect proteins: preliminary results

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Abstract

The involvement of the immunodominant membrane protein Imp of 16SrV-D phytoplasma in the interaction with insect proteins was investigated. The His-tagged partial Imp protein and a rabbit polyclonal antibody were produced and used in Far-Western dot Blot (FWdB) assays. Total native proteins were extracted from batches of six insect species (*Scaphoideus titanus*, *Euscelidius variegatus*, *Macrosteles quadripunctulatus*, *Ricania speculum*, *Metcalfa pruinosa* and *Zyginidia pullula*). FWdB showed interaction of Imp fusion protein with total proteins of *S. titanus*, *E. variegatus* (known as efficient vectors) and *M. quadripunclatulatus* (potential vector), while no interaction signal was detected with the other three species that are reported as non-phytoplasma vectors.

Keywords: Imp, leaf hoppers, grapevine yellows, planthoppers

Introduction

Phytoplasmas are phloem-limited plant pathogenic bacteria responsible for severe economic losses to agriculturally important plants (Strauss, 2009). The known phytoplasma vectors are hemipteran insects belonging to the suborder Auchenorrhyncha (Fulgoromorpha and Cicadomorpha) and the family Psyllidae (suborder Sternorrhyncha) (Trivellone, 2019). Numerous studies suggested the involvement of specific molecular phytoplasma-ligand interactions in transmission specificity the with insect hosts (Suzuki *et al.*, 2006, Galetto *et al.*, 2011, Arricau-Bouvery *et al.*, 2018). In this study, preliminary results on the role of immunodominant membrane protein Imp of 16SrV-D phytoplasmas, associated with the grapevine "flavescence dorée" subgroup 16SrV-D, in mediating the interaction with insect proteins are provided.

Materials and Methods

Insect species

To test the protein-protein interaction, 6 Auchenorrhyncha species were selected: 4 leafhoppers (*Scaphoideus titanus*, *Euscelidius variegatus*, *Macrosteles quadripunctulatus* and *Zyginidia pullula*) and 2 planthoppers (*Ricania speculum* and *Metcalfa pruinosa*). Data of acquisition (AR) and

transmission rate (TR) of 16SrV-D phytoplasma from the literature were used to define their vector capability. Accordingly, three categories of vector species were defined: efficient, potential, and non-vector (Table 1). For *S. titanus* AR was 63% (N=60) and TR was 60% (N=10) (Miliordos *et al.*, 2017). For *E. variegatus* AR was 67% (N=69) and TR was 62% (N=108) (Rashidi *et al.*, 2014). Both species are considered here as efficient vectors of 16SrV phytoplasmas. *M. quadripunclulatus* was reported in the literature to be competent vector of 16SrI and 16SrXII phytoplasmas (Trivellone, 2019), and it is considered here a potential vector of 16SrV-D phytoplasmas. For *R. speculum*, AR was 53% (N=15) and TR was 0% (N=24) (Galetto *et al.*, 2019). For *M. pruinosa* AR was 54% (N=95) and TR was 0% (N=95) (Clair *et al.*, 2001). The last two species are considered as non-vector of 16SrV-D phytoplasma similarly to the mesophyll feeder *Z. pullula*. Specimens used for the extraction of proteins were obtained from the healthy rearing of the IPSP-CNR (Turin, Italy). *S. titanus* and *M. pruinosa* neonates were obtained from two-year-old branches collected in infested vineyards, and *R. speculum* from twigs of *Clematis vitalba*, *Ligustrum lucidum* and *Rubus* spp. *M. quadripunclulatus* and *E. variegatus* were reared on oat (*Avena sativa*) in climatic chambers. Adults and plants were periodically checked for

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phytoplasma presence. Adults of *Z. pullula* were collected on graminoid species phytoplasma-free.

Fusion proteins and antibody production

The *dnaD-imp-pyrG* genomic fragment of 16SrV-D strain was amplified from FD-D infected grapevine leaves by nested PCR using primer pair DnaDFDf1/PyrGFDr1, generating a 940 bp amplicon, followed by primer pair DnaDFDf2/PyrGFDr2, amplifying a 830 bp genomic region (Italian patent no. 1429213). A total of 162 amino acids were predicted for full-length FD-D Imp protein, with residues 44-162 exposed outside the membrane. A his-tagged partial Imp protein (FD-D Imp-fp) and a rabbit polyclonal antibody (Filippin *et al.*, 2019) were synthesized.

Far-Western dot blots (FWdB)

The analysis was used to estimate the interaction between insect proteins and Imp fusion protein. Total native proteins were extracted from insect batches (5 *S. titanus*, 5 *E. variegatus*, 10 *M. quadripunctulatus*, 3 *R. speculum*, 3 *M. pruinosa* and 20 *Z. pullula*). The number of specimens for each species pool was defined using Bradford reagent (Bio-Rad) with the aim to load comparable amounts of proteins. Total proteins were extracted according the protocol described in Galetto *et al.* (2011). FD-D Imp-fp was used as probe in FWdB experiments. Forty μg of total proteins were spotted onto polyvinyl difluoride (PVDF, Bio-Rad) membranes with a Minifold I dot-blotter. All the samples were serially diluted 1: 10 and 1: 100 in PBS added with EDTA-free antiprotease cocktail Complete I (Roche) and spotted onto identical PVDF membranes with total spot proteins. One hundred ng of FD-D Imp-fp and 40 μg of BSA were spotted as positive and negative controls, respectively. Two sets of membranes were blocked (baited and no baited) following the protocol described in Galetto *et al.* (2011). Detection was performed with West Pico SuperSignal chemiluminescent substrate (Pierce) and a VersaDoc 4000 MP (Bio-Rad). The experiment was repeated three times with freshly extracted insect proteins.

Table 1. Summary of vector capability, concentration of total native proteins (TNP) extracted from insect samples, and Far-Western dot Blot (FWdB) results for the six species selected.

Species	Vector capability	TNP [μg/μl]	FWdB
Scaphoideus titanus	Efficient vector	4.3	+
Euscelidius variegatus	Efficient vector	2.7	+
Macrosteles quadripunctulatus	Potential vector	2.7	+
Ricania speculum	Non-vector	6.3	-
Metcalfa pruinosa	Non-vector	4.6	-
Zyginidia pullula	Non-vector	2.0	-

Results

The number of specimens in each pool and the protein concentration for each sample were defined by the Bradford assay (Table 1). The FWdB assays showed that Imp fusion protein interacted with total proteins extracted from the entire body of *S. titanus*, *E. variegatus* and *M. quadripunctulatus*. No signal was detected in the other three species non phytoplasma vectors. The results of FWdB assays are summarized in Table 1.

Discussion

These results suggest that Imp is involved in interactions between 16SrV-D phytoplasmas and insect vector proteins. To clarify the vector capability of *M. quadripunctulatus* additional data on transmission trials under controlled conditions will be necessary. Further proteomic analyses using Imp fusion protein and insect membrane proteins extracted from gut and salivary glands organs are ongoing.

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Interaction

The adhesin VmpA of the "flavescence dorée" phytoplasma binds insect cells in a lectin-like manner

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Abstract

Linking of adhesins to glycoproteins in a lectin-like manner is a common trait during the cycle of microbes, virus to parasites, across insects. VmpA is a "flavescence dorée" phytoplasma adhesin that binds insect cells in culture and perimicrovillar membrane of the experimental vector *Euscelidius variegatus*. It is shown that VmpA seems to attach to insect cells in a lectin-like manner using ELISA, competitive adhesion assay and Far-Western blot.

Keywords: adhesin, lectin, "flavescence dorée" phytoplasma, insect cell

Introduction

The propagation of the "flavescence dorée" (FD) phytoplasmas is naturally achieved in the vineyard by the leafhopper vector *Scaphoideus titanus* and the FD phytoplasma can be experimentally propagated in faba bean by *Euscelidius variegatus*. The surface protein VmpA is involved in the FD phytoplasma adhesion to insect cells (Arricau-Bouvery *et al.*, 2018). This constitute the initial and essential step of the propagative life cycle of the phytoplasma in its insect vector. VmpA is supposed to be involved in the endocytosis of FD phytoplasma into the midgut and salivary gland cells. As midgut and salivary gland cells are highly glycosylated, it was tested if VmpA could act as a lectin binding the surface glycoproteins of the insect vector cells in culture.

Materials and Methods

ELISA tests

Euva-1 cells (Arricau-Bouvery *et al.*, 2018) were grown in 96-well plate. When at confluence, insect cells were fixed with paraformaldehyde (Electron Microscopy Science) and incubated for 1.5 h with recombinant VmpA-His$_6$ previously incubated with αMannose (Man), αGlucose (Glc) or N-Acetylglucosamine (GlcNAc) (Sigma) at different concentrations indicated in Table 1 for 30 minutes. The presence of VmpA attached to Euva-1 cells was revealed by a first incubation with rabbit anti-VmpA polyantibodies for 1 h, a second incubation with goat anti-rabbit-IgG-PAL (Sigma) for 1 h, and a third incubation with the substrate pNPP (LCA) for 2 h at 37°C. Optical density (OD) were measured at 405 nm using an Epoch-Microplate Spectrophotometer (Biotech® Instrument).

Competitive adhesion assays

Euva-1 cells were cultivated on coverslips in 24-well plates. The lectins used for competitive adhesion assays were GNA (*Galanthus nivalis* agglutinin), LCA (*Lens culinaris* agglutinin) and LEL (*Lycopersicum esculentum* lectin) (Eurobio). The yellow-green fluorescent and amine-modified beads (Invitrogen) were covalently coated with recombinant VmpA-His$_6$ proteins according to the supplier's instructions. The Euva-1 cells were incubated with lectins for 1 h at 25°C and then incubated with VmpA-His$_6$-coated beads for 1 h at 25°C.

Fluorescence microscopy

The treated Euva-1 cells were fixed with paraformaldehyde. Cell nuclei were stained with DAPI (Sigma) and actin filaments with Alexa 568-phalloidin (Invitrogen). The cells were mounted in the antifading ProLong Gold reagent (Thermo Fisher Scientific) and analysed with a fluorescence microscope (Nikon Eclipse E800). The counting of beads per cells was performed with the software package ImageJ (http://imagej.nih.gov/ij/). To visualize the insect glycoproteins, Euva-1 cells cultivated on coverslips in 24-well plates were incubated after paraformaldehyde fixation with fluorescent FITC-conjugated lectins (Vector laboratories) for 1 h.

Far-Western assays

Euva-1 cell proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. For protein renaturation and blocking unspecific binding, membrane was incubated in PBS containing 5% non-fat dry milk for 4 h.

Table 1. ELISA showing adhesion of VmpA-His$_6$ to the Euva-1 insect cells in presence of sugar. Relative optical densities and statistical analysis were calculated considering the concentration of 0.1 M of each sugar as reference. Each experiment was repeated in 5 independent replicates, and the assays were repeated 3 times.

Sugar	0 M	0.1 M	0.25 M	0.5 M	1 M
α Mannose (Man)	1.06 (± 0.29)	1 (± 0.07)	1.22 (± 0.13)***	1.55 (± 0.26)***	1.60 (± 0.21)***
α Glucose (Glc)	0.94 (± 0.21)	1 (± 0.09)	1.04 (± 0.08)	0.92 (± 0.17)	0.87 (± 0.12)**
NAc-Glucosamine (GlcNAc)	0.87 (± 0.21)***	1 (± 0.04)	0.96 (± 0.06)	0.95 (± 0.08)	0.78 (± 0.08)***

The membrane was incubated overnight with recombinant VmpA-His$_6$ at 4°C, with anti-VmpA antibodies for 1 h at room temperature, then with HRP-conjugated goat anti-rabbit for 1 h. A signal was detected with ChemiDoc (Bio-Rad) after addition of the chemiluminescent substrate SuperSignal West PICO. For inhibition Far-Western, different concentrations of sugar (0.1, 0.5 and 1 M) were incubated at the same time as VmpA-His$_6$. For competitive Far-Western, the membrane was incubated for 1 h with lectins (20 µg/ml) prior the addition of VmpA-Hi$_6$.

Results

ELISA tests in which recombinant VmpA-His$_6$ was pre-incubated with Man, Glc and GlcNAc (Table 1) prior to incubation with Euva-1 insect cells, show that Glc and NAc-Glc decreased the adhesion of VmpA-His$_6$, whereas Man increased the adhesion in a dose dependant manner. In fluorescence microscopy, the fluorescent lectins GNA, LCA and LEL, binding Man, Man + Glc, and GlcNAc respectively, highlighted the presence of these sugars at the surface of the Euva-1 cells. A pre-incubation of Euva-1 cells with lectins significantly decreased adhesion of VmpA-His$_6$-coated beads (Table 2). These results suggest that VmpA interact with insect glycoproteins with Man, Glc and/or GlcNAc as the GNA, LCA and LEL lectins do. To determine the size of insect proteins interacting with VmpA, Far-Western assays with Euva-1 cell proteins were performed. VmpA-His$_6$ was able to interact with insect proteins with apparent mass of 210, 150, 90, 70, 40 and 28 kDa. The stronger signal observed was with a 90 kDa protein. Far-Western assays in presence of sugars and lectins showed that the pre-incubation of VmpA-His$_6$ with Man and GlcNAc modified the pattern of interaction whereas Glc had no effect. The pre-incubation of the membrane-embedded insect proteins with the lectins GNA, LCA decreased the interaction of VmpA-His$_6$ with the insect protein of 90 kDa, while LEL had only a slight effect.

Table 2. Adhesion of VmpA-His$_6$-coated beads to the Euva-1 insect cells in presence of lectins GNA, LCA and LEL. Relative adhesions were calculated considering the condition without lectin as reference. The experiments were repeated 3 times.

Lectins	none	GNA	LCA	LEL
Relative adhesion	1 (± 0.31)	0.51 *** (± 0.18)	0.55 *** (± 0.28)	0.61 *** (± 0.28)

Discussion

Transmission of virus, bacteria and parasites by insect in a circulative manner requires the crossing of the intestinal tract and salivary glands. This often implies the recognition of insect glycoconjugates by lectin-like adhesins as observed for the insect-transmitted mollicute *Spiroplasma citri* (Killiny *et al.*, 2005; Duret *et al.*, 2014). Indeed the salivary gland surface and the perimicrovillar membrane are highly glycosylated (Duret *et al.*, 2014; Gutiérrez-Cabrera *et al.*, 2016). It was thus hypothesized that at least one FD phytoplasma adhesin may act as a lectin. The results shown here indicated that VmpA could bind Man and GLcNAc residues present at the surface of insect proteins. Such adhesion to mannose or N-acetylglucosamine was previously documented for *Escherichia coli* (Krogfelt *et al.*, 1990) and *Xylella fastidiosa* (Killiny and Almeida, 2009). The identification of the insect protein(s) interacting with VmpA will improve the understanding of the specificity of FD phytoplasma transmission by vectoring leafhoppers.

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Interaction

Isolating intact phytoplasma particles from plants for sequencing applications and for testing host responses to phytoplasmas

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Abstract

'*Candidatus* Phytoplasma' species are obligate plant parasites limited to the phloem tissue. The difficulties to grow phytoplasma in culture seriously impedes the study of these prokaryotes. In this study, a protocol to extract intact phytoplasmas from infected periwinkle plants using filtration, differential and density-gradient centrifugations was optimized. Confocal and transmission electron microscopy was used to visualize intact phytoplasmas in the preparation. Quantitative-PCR and DNA-seq analyses revealed that phytoplasma DNA was significantly enriched following the extraction procedure. Challenging periwinkle plants with phytoplasma preparation induced the expression of one defence-related gene. This study provides an optimized procedure for phytoplasma DNA enrichment and for extracting intact phytoplasmas that can be used to examine host responses to them.

Keywords: phytoplasma, DNA enrichment, sequencing, microscopy

Introduction

'*Candidatus* Phytoplasma' species are obligate, insect-vectored plant-pathogenic mollicutes. Unlike most bacterial pathogens, which colonize the intercellular spaces of plants, phytoplasmas are intracellular and phloem-restricted.

Very little is known on host responses to phytoplasma infection and on phytoplasma perception by the plant immune system. The difficulties to cultivate phytoplasma *in vitro*, and to perform artificial inoculations with pure culture is impeding such studies. This also reflects on genomic studies for which phytoplasma DNA have to be obtained from plant or insect samples. To overcome the scarce amounts of phytoplasma DNA in infected samples, methods were developed to separate host from phytoplasma DNA by means of CsCl gradient centrifugation (Kirkpatrick *et al.*, 1987), by suppression subtractive hybridization (Cimerman *et al.*, 2006) and by phytoplasma particles isolation (Jiang and Chen, 1987).

In this study an optimized procedure for phytoplasma particle extraction from infected plant tissue is presented. This extract was used to obtain higher amounts of phytoplasma DNA for sequencing and to test plant response to phytoplasmas.

Materials and Methods

Sixty-five grams of "stolbur"-infected periwinkle tissue were grinded with 200 ml of extraction buffer (20 mM potassium-phosphate pH 7.4, 0.35 M sucrose, 0.05 M fructose, 1 mM calcium nitrate, 1 mM magnesium chloride, 15% BSA and 2% polyvinylpyrrolidone) using a Waring blender. The homogenate was passed through 3 layers of cheese cloth and then centrifuged for 15 min at 2,000 x g (4°C) to discard the remaining plant tissue and the cell debris. The supernatant was collected and centrifuged for 30 minutes at 22,000 x g (4°C) to pellet phytoplasma cells. The pellet was resuspended with Tris buffer (20 mM, 0.3 M sucrose, pH 7.2), loaded onto an Optiprep density gradient column and centrifuged for 20 minutes at 20,000 x g (4°C). The clear, middle fraction was collected and subjected to another, identical round of gradient centrifugation after which the middle fraction was collected, diluted with Tris buffer and pelleted for 1 h at 100,000 x g (4°C). The final pellet was resuspended with 2 ml of 1 X PBS. Phytoplasma-free periwinkle plants were used as controls and subjected to the same procedure. DNA from both samples was purified using the MasterPure™ Complete DNA and RNA kit (Epicentre), while the CTAB method (Mawassi *et al.*, 2018) was used to purify total DNA directly from plants.

Quantitative-PCR with periwinkle- and phytoplasma-specific primers was used to determine the ratio between host and phytoplasma DNA. One phytoplasma enriched sample was sent for Illumina sequencing (MiSeq 2x250bp). Reads obtained were quality filtered with Trimmomatic (Bolger *et al.*, 2014) and then classified with Kraken 2 (Wood and Salzberg, 2014) using a custom database including all the RefSeq complete bacterial genomes, the peanut witches' broom phytoplasma NTU2011 (GenBank accession numbers GCA_000364425.1), and the periwinkle nuclear and chloroplast genomes (GenBank acc. nos. GCA_000949345.1 and NC_021423, respectively). Confocal microscopy (Leica SP8) was used in conjunction with FM4-64 and DAPI staining to observe membranes and DNA, respectively, in the samples. Transmission electron microscopy (TEM) (JEOL 7800 HRSEM) was used to observe phytoplasma cells.

In vitro grown periwinkle plants were challenged with phytoplasma-enriched samples using a cotton wool clip-inoculation method. Leaf samples were collected 48 h post inoculation for RNA extraction followed by cDNA synthesis and qPCR with periwinkle primers to estimate the expression level of host defense-related gene.
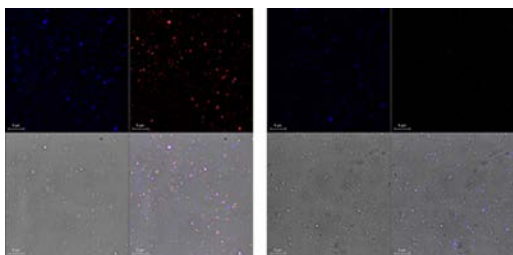
Figure 1. Confocal microscope observations of phytoplasma enriched samples purified from infected (left) and healthy (right) periwinkle plants. Samples were stained with DAPI (purple, upper left) and FM4-64 (red, upper right). Bright field (bottom left) and merged (bottom right) images are also shown.
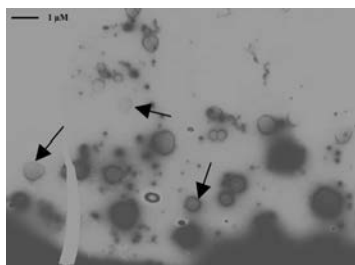
Figure 2. Transmission electron microscope image of a phytoplasma-enriched sample extracted from a periwinkle plant. Black arrows indicate phytoplasma-like particles.

Results

Phytoplasma DNA was detected in all phytoplasma enriched samples. The qPCR analysis yielded a ΔΔCt value of 8, indicating that the phytoplasma/plant DNA ratio was about 250-fold higher (2^8) in the enriched sample *versus* the total DNA extraction. Illumina sequencing of one enriched sample and one total DNA sample revealed about 20-fold increase in phytoplasma reads in the enriched sample.

Particles with the size range of phytoplasma cells were co-stained with both membrane and DNA dyes, suggesting that intact cells were extracted (Figure 1). Intact phytoplasma-like particles were also observed in the enriched sample by TEM (Figure 2), while none were detected in the control sample (data not shown). Periwinkle plants, challenged with phytoplasma preparations from infected plants, induced the expression of one of the two defense-related genes tested. Induced expression was also observed in response to preparations from healthy plants, but to a lesser extent.

Discussion

The ability to obtain a pure culture of the disease agent is paramount to every phytopathological study. As phytoplasma can be difficultly cultivated *in vitro*, alternative approaches have to be developed to allow a more thorough study of these pathogens. In this study the optimization of a method to extract intact phytoplasma cells from infected plants was developed. This extraction resulted in a significantly higher phytoplasma DNA content as compared with the total DNA extraction. Furthermore, it yielded approximately 20-fold enrichment in phytoplasma reads with Illumina sequencing, showing the applicability of this procedure for whole genome sequencing of phytoplasmas. Very little is known on host responses to phytoplasma infections. Obtaining intact phytoplasma cells allowed to inoculate plants and to test their response to phytoplasma cells. An immune-related gene was upregulated in response to this phytoplasma preparation suggesting that plants are capable of detecting phytoplasma cell presence. Further studies using this extraction method are underway to verify which parts of phytoplasmas are detected and which immune genes are involved.

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Interaction

Structural analysis of phyllogen, a phyllody-inducing effector, revealed the importance of two conserved α-helices

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Abstract

Phyllody is one of the most characteristic symptoms associated with phytoplasmas, in which flower organs are transformed into leaf-like structures. A phytoplasma conserved effector, called phyllogen (phyllody-inducing gene family), works as a phyllody inducer by degrading floral MADS-domain transcription factors (MTFs). In this study the crystal structure of $PHYL1_{OY}$, a phyllogen of '*Candidatus* Phytoplasma asteris' onion yellows strain, was elucidated at a resolution of 2.4 Å. $PHYL1_{OY}$, is formed by two α-helices connected by a random loop in a coiled-coil manner. Other phyllogens were also predicted to contain two consensus α-helices. Amino acid insertion mutations into either α-helix of $PHYL1_{OY}$ disrupted its phyllody-inducing activity and ability to degrade MTF, although the same insertion in the loop region did not affect either. These results indicated that both conserved α-helices are important for the function of phyllogen.

Key words: phyllody, phyllogen, crystal structure, MADS-domain transcription factors, α-helix

Introduction

Phyllody is one of the most characteristic symptoms induced by phytoplasma infection. Recently, a phytoplasma secreted protein, SAP54/PHYL1, was reported to induce phyllody in plants (MacLean *et al.*, 2011; Maejima *et al.*, 2014; Yang *et al.*, 2015). Their homologs are conserved among phytoplasmas and designated as phyllogens (phyllody-inducing genes) (Maejima *et al.*, 2014). Phyllogen induces phyllody by interacting with and degrading the A- and E-class floral MADS-domain transcription factors (MTFs) via the 26S proteasome (MacLean *et al.*, 2014; Maejima *et al.*, 2014). Rad23 proteins, which translocate ubiquitylated proteins to the 26S proteasome, were reported as another interactor of phyllogen required for phyllody induction (MacLean *et al.*, 2014). The keratin-like (K) domain in floral MTF was reported as the binding region for phyllogen (MacLean *et al.*, 2014). The domain of SEPALLATA3 (SEP3; E-class MTF of *Arabidopsis thaliana*) consists of two amphipathic α-helices responsible for the oligomerization of MTFs (Puranik *et al.*, 2014). In this study the crystal structure of $PHYL1_{OY}$, a phyllogen of '*Candidatus* Phytoplasma asteris' onion yellows (OY) strain was determined and a structure–function relationship for $PHYL1_{OY}$ was demonstrated.

Materials and Methods

Crystals of $PHYL1_{OY}$ were prepared by a custom structure analysis service (Protein Wave) using pGEX-4T-1 (GE Healthcare)-cloned *$PHYL1_{OY}$*. Consensus secondary structure element prediction of phyllogen homologs was performed by the PROMALS3D. Mutations of $PHYL1_{OY}$ were introduced by GeneArt site-directed mutagenesis system (Invitrogen). Yeast two hybrid assay (Y2H) was performed according to a previous report (Kitazawa *et al.*, 2017). For transient *in planta* expression, *Agrobacterium tumefaciens* cells (OD_{600}=1.0) carrying the viral silencing suppressor P19, myc-SEP3, each of 3flag-$PHYL1_{OY}$ constructs were co-infiltrated at a ratio of 1:10:1 into *Nicotiana benthamiana* leaves. Protein extraction and detection were performed based on Kitazawa *et al.* (2017). For the stable protein expression in *A. thaliana*, a modified tobacco rattle virus (TRV)-based gene expression vector system (Tian *et al.*, 2014) was employed.

Results

Crystal structure of $PHYL1_{OY}$ consists of two amphipathic α-helices connected by a random loop in coiled-coil manner (Figure 1A), wherein the hydrophobic face of each helix is oriented toward the protein interior.
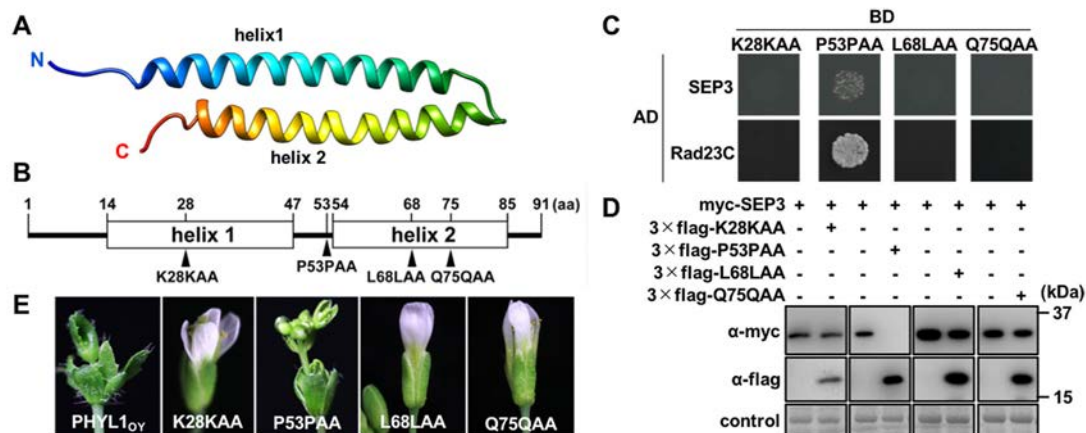
Figure 1. (A) Crystal structure of the PHYL1$_{OY}$ monomer drawn by UCSF chimera. (B) Secondary structure of PHYL1$_{OY}$. The arrowheads indicate insertions-introduced sites. (C) Yeast two hybrid assay of PHYL1$_{OY}$ mutants with SEP3 and Rad23C. (D) The accumulation of transiently expressed myc-SEP3 upon co-expression of 3×flag-fused PHYL1$_{OY}$ mutants. (E) Flower phenotypes of PHYL1$_{OY}$-expressing *Arabidopsis* plants.

Secondary structure predictions suggested that other phyllogens also contain two á-helices with highly conserved hydrophobic residues (data not shown), suggesting that phyllogens shared structural features. To examine the structure–function relationships of phyllogen, four PHYL1$_{OY}$ mutants were constructed (Figure 1B), in which two alanine residues were inserted into the helix 1 region (K28KAA), loop region (P53PAA), or helix 2 region (L68LAA and Q75QAA). Y2H showed that P53PAA interacted with SEP3 and RAD23C, while the others did not (Figure 1C). Transient co-expression of myc-SEP3 and each of the 3 X flag-tagged PHYL1$_{OY}$ mutants showed that P53PAA decreased the accumulation of SEP3, while the others did not (Figure 1D). TRV-based expression showed only P53PAA induced phyllody as in the case of PHYL1$_{OY}$ (Figure 1E). These results indicated that both á-helices are essential for phyllody-inducing activity of PHYL1$_{OY}$.

Discussion

Crystal structure of PHYL1$_{OY}$ and the following structure prediction of homologs strongly suggested that phyllogens consists of two α-helices with highly conserved repeating series of hydrophobic residues. Amino acid insertion into either helix resulted in a loss of the biological activities of PHYL1$_{OY}$, while insertion into the loop region did not. These results indicate the importance of both α-helices for the function of PHYL1$_{OY}$. Interestingly, similar to phyllogen, K domain of MTFs consists of two conserved amphipathic α-helices. These act as hydrophobic interaction surfaces for the oligomerization of MTFs (Puranik *et al.*, 2014). Considering with importance of both α-helices of phyllogen for the interaction with SEP3, this interaction can be mediated by a mechanism similar to the one mediating oligomerization among MTFs. Y2H assay indicated that each α-helix of phyllogen was also important for interactions with Rad23. Further structure-based mutagenesis studies will provide additional insights on how phyllogen establishes the interactions with both MTF and Rad23 to degrade MTF.

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Interaction

Flower traits change in response to infection with 'Candidatus Phytoplasma mali' in *Nicotiana tabacum* as model system

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Abstract

Flower traits such as flower colour, shape and volatile bouquet are shaped by a number of different factors, among them the interactions with pathogens. Infection with the 'Candidatus Phytoplasma mali' is associated with apple proliferation disease in *Malus* x *domestica*, a serious threat for commercial apple farming. Apple proliferation induces a range of symptoms on the leaves and a very reduced fruit size. However, the effects of this phytoplasma infection on floral traits are unknown. The phytoplasma presence was traced in different flower parts of the model system *Nicotiana tabacum* and changes in the floral reproductive tissue and other floral traits were explored. 'Ca. P. mali' was detected in floral tissues and seeds, and the floral traits changed in response to the infection. If this finding holds for *M. domestica*, pollinators may be able to discriminate between healthy and infected apple flowers, and their behaviour and thus pollination service may change as a consequence.

Keywords: apple proliferation, phytoplasma, *Nicotiana tabacum*, floral traits

Introduction

Apple proliferation, a disease associated with the presence of the bacterium 'Candidatus Phytoplasma mali', is a serious economic threat in apple-growing regions across Europe. The range of symptoms altering vegetative traits and fruit development of apple (*Malus* x *domestica*) are well-documented and include witches' broom, foliage reddening, enlarged stipules and dwarf fruits (Seemüller and Schneider, 2007). However, it is unknown whether 'Ca. P. mali' also infects floral tissue and potentially changes reproductive function, *e.g.* affecting pollen as the male gametophyte. Moreover, 'Ca. P. mali' in floral tissue could influence floral traits such as the flower colour and shape or the floral volatiles. Visual and olfactory cues are essential attractants for pollinating insects. Apple trees depend on cross-pollination for successful fruit set due to their self-incompatibility, *i.e.* pollen has to be transferred between flowers of different varieties. If floral traits change in response to phytoplasma infections, this could have an influence on pollinator behaviour and pollination success.

The model system *Nicotiana tabacum* (Solanaceae, Nicotianoideae) was used to investigate potential effects of an infection with 'Ca. P. mali' on floral organs. Tobacco was chosen because it is a convenient host for a range of different phytoplasma strains (Rid *et al.*, 2016). The work was focused on two major questions: i) can phytoplasma presence be detected in different flower tissues and seeds? ii) Are there any changes in flower traits induced by the bacterial infection?

Materials and Methods

Plant material

N. tabacum plants were kept in pots in the greenhouse of the Julius Kuehn-Institute (Dossenheim, Germany). Plants were infected by grafting with either an avirulent "1/93" or a virulent "12/93" apple proliferation phytoplasma strain. Control plants of the same age were pruned but not grafted.

DNA extraction

Flowers of *N. tabacum* were collected (Figure 1) and dissected in male and female flower parts. Stamens were washed in Doyle-buffer (Doyle and Doyle, 1990) to collect the pollen grains. Seeds were collected from mature seed capsules. DNA from floral tissues (except pollen) and seeds was extracted with a simple modified Doyle and Doyle extraction. Pollen grains were mechanically fragmented in a FastPrep-24 Homogenisor (MP Biomedicals).

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Figure 1. A) Inflorescence and B) single flower of *N. tabacum*. Visible are the corolla tube, the gynaeceum, with the orange nectar producing tissue and the stamens.

PCR

Quantitative PCR was used to detect phytoplasma DNA. Primers and conditions followed Christensen *et al.* (2004).

Size measurements

A digital calliper was used to measure the diameter and length of the corolla tube. Seeds were examined with a dissecting microscope and weighed, the size of pollen grains was measured under a compound microscope, each with an attached camera system.

Pollen number and pollen tube germination

Pollen grains per anther were counted following Costa and Yang (2009). The rate of pollen tube germination was tested with different sucrose solutions (concentrations between 10-30%) according to Dafni *et al.* (2005).

Colour measurement

The colour of the attractive parts of the corolla tube was measured with a spectrometer (Ocean Optics Flame S UV-VIS) combined with a xenon light source (Ocean Optics PX-2).

Nectar analysis

The sucrose concentration in nectar was measured with a hand-held refractometer (Bellingham and Stanley) and 2 µl of nectar were collected from four flowers per plant.

Results

Quantitative PCR revealed DNA of '*Ca.* P. mali' in different flower tissues as well as in seeds of the infected plants. Furthermore, there was evidence of change in a number of parameters in response to the phytoplasma infection, *e.g.* pollen grains were larger in infected flowers where nectar sucrose concentration also dropped.

Discussion

These preliminary results indicate that '*Ca.* P. mali' can be present in flower tissues of infected plants, as confirmed by qPCR. Furthermore, floral traits of infected *N. tabacum* plants changed in response to the presence of '*Ca.* P. mali' in a number of ways. Currently a broad range of experiments with the original host *Malus* x *domestica* are being carried out. These data will reveal if the findings in *N. tabacum* are applicable to *Malus* x *domestica*. However, the finding that phytoplasmas infect floral tissue and alter the floral phenotype in subtle ways as opposed to the well-known phyllody phenomenon itself opens up a range of intriguing possibilities. For instance, if an infection with '*Ca.* P. mali' is associated with similar changes in apple flowers, pollinators may be able to discriminate between flowers of infected and healthy trees, which in turn could influence pollination and fruit set. Thus, follow-up experiments will not only explore interactions between '*Ca.* P. mali' and apple flowers, but also focus on pollinator behaviour in response to phytoplasma-induced changes in flower phenotypes.

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Interaction

Small RNA profiling of aster yellows infected *Catharanthus roseus* plants

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Abstract

Aster yellows phytoplasma strain Hyd35 (16SrI-B) in micropropagated periwinkle shoots in collection was used to produce infected plants in pots that were separated according to the diverse symptomatology *i.e.* phyllody and witches' broom. Small RNA high-throughput sequencing (HTS) was then used to determine the small RNA pattern of these plants. Bioinformatics analysis revealed the presence of expression changes of different miRNA classes and the presence of phytoplasma derived small RNAs. These results could complement previous studies and serve as a starting point for small RNA omics in phytoplasma research.

Keywords: aster yellows, *Catharanthus roseus*, miRNA, small RNA, HTS

Introduction

Phytoplasmas are obligate bacterial plant pathogens restricted to the host phloem (Bertaccini and Duduk, 2009). They seems to have a limited coding capacity in their genomes (Bendix and Lewis, 2018), and often interfere with the hormone metabolism of the host inducing developmental abnormalities like witches' broom, phyllody, irregular flowering, decreased fruit size, yellowing, decline and stunting. Developmental processes and hormone metabolism are regulated by transcriptional factors, which can be further regulated by endogen small RNAs known as microRNAs (miRNAs). RNA interference (RNAi) based plants defence mechanisms, directed by small interfering RNAs, control the pathogen concentration on a sequence specific way. These small RNAs originates from the pathogens, therefore their sequence is the same as the sequence of the pathogens. RNAi was proved to work against viruses, fungi and bacteria, but no report are available on phytoplasma derived small RNAs.

In recent years with the fast development of sequencing techniques, it is possible to determine the complete profile of the small RNAome in one organism. The miRNAs, showing significant changes during the phytoplasma infection were identified in different plant hosts: grapevine, Mexican lime, mulberry and paulownia (Ehya *et al.*, 2013; Gai *et al.*, 2014; Snyman *et al.*, 2017; Fan *et al.*, 2015). As the pathogen presence is limited to the phloem, its effect can be different in stems and in distinct parts of the plant. This feature could explain why different changes (even the opposite trend) could be detected for the same miRNAs. Molecular characterization

of phytoplasma-induced changes were also studied in *Catharanthus roseus* as phytoplasma model host plant (Su *et al.*, 2011). Although miRNA profiling of *C. roseus* is available (Pani and Mahapatra, 2013; Prakash *et al.*, 2015; Shen *et al.*, 2017), there are no studies dealing with changes in the small RNA pattern during the phytoplasma infection on this plant species. In this work the characterization of small RNA pattern in aster yellows (AY) phytoplasma infected *C. roseus* plants showing phyllody or witches' broom symptoms and the comparison of changes with healthy plants using small RNA HTS was performed.

Materials and Methods

Potted cuttings from one micropropagated clone of *C. roseus* infected with aster yellows (strain Hyd35, 16SrI-B) from collection (Bertaccini, 2014) were used. Plants with different phenotypes were selected: three showing phyllody, three showing witches' broom and three asymptomatic and phytoplasma negative (Figure 1). They were grown in the same glasshouse, under insect proof conditions.

Figure 1. Periwinkle plants used for small RNA NGS. From left to right: phyllody, witches' broom and asymptomatic types.

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Total nucleic acid was extracted from the leaves. Purified fractions of small RNAs, separated on 8% polyacrylamide gels, were used for small RNA sequence library preparation (one library/plant), using Illumina Trueseq small RNA library preparation kit and an in-house modified protocol (Czotter *et al.*, 2018). After sequencing CLC genomic workbench and Geneious software were used for bioinformatics analysis. Significant changes were validated by small RNA Northern blot analyses.

Results and Discussion

The principal component analysis of the small RNA profiles showed that the periwinkle plants not infected clustered together, while the plants infected with phytoplasmas and showing different symptoms had a distant correlation from the healthy ones (Figure 2).
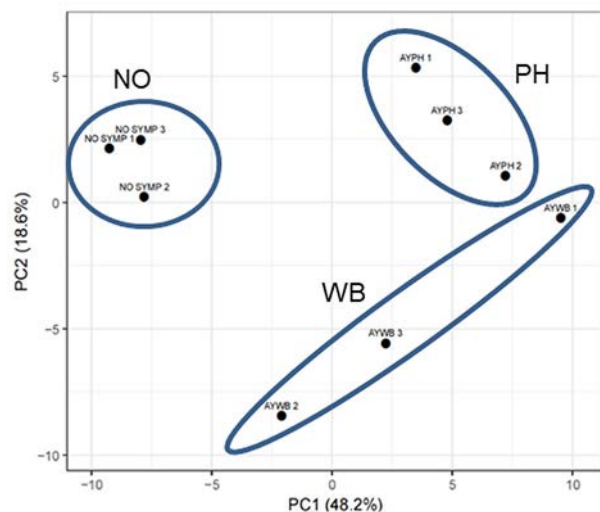
Figure 2. Principal component analysis of the expression changes in the miRNA profile in non symptomatic plants (NO) compared to plants showing phyllody (PH) or witches' broom (WB).
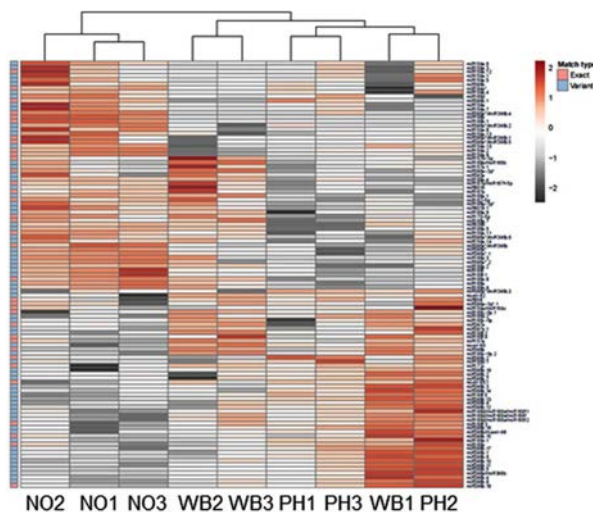
Figure 3. Heat map of expression changes of conserved miRNAs.

The heat map analysis of the expression profile (Figure 3) of significantly changed miRNAs highlighted that it is not straightforward to conclude that expression change of a miRNA is related to a specific symptom. In accordance to previous reports the up-regulation of miR156, mir157, miR166, miR171, miR172, miR397 and miR398 was found, while the most down-regulated miRNAs were miR159, miR162, miR168, miR169 and miR396. The analysis also showed that small RNAs, originating from different parts of the phytoplasma genome were generated, and their number was higher in the plants showing witches' broom symptoms.

Acknowledgements

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Interaction

Small RNA analysis reveals different profiles among 'Candidatus Phytoplasma mali' strains in *Malus x domestica* cultivar Golden Delicious *in vitro* plants

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Abstract

In plant hosts phytoplasmas were shown to affect the expression of a broad range of genes and RNA regulatory molecules acting at a post-transcriptional level and leading to changes in several metabolic pathways. Effector proteins secreted by phytoplasmas play a paramount role in the metabolic changes resulting in disease symptoms development. It is shown here that in 'Candidatus Phytoplasma mali'-infected *in vitro* plants of *Malus x domestica* cultivar Golden Delicious another class of pathogen-derived molecules represented by small RNA can be detected through specific sequencing. The profiles obtained from seven different phytoplasma strains revealed differences that could be associated to their capacity to multiply in the plant host or due to differences in the expression level of the phytoplasma transcripts.

Keywords: 'Candidatus Phytoplasma mali', small RNA, phytoplasma strains, apple proliferation

Introduction

Identified for the first time in the middle 1960s (Doi *et al.* 1967) phytoplasmas still represent a challenge for the researchers trying to understand the mechanisms underlying their interaction with plant and insect hosts. In the last two decades, many advances have been achieved allowing a better understanding of this complex system. Studies focusing on the changes occurring in the plant host during the infection showed that the expression of several genes as well as the expression of post-transcriptional regulatory molecules as micro RNAs are affected by the phytoplasma presence (Ghayeb Zamharir *et al.*, 2011, Gai *et al.*, 2014). However insights into the phytoplasma biology, as gene expression and encoded proteins, are still limited. In this work an *in vitro* system was used to analyse the interaction between *Malus x domestica* cultivar Golden Delicious and 'Candidatus Phytoplasma mali' associated with apple proliferation disease (AP). It is shown for the first time, that another class of molecules represented by 'Ca. P. mali'-derived small RNA can be detected in apple and that their profile is different among seven 'Ca. P. mali' strains maintained *in vitro*. This opens new ways to analyse this pathogen in its plant hosts.

Materials and Methods

'Ca. P. mali'-infected and healthy *Malus x domestica* cultivar Golden Delicious shoots were maintained *in vitro* as described in Jarausch *et al.* (1996). Healthy and 'Ca. P. mali'-infected plant material was collected, frozen in liquid nitrogen and ground to powder. Small RNA extraction was carried out starting from 100 mg of frozen powder using the mirPremier™ microRNA Isolation Kit (Sigma Aldrich) following the manufacturer's instructions. Small RNA were then subjected to the TruSeq Small RNA Library Preparation protocol (Illumina) followed by sequencing on an Illumina HiSeq2500. Sequences were then trimmed from adapters and the subset of small RNA between 15 nt and 40 nt were aligned against a dataset of rRNA, tRNA, snRNA and snoRNA (rfam.xfam.org) to retrieve non-mapping sequences. Afterwards, the cleaned sequences were aligned against the apple genome. Again, the non-mapping sequences were retained and aligned this time against the 'Ca. P. mali' AT genome (GenBank accession number: GCF_000026205.1). Alignments were performed using bowtie (version 1.2.2) allowing 3 mismatches for the cleaning steps and 0 mismatches for the alignments against the AT genome.

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Results

The subsets of raw reads with a size length from 15 to 40 nt were retrieved from the small RNA dataset and their number was comprised between 8,922,207 and 18,083,724 (Table 1). The number of reads aligning on the '*Ca*. P. mali' genome ranged from 2,217 (PM7) to 15,951 (PM11).

Table 1. Raw reads and reads aligning on AT genome (size between 15 and 40 nt).

	Raw reads	Aligning vs AT
PM5	17,011,154	9,325
PM6	17,813,196	2,424
PM7	8,922,207	2,217
PM11	15,279,185	15,951
PM13	18,083,724	6,998
PM40	15,323,253	11,580
PM44	16,959,413	5,443
GD	15,178,177	0
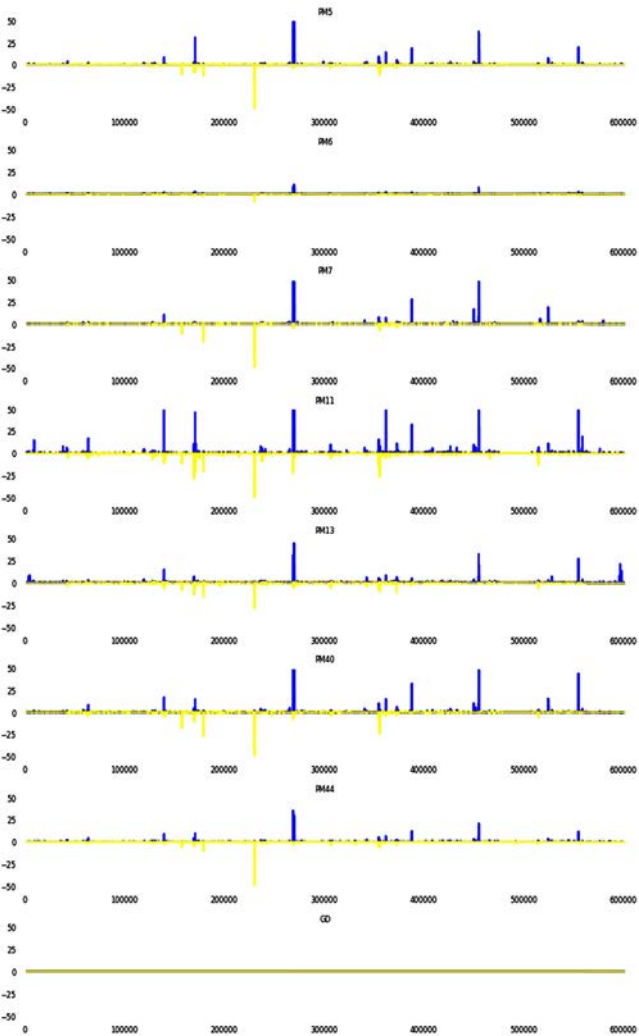
Figure 1. Alignment profiles of small RNAs of 7 '*Ca*. P. mali' strains on the '*Ca*. P. mali' AT genome (blue: plus strand, yellow: minus strand).

The analysis of the small RNAs alignment profiles on the '*Ca*. P. mali' genome showed the presence of coverage peaks where a higher number of reads accumulated mapping on certain genomic positions on both DNA strands (Figure 1). The major part of the peaks were common to all the analysed strains whereas several peaks were present only in certain strains, resulting in a different peak distribution differentiating the seven strains.

Discussion

The results presented in this work showed that short RNAs aligning uniquely on the '*Ca*. P. mali' genome were present in the small RNA fraction extracted from '*Ca*. P. mali'-infected *in vitro* plants and subjected to high throughput sequencing. Being that the detected signal derives from RNA molecules, it is hypothesized that these sequences are captured from the phytoplasma transcripts. Different small RNA profiles among the seven analysed strains suggest that there are differences either in the way these reads are generated or due to variants at the sequence level among the strains. This study provides new insights on the phytoplasma-host interaction opening for further analyses on this pathosystem biology.

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Interaction

RNAi silencing to validate the role of insect genes in phytoplasma transmission

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Abstract

The leafhopper *Euscelidius variegatus* is a known phytoplasma vector. Previous studies indicated a crucial role for insect ATP synthase during the phytoplasma infection process. Gene silencing of ATP synthase β was obtained by injection of specific dsRNAs in *E. variegatus*. The significant reduction of corresponding protein expression and preliminary results on significant differences found in phytoplasma quantity in silenced insects compared to the controls, following injection with ATP synthase β and successive phytoplasma acquisition on infected plants are presented. An *in vivo* role for insect ATP synthase β could be hypothesized during phytoplasma infection process.

Keywords: *Euscelidius variegatus*, ATP synthase β, gene silencing, insect vector

Introduction

Phytoplasmas are plant pathogenic bacteria transmitted by sap-feeding insects belonging to the order Hemiptera and associated with many diseases that cause severe economic impacts on many crops worldwide (Tomkins *et al.*, 2018). The leafhopper *Euscelidius variegatus* is a natural vector of '*Candidatus* Phytoplasma asteris' chrysanthemum yellows (CY) strain and an efficient vector of "flavescence dorée" phytoplasma (FD) under laboratory conditions (Galetto *et al.*, 2018). Previous studies demonstrated the *in vitro* interaction between insect ATP synthase β and the CY antigenic membrane protein (Amp) as well as the *in vivo* role of CY Amp in phytoplasma transmission (Galetto *et al.*, 2011; Rashidi *et al.*, 2015). RNAi gene silencing reduced the transcription of ATP synthase β of *E. variegatus*, following insect abdominal microinjection with dsRNAs targeting the corresponding gene product (Abbà *et al.*, 2018). Preliminary results on the effect induced by RNAi of vector ATP synthase β in terms of phytoplasma quantity in silenced insects, following acquisition on infected plants are presented.

Materials and Methods

The complete coding sequence of ATP synthase β (target mRNA) can be found in the TSA sequence database (BioProject: PRJNA393620) at NCBI under the accession number GFTU01013594.1. A fragment of the target sequence was obtained from total RNA from adult insects using a reverse transcription polymerase chain reaction (RT-PCR). A control template corresponding to a fragment of the gene sequence of green fluorescent protein (GFP) was PCR-amplified from the plasmid pJL24. Molecules of dsRNA were synthetized and delivered by microinjection as described in Abbà *et al.* (2018). Primers used to generate the dsRNA templates are indicated in Table 1. Western blots were performed on insects collected at different days post injection to evaluate the ability of the injected dsRNAs to reduce over time the expression of the ATP synthase β at the protein level, as described in Galetto *et al.* (2018). Following dsRNA (targeting either GFP as a control or ATP synthase β) injection, the insects were allowed to acquire the phytoplasmas by feeding on infected plants for a few days, then isolated on oat plants for a brief latency and thus collected for molecular analyses. Collected insects were sexed and total RNA was extracted from each individual. Quantitative RT-PCR (qRT-PCR) was used to quantify the ability of the injected dsRNAs to knockdown target mRNA and to measure the effect of gene silencing on phytoplasma multiplication. The cDNA was synthesized and used as a template for qPCR as described in Abbà *et al.* (2018). All the primer pairs used for qRT-PCR are listed in Table 1.

Table 1. List of primers used in this work. The T7 promoter sequence is in grey.

Application	Primer name	5′ –3′Sequences	Target gene
dsRNA synthesis	Eva_T7ATPsynBetaf Eva_T7ATPsynBetar	TAATACGACTCACTATAGTACGGCCAGATGAACGAGCC TAATACGACTCACTATAGGGACACCACGAGCAATGTTG	ATP synthase β
	T7GFPf T7GFPr	TAATACGACTCACTATAGCTTTTCACTGGAGTTGTCCC TAATACGACTCACTATAGGGTTTGTGTCCGAGAATGTTTC	GFP
Quantitative PCR (insect target gene)	ATP β Fw622 ATP β Rv792	CGCTTTACTCAGGCTGGTTC GTCATCAGCTGGCACGTAGA	ATP synthase β
Quantitative PCR (insect housekeeping gene)	EF1-α_F215 EF1-α_R325	CCATCGACATTGCCCTGTGG CCTGTGAGGTTCCAGTGATCATG	Elongation factor-1α
Quantitative PCR (phytoplasma gene)	CYS2Fw CYS2Rv	AGGTTGAACGGCCACATTG TTGCTCGGTCAGAGTTTCCTC	Phytoplasma 16S rRNA

The amount of the phytoplasma gene transcript was calculated using a standard curve made of serial dilutions of a plasmid harbouring a target phytoplasma amplicon as described in (Marzachì and Bosco, 2005). The Mann-Whitney rank sum test ($P < 0.05$) was used to analyse ATP synthase β expression levels and numbers of phytoplasma transcripts measured in silenced and in control insects.

Results

Microinjection of ATP synthase β dsRNAs into adult *E. variegatus* insects caused an exponential reduction in the expression of the corresponding gene, which began within 72 h from dsRNA injection and lasted for over 20 days. A significant reduction in the expression of the corresponding protein was also observed, but occurring only after 12 days post injection. Preliminary data indicated that a significant difference could be observed in phytoplasma mean quantities measured in the insects injected with ATP synthase β dsRNAs compared with those measured in insects injected with GFP dsRNAs.

Discussion

RNAi induced gene silencing was confirmed to occur in *E. variegatus* phytoplasma vector and turned out to be a powerful molecular tool to study the phytoplasma transmission mechanism. Beside the gene silencing observed at the transcript level, a significant decrease of corresponding protein was also observed. Determining the effect of dsRNA injection on protein expression allowed to perform optimized experiments for phytoplasma acquisition with a proper time scale. Indeed, the phytoplasma acquisition was performed only after the effect of gene silencing was evident also at the protein level. A significant difference was found in phytoplasma quantity measured in silenced insects compared with the controls. These preliminary results seem to demonstrate an *in vivo* role in phytoplasma infection

mechanism for ATP synthase β. Interestingly this protein is already known to interact *in vitro* with pathogen membrane proteins (Galetto *et al.*, 2011; Paingankar *et al.*, 2010).

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Interaction

Metabolic and physiological changes induced in *Sesamum indicum* infected by phytoplasmas

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Abstract

Sesame is one of the oldest oil seed crops grown around the world and phytoplasmas are one of the most destructive plant pathogens associated with different morphological, anatomical and physiological changes leading to huge losses in oil seed crop production. The objective of the present study was to quantify and study various primary and secondary metabolites produced in response to phytoplasma infection to understand how phytoplasma affects the physiology and metabolic processes of infected plants. In the infected sesame plants increased catalase, superoxide dismutase and peroxidase activity along with phenolic contents and soluble proteins was detected. The levels of proline and glycinebetaine also increased. Higher levels of malondialdehyde and hydrogen peroxide were measured and infected plants showed lower contents of soluble sugars.

Keywords: physiology, morphology, anatomy, Pakistan

Introduction

Sesame (*Sesamum indicum* L.) is one of the oldest crops used for its oil producing seeds. Because of its great stability, resistance to drought, and easiness of extraction it was considered as a major oil seed crop in ancient world. Archaeological remnants were found in the Harappa valley which date back to 5500 BC (Torres *et al.*, 2006). Even now, India is one of the world's largest producer of sesame (Wei *et al.*, 2013). Sesame seeds contain antioxidants and lignans and it is widely used for medicinal and cooking purposes. Phytoplasma infection is severely damaging the sesame crop due to its high susceptibility and this is a serious problem also in Pakistan (Akhtar *et al.*, 2009). It was reported that phytoplasmas affected the hormonal balance and the transportation of carbohydrates and amino acids (Bertamini *et al.*, 2002). To manage phytoplasma diseases the main focuses are on controlling their insect vectors and planting disease resistant cultivars. However since it is also necessary to understand the effect of phytoplasmas on plant's physiology this was the objective of the present study.

Materials and Methods

Sesame plants were inoculated with phytoplasma through insect transmission and grafting. The diseased plants showed characteristic symptoms of phyllody, virescence and proliferation. Samples collected from healthy (control) and symptomatic plants were tested through PCR using universal primers. The infected and healthy plant samples were analysed and compared for various physiological attributes such as chlorophyll and carotenoid contents by following the method of Arnon (1949), proline by Bates *et al.* (1973), glycinebetaine (GB) by Grieve and Grattan (1983), total soluble proteins by Lowery *et al.* (1951), hydrogen peroxide (H_2O_2) by Velikova *et al.* (2000), soluble sugars by Yemm and Wills (1954), phenolics by Julkenon (1985), and malondialdehyde (MDA) by Heath and Packer (1986). The ascorbic acid contents, enzyme activity of CAT (catalase), SOD (superoxide dismutase), POD (peroxidase), antioxidants was determined by Mukherjee and Choudhuri (1983), Chance and Maehly (1955), Giannopolitis and Ries (1977) and Asada and Takahashi (1987) respectively. The experimental data were analysed as Mean Standard Deviation (Mean ± SD) and variables were compared using ANOVA.

Results

The results of the analysis of variance of chlorophyll a, b, carotenoids, total chlorophyll, total phenolics, proline, (H_2O_2) GB, MDA, ascorbic acid, total soluble proteins and activity of SOD, POD, CAT and APX enzymes showed that the mean values differed significantly between healthy and infected samples (Table 1). The infected *S. indicum* plants showed lower contents of chlorophyll b and soluble sugars while high levels of proline, total soluble proteins, H_2O_2, phenolics, MDA, GB and antioxidant enzyme activity as compared to the healthy plants.

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Table 1. Mean values of physiological parameters studied.

Parameter	Healthy sample	Infected sample
Chlorophyll a	0.5 mg/g	0.46 mg/g
Chlorophyll b	0.5 mg/g	0.2 mg/g
Carotenoids	0.05 mg/g	0.01 mg/g
Phenolics	0.05 mg/g	nd
Proline	60 µmol/g	110 µmol/g
Glycinebetaine	27 µg/g	32 µg/g
MDA	13 µmol/g	22 µmol/g
H_2O_2	3.6 µmol/g	9 µmol/g
Soluble sugars	36 µmol/g	55 µmol/g
Soluble proteins	160 mg/g	210 mg/g
Ascorbic acid	12,000 µmol/l	180,000 µmol/l
SOD	2.1 mg/g protein	2.4 mg/g protein
POD	3.9 µmol/mint/mg protein	5.9 µmol/mint/mg protein
CAT	32 µmol/mint/mg protein	35 µmol/mint/mg protein

Discussion

The remarkable reduction in PS II activity in infected leaves was due to the decrease in chlorophyll and light harvesting chlorophyll protein complexes. The reduction in chlorophyll content shows that the phytoplasma can interfere in photosynthesis and induce senescence in the leaf (Zafri *et al.*, 2012). Under stress conditions the accumulation of osmolytes like GB in cells protects the organisms against biotic stresses via osmoregulation or osmo protection (Giri *et al.,* 2011). Phytoplasma infected sesame plants in this study showed higher proline and GB contents as compared to the healthy ones. Under biotic stress conditions, the proline overproduction bring ROS concentrations to a normal concentration, stop electrolyte leakage by maintaining cell turgor and osmotic balance and stabilize the membranes thereby improving the plant tolerance to biotic stress (Heath and Packer, 1986). Phytoplasma infected sesame plants also showed greater SOD contents. Bertamini *et al.* (2002) found that the ROS and SOD concentration increased in infected plants as compared to healthy plants. The activation of antioxidant defence system in plants protect them against oxidative stress damage (Torres *et al.*, 2006). The results of present study showed that phytoplasma infected plants had higher POD and CAT contents similar to the results reported by Lepka *et al.* (1999). The outcomes of the present study also showed that phytoplasma infected sesame plants had significantly higher soluble protein contents. Abdollahi *et al.* (2013) and Junqueira *et al.* (2004) found similar results in lime and maize plants infected by phytoplasmas. Zafari *et al.* (2012) reported that phytoplasma infection decreases the contents of total soluble sugars in infected plants, possibly due to a reduction in the photosynthesis. The results of the present study verified also that soluble sugars were significantly lower in phytoplasma infected plants.

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Mixed infection

A survey in carrot reveals a widespread aster yellows infection, and a first case of '*Candidatus* Liberibacter solanacearum' in Belgium

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Abstract

In an attempt to identify the agent of widespread yellowing and reddening symptoms, as well as the presence of adventitious roots in commercial carrot in Belgium, an extensive survey on the presence of phytoplasmas and '*Candidatus* Liberibacter solanacearum' was organised in 2015 and 2016. The survey on 29 fields in the regions Flanders and Hainaut revealed a widespread aster yellows (AY) phytoplasma presence associated to the majority of the symptoms. A second survey, specifically focused on identifying (potential) plant- and leafhopper vectors was set-up in selected carrot fields. This vector survey resulted in identifying *Macrosteles sexnotatus* as the most likely vector, and several other plant- and leafhoppers (notably *Philaenus spumarius* and *Typhlocyba quercus*) potentially playing a role in the spread of AY. During the insect survey, '*Ca.* L. solanacearum' haplotype D, was found in two commercial carrot fields, however its known psyllid vectors were not found.

Keywords: aster yellows phytoplasma, 16SrI-A, leaf- and planthoppers, haplotype

Introduction

Following a general concern on the a possible introduction of '*Candidatus* Liberibacter solanacearum' (CaLsol), an organism included in the EPPO A1 list, a project was initiated to identify the cause of leaf yellowing and reddening, and the presence of hairy roots in several commercial carrot (*Daucus carota* L.) productions. In carrot, mainly aster yellows phytoplasmas ('*Candidatus* Phytoplasma asteris'; AY) with strains classified in several subgroups are associated with yield losses (Duduk *et al.*, 2008), however, also mixed phytoplasmas and CaLsol presence has been reported (Satta *et al.*, 2017). Phytoplasma diseases in carrot and potato are known to be transmitted by a number of plant-sucking insects (plant- and leafhoppers) of the Auchenorrhyncha (Hymenoptera suborder), whereas CaLsol transmission is associated with psyllid presence. Both a pathogen and insect survey were organised.

Materials and Methods

For two growing seasons both commercial carrot and potato (*Solanum tuberosum* L.) fields were sampled and screened for the presence of phytoplasmas and CaLsol. All carrot and potato samples underwent sub-sampling (1 g of homogenised leaf and petiole, for carrot also root tissue) and subsequent DNA extraction using an adapted CTAB method (Doyle and Doyle, 1990). A generic nested PCR was used for phytoplasma

detection (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and quantitative PCR according to Li *et al.* (2009) for CaLsol screening. Further identification and characterisation by haplotyping was done according to the methods described by Liefting *et al.* (2009), Munyaneza *et al.* (2009), and Nelson *et al.* (2011).

Results

The initial carrot survey (2015/2016) used a symptom-based sampling strategy in geographically dispersed commercial fields. In total, 316 samples were analysed, collected from 29 locations. None of the samples tested positive for the presence of CaLsol during this survey. However, '*Ca.* P. asteris' was found in 10 locations at an overall disease incidence of 19%. General leaf yellowing and reddening was observed, and in some cases, deformation of the leaves and moderate to severe secondary root hair formation on the pen root was observed (Figure 1). Virtual RFLP (on the GenBank accession number MH883790) and phylogenetic analysis on the amplified 16S rDNA fragments of the Belgian strains from two carrot plots and a strain retrieved from *Macrosteles sexnotatus*, indicated that the phytoplasma clusters with those in the 16SrI-A ribosomal subgroup (Figure 2). During a follow up survey which focused on the identification of (potential) vectors in a selection of the AY affected carrot fields, a first detection and identification of the presence of CaLsol in two separate fields, at a distance of about 10 km apart, was

evidenced. No clear difference could be made between the symptoms on carrots affected by the AY phytoplasma or CaLsol (Figure 1). In both locations, several individual plants tested positive for both AY phytoplasma and CaLsol. The cultivar Nerja seed lots, tested positive for the presence of CaLsol. The obtained ribosomal sequences of the 50S, and partial ISR-23S fragments of CaLsol strain GBBC_CLso_03 from carrot were identified as haplotype D (GenBank accession numbers MH748578 and MH734515, respectively).

Phytoplasma detection on a selection of potential insect vectors, identified from a yellow sticky plate survey (growing season 2017) only resulted in positive signals for *Macrosteles sexnotatus*. A partial 16S rDNA fragment was deposited in GenBank under the accession number MH764575. Only a very limited amount of psyllids (12 in total) were caught during the survey. They all belonged to the families of the Triozidae and Aphalaridae, and none tested positive for CaLsol. *Aphalara freji* was identified as the prevailing psyllid, in one location, *Bactericera albiventris* was also identified. Both *A. freji* and *B. albiventris* are not known to feed on carrots and were probably feeding on weeds in the area.

Figure 1. From left: during harvest, a considerable percentage of poor quality carrots are manually sorted out; leaf yellowing and reddening in the field; excessive secondary ("hairy") roots formation.
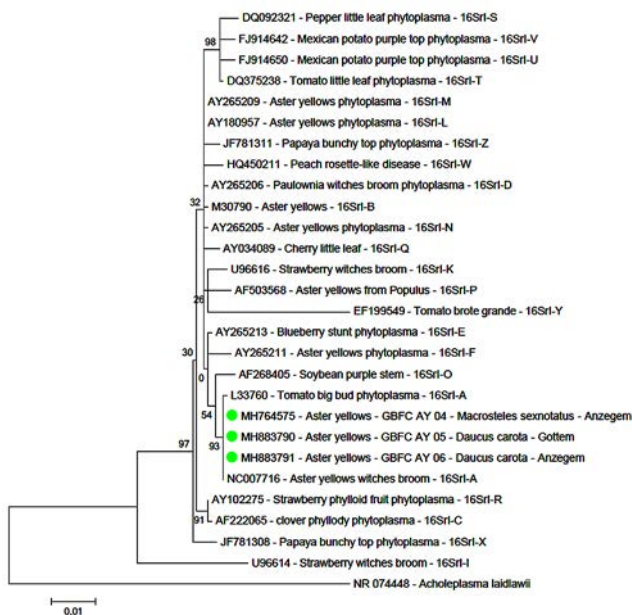
Figure 2. Phylogenetic tree of the 16S rDNA fragment (P1/P7 primers) constructed with the Maximum Likelihood method (Tamura 3-parameter model). Belgian strains are indicated with a green dot. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

Discussion

This study demonstrated that '*Ca*. P. asteris' is a widespread pathogen in the Belgian carrot fields. Additionally, the monitoring also revealed the presence of CaLsol in two commercial carrot fields. Even if the remaining seed lots also tested positive for the presence of CaLsol, it is not certain that the outbreak was the result of this seed lot infection. Previous studies indicate high '*Ca*. L. solanacearum' incidence rates in carrot seeds, and even if one report indicates high seed transmission rates (Bertolini *et al.*, 2015), later studies indicate a low transmission risk through seed (Loiseau *et al.*, 2017). This is the first report of the presence of a '*Ca*. L. solanacearum' in commercial carrot fields in Belgium.

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Mixed infection

Simultaneous evaluation of '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter solanacearum' seed transmission in carrot

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Abstract

Analysis on commercial carrot seeds and derived plants were performed to evaluate the '*Candidatus* Liberibacter solanacearum' and '*Candidatus* Phytoplasma' presence. The analyses on the seeds demonstrated the presence of both prokaryotes in nearly 100% of the seed batches tested. Sequencing confirmed that '*Ca.* L. solanacearum' strains detected in the seed samples belong to the haplotype D. Seedlings were grown in an insect proof greenhouse and in a growing chamber under controlled conditions. Symptoms of root malformation and leaf whitening and browning were observed in some of the carrot plants obtained from seed. DNA extraction and PCR assays were performed on both symptomatic and asymptomatic samples and diverse '*Candidatus* Phytoplasma' species were detected in the seedlings at different growth stages. DNA belonging to the ribosomal groups 16SrI and 16SrXII-A was detected and confirmed by RFLP analysis and sequencing indicating the seed transmission of these phytoplasmas in carrot. The analyses to detect '*Ca.* L. solanacearum' in these seedlings resulted always negative.

Keywords: '*Candidatus* Phytoplasma', '*Candidatus* Liberibacter solanacearum', seed transmission, carrot

Introduction

'*Candidatus* Liberibacter solanacearum' and '*Candidatus* Phytoplasma' are bacteria which share the phloematic habitat and are associated with economically relevant plant diseases, however the symptoms associated to their presence are analogous. The first are Gram negative with thin cell walls (Liefting *et al.*, 2009), associated (in their aplotype A and B) with the "zebra chip" disease of potato in America (Munyaneza, 2012) and with Apiaceae diseases in Europe (Munyaneza *et al.*, 2010). Phytoplasmas are associated to a multitude of diseases involving hundreds of cultivated and spontaneous plants, both woody and herbaceous. The study has been focused on the verification of seed transmission of both prokaryotes in carrot species.

Materials and Methods

Twenty nine carrot commercial seeds batches were tested for phytoplasma and '*Ca.* Liberibacter' presence. Seeds from 14 of these batches were sowed in an insect proof greenhouse and in a growing chamber under controlled conditions, in order to produce the new-born plants needed in two different times (4 and 10 samples each time) and sampled different times after sprouting for testing (Figure 1). Additionally, some plants were sampled after being placed in long-term condition of water shortage (stressed samples). DNA was extracted with CTAB and CTAB+Kit Qiagen methods

(Angelini *et al.*, 2001; Ilardi *et al.*, 2019) according with the tissue type. Extracted DNAs were tested by PCR using the primers pairs reported in Table 1 to verify the presence of '*Ca.* L. solanacearum' and '*Ca.* Phytoplasma' under the reported cycling conditions. For the confirmation of the phytoplasma presence the *tuf* gene amplification was also employed (Makarova *et al.*, 2012). DNA samples of phytoplasma strains maintained in collection (Bertaccini, 2014), and of '*Ca.* Liberibacter' (Satta *et al.*, 2016) and samples containing sterile distilled water were employed as positive and negative controls. Phytoplasma identification was performed by RFLP analysis with *Tru*1I restriction enzyme, whereas '*Ca.* L. solanacearum' was identified by amplicon sequencing and comparison with nucleotide sequences in the GenBank database to determine the aplotype (Nelson *et al.*, 2011, 2012).
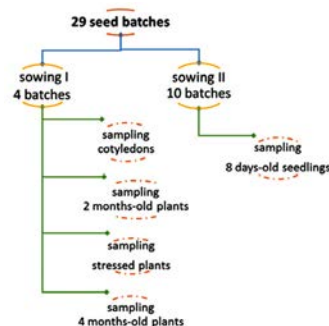
Figure 1. Scheme of sowing and samplings the carrot seedlings.

Table 1. PCR primers used to detect '*Ca*. L. solanacearum' and '*Ca*. Phytoplasma' species in carrot seeds and seedlings.

Name	Literature
'*Candidatus* Liberibacter solanacearum'	
Lso TX 16/23 (f)	Ravindran A *et al.*, 2011.
Lso TX 16/23 (r)	*Plant Disease*, 95(12): 1542-1546.
CL514 (f)	Munyaneza JE *et al.*, 2009.
CL514 (r)	*Plant Disease*, 93(5): 552-552.
OA2	Liefting LW *et al.*, 2008.
OI2c	*Plant Disease*, 92(10): 1474-1474.
'*Candidatus* Phytoplasma' species	
R16mF2	Gundersen DE and Lee I-M 1996.
R16mR1	*Phytopathologia Mediterranea*, 35: 114-151.
R16F2n	Gundersen DE and Lee I-M 1996.
R16R2	*Phytopathologia Mediterranea*, 35: 114-151.
U5	Lorenz KH *et al.*, 1995.
U3	*Phytopathology*, 85:771-776.
16R758f	Gibb KS *et al.*, 1995.
16S1232r	*Phytopathology*, 85: 169-1-74.

Results

In the DNA extracted from the seed batches the presence of both prokaryotes was found to be nearly 100%. In fact, 27 out of 29 batches tested resulted positive to '*Ca*. L. solanacearum' and 24 resulted positive to phytoplasma presence. '*Ca*. L. solanaceaum' DNA sequence analyses allow to classify it as haplotype D. All the plants in all growth stages resulted negative for '*Ca*. L. solanacearum' presence. Instead, phytoplasmas belonging to the ribosomal groups 16SrI and 16SrXII-A were detected in both the seed batches and the seedlings, with primers for 16S rRNA (Table 1) and *tuf* genes. Phytoplasma presence was registered in seedlings as decreasing with the time (with the exception of the stressed samples). In fact, in the seedlings the analysis on 16S rRNA and *tuf* genes registered 1 positive out of 10 batches at the sprouting time, respectively, for the two genes, 5 and 1 out of 14 in the cotyledons phase, 4 and 1 out of 14 in the 2-monts-old plants, 5 and 3 out of 14 in the stressed plants and 1 out of 52 in the 4-month-old plants. In this study has been firstly demonstrated that the '*Ca*. P. asteris' (16SrI) can be transmitted by seeds to the carrot plants and it was detected until the 4-month-old plants. Moreover, also '*Ca*. P. solani' (16SrXII-A) is transmitted by seed but it was detected only in the cotyledon stage and sometimes in mixed infection with '*Ca*. P. asteris'. The '*Ca*. L. solanacearum' resulted not transmitted from the seed to the deriving plant, but further studies are necessary to increase the statistical robustness of the test since about 100 tests were carried out for a total of about only 500 carrot seedlings in the diverse growth stages.

Discussion

While the '*Ca*. Phytoplasma' species seems to be effectively transmitted by seed in the carrot seedlings, the '*Ca*. L. solanacearum' has not been detected in the daughter plants. Considering that these prokaryotes share the same habitat,

the '*Ca*. L. solanacearum' is likely not able to be transmitted through the seeds or is not viable. The fact that only '*Candidatus* Phytoplasma' has been showing capable of seed transmission could be related to his lack of cell wall. It seems also plausible that in the seed (which in carrot is an achene) '*Ca*. L. solanacearum' fails to cross over maternal tissue up to the embryo, remaining therefore localized in the dry tissue wrapping the true seed. This work is confirming the phytoplasma seed transmission in carrot as demonstrated for other plant species (Satta *et al.*, 2019).

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Mixed infection

Potato purple top disease in Ecuador

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Abstract

In 2013 potato purple top symptoms appeared in Ecuador affecting potato crops, since then the potato production has been decreasing. In 2015 started the molecular analyses to identify the possible pathogen/s involved. Amplification with primers targeting as agent of this potato purple top diverse possible agents were used without obtaining clear results. Only in 2018 a phytoplasma of the subgroup 16SrI-F was detected and identified in the symptomatic plants. In the late 2018 the presence of the potato psyllid *Bactericera cockerelli* was for the first time observed in Ecuador and the hypothesis is that it might be involved in the purple top disease spreading. Several trials were performed to corroborate the appearance of the symptoms when potato psyllids are present. No '*Candidatus* Liberibacter solanacearum' has been found in plant potato samples or potato psyllids so far.

Keywords: phytoplasma, zebra chip, *Bactericera cockerelli*, PCR

Introduction

In 2013 in the northern of Ecuador, symptoms of potato purple top disease (PPT) started to appear. In a year, the diseased plants resulted to be more than 80% in the fields, with losses reaching up to the 50%. In 2015 '*Candidatus* Phytoplasma aurantifolia' (16SrII group) was reported associated with PPT in Ecuador (Caicedo *et al.*, 2015). The characteristic symptoms of this disease are yellow and purple coloration of the upper leaflets, apical leaf curling, axillary buds and aerial tubers (Figure 1). Diseases caused by other plant pathogens like *Rhizoctonia*, which produces aerial tubers as well, mislead to identify the causal agent of PPT in Ecuador. The rapid spread of the PPT did not correspond to the *Rhizoctonia* epidemiologic behaviour that was in fact isolated only from some scattered plants (Castillo Carrillo *et al.*, 2018). The tomato potato psyllid (TPP), *Bactericera cockerelli*, has been reported in North America, in some Central American countries, and in New Zealand (Crosslin *et al.*, 2010; Johnson *et al.*, 2017; Liefting *et al.*, 2008). The TPP is the vector of '*Candidatus* Liberibacter solanacearum' (CaLsol). The objective of the research was to identify the causal agent of PPT and its possible insect vector.

Materials and Methods

DNA from PPT symptomatic plants was extracted using a CTAB method. Several primer pairs were tested in PCR reactions (Table 1) however the successful combination was the universal phytoplasma primer pair P1/P7 (Deng and

Hiruki, 1991; Schneider *et al*, 1995) and the nested PCR with primer pair R16(I)F1/R1 (Lee *et al.*, 1994). RFLP analyses were performed using the estriction enzyme *Rsa*I. Further analyses were carried out to verify the presence of CaLsol using primers OA2 and OI2c and primers CaLsol TX 16/23 F/R (Castillo Carrillo *et al.*, 2018). Potato psyllids (PPs) were collected in two potato fields in the Pichincha province. The specimens were identified morphologically using colour and structural characters (Yen and Burckhardt, 2012), and by DNA extraction according to Crosslin *et al.* (2013) and PCR using the primer pair COIF3/COIR3. PCR amplicons were sequenced and compared to the sequences obtained by Swisher *et al.* (2012). The presence of CaLsol was verified by PCR with primers OA2/OI2c (Castillo Carrillo *et al.*, 2019). Trials in the greenhouse were performed to determine if the PP was the vector for PPT. Healthy plants were exposed to PPs collected from PPT infected fields. Furthermore, potato seeds from PPT infected plants were planted in two groups. One group was heavily sprayed with insecticides and the other group was exposed to PPs reared on PPT infected plants.

Figure 1. Potato field affected by purple top disease in Ecuador.

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Table 1. Primer used in the diagnostic of phytoplasmas in Ecuador.

Primers for PCR	Primers for nested PCR
P1/P7	R16mF2n/R16mR2
P1/P7	R16mF2/R16mR1
P1/P7	T16end/Tint
P1/P7	P3/P7
P1/P7	U5/RU
R16mR2/R16mR1	R16F2n/R16R2
P1/16S-SR	P1A/16S-SR
P1/P7	R16(I)F1/R1

Results

Several primer pairs were tested (Table 1) without obtaining clear results. The successful combination that yielded the presence of phytoplasma DNA was the use of P1/P7 primers followed by R16(I)F1/R1 in nested PCR. RFLP analyses on these amplicons with the enzyme *Rsa*I identify a phytoplasma of the 16SrI-F subgroup. No CaLsol was detected (Castillo Carrillo *et al.*, 2018). Potato psyllids are first reported in Ecuador and in South America. The specimens were identified as *B. cockerelli* central haplotype similar to the one reported by Swisher *et al.* (2012) and no CaLsol was detected (Castillo Carrillo *et al.*, 2019). The trials in the greenhouse showed the development of the PPT symptoms in the potato plants when the PPs collected in the symptomatic fields were present.

Discussion

The epidemiology of PPT in the Ecuadorian fields may be associated with 16SrI-F phytoplasmas, spread by TPP psyllids in PPT affected potato fields. *Rhizoctonia*-like plant pathogens were ruled out as a possible cause of PPT in potato fields. A previous study detected the presence of '*Ca.* P. aurantifolia' (16SrII group) (Caicedo *et al.*, 2015), but another study allowed verifying the presence phytoplasmas enclosed in 16SrI-F subgroup (Castillo Carrillo *et al.*, 2018). Phytoplasmas enclosed in this subgroup has only been reported in Spain (strain ACLR-AY = A-AY) and in Germany (strain CVB) in apricot and periwinkle respectively (Lee *et al.*, 1998; Bertaccini, 2014).

TPP is one of the most devastating pests of solanaceous crops (Vereijssen *et al.*, 2018) in western North America, Central America and New Zealand. It has not been reported in South America. Sequencing of the cytochrome oxidase (COI) gene confirmed that all psyllid samples were central haplotype. This suggests that the TPPs from Ecuador are genetically related to those from Texas (USA) and Central America. It is interesting to note that TPP has not been reported from Costa Rica, Panama and Colombia, three adjacent countries that connect Central and South America. Lastly, it is also possible that TPPs are present in other South American countries. TPP is a minute insect which can be easily overlooked or misidentified. The presence of the TPP in South America is particularly alarming due to the availability of a large number of cultivated and wild

Solanaceae plants. No CaLsol was detected in the psyllids or in the potato plants. Even though '*Ca.* L. solanacearum' has not been found in the current study, the presence of the vector poses a higher risk for CaLsol arrival (Castillo Carrillo *et al.*, 2018; 2019). More research should be done to demonstrate that TPP might be involved in the epidemiology of PPT in potato fields in Ecuador.

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Mixed infection

Phloem limited bacteria: a threat to Khasi mandarin cultivation in Assam, India

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Abstract

Phloem limited bacteria *i.e.* phytoplasmas were detected in Khasi mandarin samples during a roving survey conducted for citrus greening disease (CDG) incidence in Assam and North East India. Dual infection of CGD and phytoplasmas was detected by molecular methods in the collected leaf samples. PCR amplification of the 16S rDNA of phytoplasmas yielded 1,500 nucleotides product in direct PCR and 1,250 nucleotides product in nested PCR assays, respectively, in some Khasi mandarin samples. All the CGD infected samples yielded a band of 703 base pair in Khasi mandarin samples. Identification and characterization of the detected phytoplasmas at molecular level, incidence and associated loss in yield together with the identification of the insect vector is a future research area.

Keywords: Khasi mandarin, citrus greening, phytoplasma, dodder, periwinkle

Introduction

Citrus is one of the major fruit crops grown in India and it also occupies the place of highest value in terms of international trade. The North Eastern states of India harbors a vast reservoir of diverse *Citrus* species. Khasi mandarin (*Citrus reticulata*) covers an area of about 112,500 ha and 15,650 ha in North-east and Assam state respectively. One of the major constraints in citrus production is the citrus decline which is a complex syndrome associated with *Citrus tristeza virus*, citrus canker and citrus greening. Citrus greening disease (CGD) or "huanglongbing" is associated with the presence of uncultured fastidious bacteria belonging to diverse 'Candidatus Liberibacter' species (Bové *et al.*, 2008; Teixeira *et al.*, 2009). Phytoplasmas are small sized mollicutes possessing small genome and known to be pathogenic to more than a thousand plant species (Marcone *et al.*, 1999). Phytoplasma infected plants produces different types of symptoms such as, yellowing, stunting, shoot proliferation, distorted twigs, shortening of internodes, witches' broom and lack of vigour (Bertaccini, 2007). In the surveyed areas yellowing and small upright leaves were suspected to be associated with phytoplasmas. Therefore, a systematic study was done to verify the possible phytoplasma association with citrus greening disease in symptomatic plants.

Materials and Methods

A roving survey was conducted in four major citrus growing

districts of Assam: Tinsukia, Jorhat, Golaghat and Dibrugarh. The presence of CGD was assessed by visual inspection for the presence of typical symptoms such as leaf asymmetric blotch (Figure 1) and by specific PCR assays.

Figure 1. A) Chlorotic and mottled yellow shoots, B) witches' broom symptoms with excessive shoot proliferation and distorted twigs.

From the plants showing also distorted twigs resembling witches' broom leaf samples were collected and stored at -45°C. Total DNA was extracted from the midribs of the collected symptomatic as well as asymptomatic leaf samples using the cetyl trimethylammonium bromide (CTAB) method described by Kollar *et al.* (1990). Polymerase chain reaction (PCR) amplification using phytoplasma-specific primer P1/P6 (Deng and Hiruki, 1991; Schneider et al., 1995) for PCR amplification and R16F2n/R2 (Gundersen and Lee, 1996) for nested PCR was then carried out. The PCR products were resolved in 1.5% agarose gel in 1X Tris EDTA (TAE) with ethidium bromide (Sambrook and Russell, 2001). Dodder

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transmission was carried out using phytoplasma infected periwinkle plants maintained in the net house of the Department of Plant Pathology, AAU, Jorhat. The dodder bridge between the citrus plants and the infected periwinkle plants were detached after 4 weeks and observed regularly for symptom development.

Results

In citrus trees blotchy mottle and small yellow chlorotic leaves appearance are the main symptom of CGD, while distorted twigs resembling witches' broom are an indication of phytoplasma infection (Figure 2, Table 1).

Table 1. Presence of phytoplasmas and CGD in Khasi mandarin trees in the surveyed areas.

No.	Districts	Symptoms observed	
		Yellowing and mottling	Witches' broom and short internodes
1	Tinsukia	+++	++
2	Jorhat	++	+
3	Golaghat	+++	++
4	Dibrugarh	++	++

+ Mild,   ++ Moderate,   +++ Severe
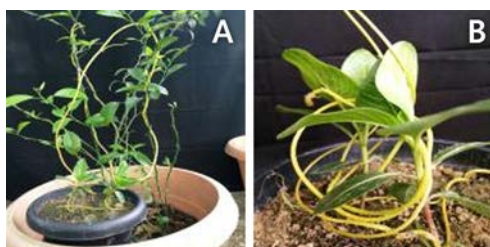
Figure 2. A) Dodder transmission from periwinkle to Khasi mandarin plant, B) infected periwinkle plant with dodder.
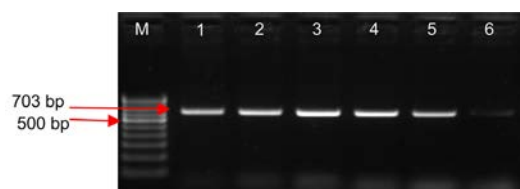
Figure 3. Agarose gel electrophoresis showing amplified PCR products of 703 bp from CGD infected samples. M, 100 bp ladder; 1-2, Tinsukia; 3-4, Jorhat; 5, Golaghat and 6, Dibrugarh samples.
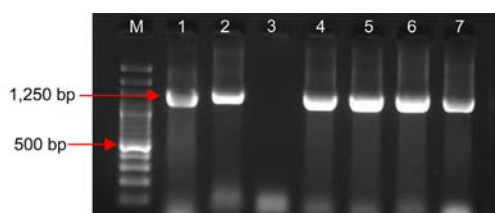
Figure 4. Amplified nested PCR products from phytoplasma infected samples. M, 100 bp ladder; 1, positive control; 2, citrus plant used in transmission trials; 3, negative control, 4-7, Tinsukia, Jorhat, Golaghat and Dibrugarh samples respectively.

The collected leaf samples from surveyed areas were found positive to CGD through PCR analysis and in the gel electrophoresis a clear 703 bp band was observed (Figure 3). The same samples were tested for phytoplasma and PCR amplification of the 16S rDNA from some of the positive citrus samples yielded 1,500 bp products (Figure 4) in PCR with primer pair P1/P6 and 1,250 bp products when amplified in nested PCR assays. The dodder bridge transmission from phytoplasma infected periwinkle to healthy citrus is under observation for symptom appearance.

Discussion

Phytoplasmas are known to be associated with CGD in citrus. The 78% of CGD infected plants showed the presence of phytoplasmas in China (Chen *et al.,* 2008; Texeira *et al.,* 2009). In this study the phytoplasma presence in CGD infected Khasi mandarin plants was detected using phytoplasma specific primers. The association of phytoplasma with CGD infected citrus confirms the results of Bové *et al.* (2008). Identification and characterization of the detected phytoplasmas at molecular level, incidence and associated loss in yield together with the identification of the insect vector is a future research area.

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Mixed infection

Association of phytoplasmas with banana bunchy top viral disease in banana plants in Assam, India

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Abstract

The possible association of phytoplasmas with the *Banana bunchy top virus* (BBTV) and little leaf symptoms in banana was investigated. Symptomatology showed presence of characteristic little leaf symptoms in BBTV infected banana plants. Six banana samples showing little leaf were assayed for the presence of phytoplasmas and their association with BBTV using polymerase chain reaction (PCR). Five out of thr six tested genotypes were found positive to phytoplasmas while all were positive to BBTV. The 1.5 kb product from the amplification of the 16S rRNA in PCR with primer pair P1/P6 and about 1.25 kb product in nested PCR assay confirmed the association of phytoplasmas with BBTV infected banana genotypes.

Keywords: banana bunchy top disease, mixed infection, PCR, plant disease

Introduction

Banana is one of the important commercial horticultural crops. Bunchy top disease of banana caused by *Banana bunchy top virus* (BBTV) (Babuvirus; Nanoviridae) is considered among the most important constraints in production of banana in different parts of the world. The disease was first recognized in Fiji in 1889. Due to high destructive potential, BBTV is regarded as quarantine pest of very high importance and Invasive Species Specialist Group (ISSG) of the IUCN listed BBTV in the 100 of the world's worst invasive alien species (Wickramaarachchi *et al.*, 2016).

Phytoplasmas having a broad host range are obligate plant pathogenic prokaryotes without cell wall, belonging to the class *Mollicutes* of phylum *Tenericutes* (Hogenhout *et al.*, 2008). In the experimental field of Assam Agricultural University (AAU), Jorhat, Assam little leaf symptoms were observed in different BBTV infected banana genotypes. Therefore, an effort was made to investigate the association of phytoplasma with the BBTV and little leaf symptoms.

Materials and Methods

The banana leaf samples were collected from different genotypes maintained at the experimental field of AAU, Jorhat during July-October, 2018, transported in plastic bags and stored at -45°C. The samples were at first screened for presence of BBTV and suspected genotypes showing little leaf symptoms along with bunchy top were assayed for presence of phytoplasmas. Total DNA from banana plant samples was extracted following a CTAB method (Kakati, 2017) with some modification. The phytoplasma PCR analysis was done using the primer pair P1 (Deng and Hiruki, 1991) and P6 (Schneider *et al.*, 1995) for direct PCR amplification and R16F2n/R2 (Gundersen and Lee, 1996) for the nested amplification. For the *Banana bunchy top virus* the PCR analysis was done using the primer pair BBTV-DNA1F/BBTV-DNA1R following 30 cycles of reaction with primer specific annealing temperature (Wickramaarachchi *et al.*, 2016). The products were resolved on 1.5% agarose gel stained with ethidium bromide (Sambrook and Russell, 2001).

Figure1. Overview of the banana field showing bunchy appearance and little leaf symptoms.

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Results

Banana plants showed typical bunchy top appearance represented by the characteristic symptom and amongst them some plants showed little leaf symptoms and were tested for the presence of phytoplasmas (Figure 1). Out of the 36 banana genotypes assayed for BBTV, 20 were positive through PCR analysis since they were producing the expected 1,111 bp bands in agarose gel electrophoresis (Figure 2). Six of BBTV infected genotypes suspected for the presence of phytoplasmas were assayed and five were found positive since they yielded a 1.5 kb product from PCR amplification of the 16S rRNA (Figure 3) in the direct PCR with primer pair P1/P6 and a 1.25 kb product in nested PCR assays. The results of PCR analysis for *Banana bunchy top virus* and phytoplasmas are presented in the Table 1.

Table 1. Banana genotypes under study and results of PCR analysis for BBTV and phytoplasmas.

No.	Genotype	BBTV	Phytoplasma
1	Chenichampa (AAB)	+	-
2	Assam Malbhog (AAB)	+	+
3	Grand Naine (AAA)	+	+
4	Suti jahaji (AAA)	+	+
5	Tulaathiya (BB)	+	+
6	Bhimkol (BB)	+	+
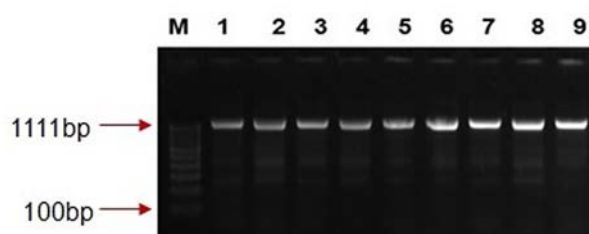
Figure 2. Agarose gel electrophoresis showing positive amplified PCR products of BBTV infected samples. M, 100 bp ladder; 1-9, banana plant samples from different genotypes.
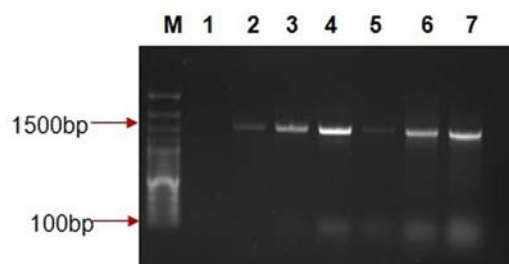
Figure 3. Agarose gel electrophoresis showing amplified PCR products from phytoplasma infected samples. M, 100 bp ladder, 1-6, banana plant samples from different genotypes; 7, phytoplasma positive control.

Discussion

Banana genotypes infected with BBTV showing little leaf symptoms were found to be infected also with phytoplasmas

by PCR analysis (Figure 4). Phytoplasmas have a wide host range and were identified from many host plant species. Recently they are reported as associated with severe banana wilting diseases worldwide (Aliaga *et al.*, 2018; Miyazaki *et al.*, 2018). This finding has provided a new field of research to be carried out to screen different banana genotypes for phytoplasma presence in mixed infection with other banana viruses. These research should be able to elucidate the presence of still unknown banana pathogen helping in the improvement of management of infected plants especially in genotype collections in order to prevent the dissemination of very harmful pathogen to new countries.

Figure 4. Appearance of little leaf symptoms (central plant) in *Banana bunchy top virus* infected banana plants maintained in pot.

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Mixed infection

Mixed infection of virus and phytoplasma in gladiolus varieties in India

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Abstract

Gladiolus grandiflorus is a bulbous ornamental plant popularly grown for its cut flowers of high commercial value. On a survey to the experimental fields at IARI, New Delhi, during 2016-2018. About 8% to 96 % disease incidence of leaf yellowing and streaks were observed in eleven varieties of gladiolus. The presence of '*Candidatus* Phytoplasma trifolii' (16SrVI-D) subgroup in all the eleven symptomatic varieties was confirmed by PCR amplification using phytoplasma specific primer pairs P1/P7 and R16F2n/R16R2 and amplified 16Sr DNA sequence comparison. The presence of mixed infection with *Cucumber mosaic virus* (CMV) was also confirmed by electron microscopy and coat protein sequence analysis of gladioli samples. The results suggest the presence of mixed infection of 16SrI-D subgroup phytoplasmas and CMV associated with leaf yellowing and streaks symptoms in gladiolus varieties.

Keywords: gladiolus, phytoplasma, 16SrVI-D subgroup, *Cucumber mosaic virus*

Introduction

Gladiolus (*Gladiolus grandiflorus* L.) is a bulbous flowering plant that belongs to the family Iridaceace. It is popularly grown for its magnificent spikes of varying colors used as cut flowers. The major gladiolus producing states in India are Uttar Pradesh, West Bengal, Odisha, Chhattisgarh, Haryana and Maharashtra. The presence of '*Candidatus* Phytoplasma asteris' (16SrI group) in gladioulus has been reported from Italy, Portugal, Poland and India associated with symptoms of chlorosis, yellowing, malformation, twisting, discoloration and virescence of flower spikes and faded colour corms (Bellardi *et al.*, 2018).

Materials and Methods

Survey of experimental fields of Division of Floriculture and Landscaping, Indian Agricultural Research Institute, New Delhi, was made in 2016-2018, to verify the virus and phytoplasma presence in gladiolus cultivars exhibiting symptoms of leaf yellowing and streaks (Figure 1). Symptomatic gladiolus plants were subjected to total RNA extraction from asymptomatic and symptomatic samples and used as a template in cDNA synthesis for PCR amplification of CMV coat protein with specific primers (Baranwal *et al.*, 2016). For phytoplasma identification, the total DNA was

extracted by a CTAB method from symptomatic and asymptomatic gladiolus leaves subjected to PCR amplification with phytoplasma universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by primers R16F2n/R16R2 (Gundersen and Lee, 1996) in nested reaction. The amplified fragments were purified and sequenced in both directions at Agri genome, India. The sequences were assembled using DNA Base V.4 (http://www.dnabaser.com) and aligned with representative sequences available in GenBank using ClustalW. These consensus sequences were submitted to GenBank. The phylogenetic trees were constructed using the neighbour-joining method with MEGA 7.0 with 1,000 bootstrap replications (Kumar *et al.*, 2016). The 16S rDNA sequences (~1.25 kb) from gladiolus phytoplasmas were subjected to in silico RFLP comparison using *i*PhyClassifier (Zhao *et al.*, 2009).
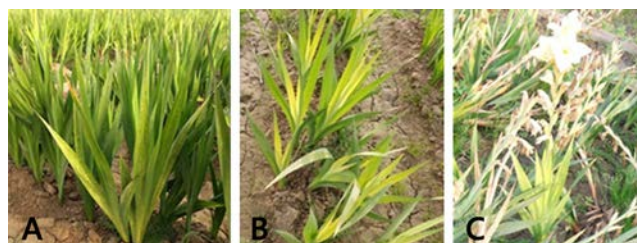
Figure 1. Gladiolus varieties showing symptoms a: streaks ("Bindiya"), b: leaf yellowing ("P-16-1 x Eurovision"), c: stunting and leaf yellowing ("Suchitra x Melody").

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Results and Discussion

The disease was recorded in 11 varieties of gladiolus with presence ranging from 7.91% to 96.03% (Table 1). The PCR amplification with coat protein specific primers of CMV showed amplification of 650 bp in all the symptomatic samples and amplification of about 1,250 bp was obtained with primers specific for phytoplasmas.

Sequences of two gladiolus virus and phytoplasma strains from "P-16-1 x Eurovision" and "Bindiya" were submitted to GenBank. Phytoplasma sequences comparison and phylogenetic trees showed 99.6% identity among themselves and clustering with members of 16SrVI group (Figure 2).

Table 1. Presence of phytoplasmas and virus in gladiolus.

Gladiolus genotypes	Disease presence %
P-16-1 × Eurovision	96.03
Melody open seedling	5.88
Berlew open seedling-I	18.19
Suchitra × Melody	93.47
(Melody× Mayur) × Headywine	8.38
Bindiya	81.75
Berlew open seedling-II	7.91
Smokey Lady × Oscar	10.1
(Snow Princess × Berlew) ×Headywine	59.54
Surekha hybrid	23.35
Salmon Queen open seedling	26

Figure 2. Phylogenetic tree showing phylogenetic relationship of gladiolus phytoplasmas with other strains of *16S rRNA* gene.
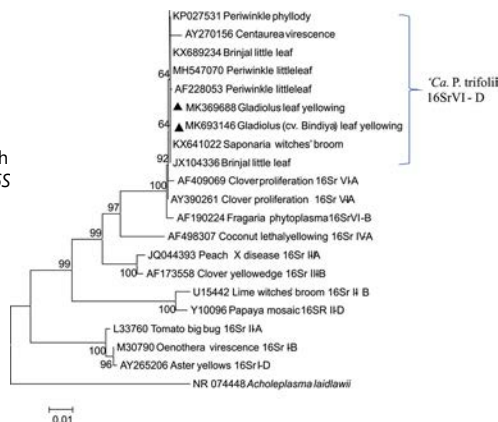
Figure 3. Phylogenetic tree showing phylogenetic relationships of CMV from gladiolus with reported CMV strains.
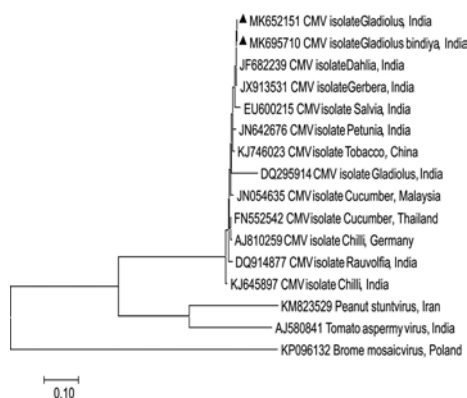
The virtual RFLP analysis of gladiolus phytoplasma strains sequences (GenBank accession numbers, acc. nos. MK369688 and MK693146) classified them in the 16SrVI-D subgroup. The sequence comparison and phylogenetic analysis of partial CP genes of the CMV strains (acc. nos. MK652151 and MK695710) revealed 99% identity with *Cucumber mosaic virus* strains (Figure 3). These results indicate mixed infection of a '*Candidatus* Phytoplasma trifolii'-related strain and CMV in gladiolus plants showing leaf yellowing and streaks disease. Earlier phytoplasma disease on gladioli has been reported from India and abroad to be associated with '*Ca*. P. asteris' (Bertaccini *et al.*, 1990, Louro *et al.*, 1996, Kaminska *et al.*, 1999, Raj *et al* 2009).

This is therefore, the first report of the presence 16SrVI-D subgroup phytoplasmas in gladiolus in mixed infection with CMV. Since the losses due to mixed infection are significant, further studies are required to understand the disease epidemiology and planning suitable control strategy.

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Mixed infection

Mixed infection among phytoplasmas, *Potato virus Y* and *Cucumber mosaic virus* in *Xanthosoma* plants in Assam, India

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Abstract

The occurrence of phytoplasmas, *Cucumber mosaic virus* (CMV) and *Potato virus Y* (PVY) disease complex was studied in *Xanthosoma* plants in Assam, India. Through PCR analysis the disease complex was detected. Total DNA and RNA were extracted following standard protocols with some modifications. PCR analysis was done with primer specific annealing temperature. This is the first report that phytoplasmas are associated with CMV and PVY in *Xanthosoma* plants.

Keywords: phytoplasmas, molecular detection, PVY, CMV, PCR

Introduction

Xanthosoma is a genus of flowering plants in the arum family, Araceae grown for their starchy corms, an important food staple in the tropical regions. The genus is native to tropical America but it is widely cultivated and naturalized in several tropical regions. *Potato virus Y* (PVY) is one of the most important virus from the family *Potyviridae* characterized by a broad range of host plants that includes both monocotyledonous and dicotyledonous species. PVY has been classified into different strains according to host (de Bokx and Huttinga, 1981). *Cucumber mosaic virus* (CMV) belongs to the family *Bromoviridae* is a widely prevalent plant virus because of its large host range that spans around 800 plant species (Palukaitis *et al.*, 1992).

Phytoplasmas are phloem inhibiting, wall-less, obligate bacteria belonging to the class *Mollicutes*. They are known to infect more than 1,000 plant species including many agriculturally important crops across the world (Sridhar *et al.*, 2013; Rao *et al.*, 2018). In the field symptoms such as yellowing, mosaic and curling were noticed in the *Xanthosoma* leaves that could be associated with the presence of viruses or phytoplasmas or both. Therefore an effort was made to verify the presence of phytoplasmas and other viruses in these symptomatic *Xanthosoma* plants.

Materials and Methods

During January-February, 2019 symptomatic *Xanthosoma* leaf samples were collected from the Horticultural Orchard, AAU, Jorhat and stored at -45°C. Total DNA was extracted following the standard protocol of Kollar *et al.* (1990). For phytoplasma PCR detection the P1/P6 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) primers were employed following 32 cycles of reaction and using primer specific annealing temperature. Total RNA was extracted from leaf samples using standard Tri-Reagent method as described by Akad and Czosnek (2002) with slight modifications. Primer pair used for analysis are listed in Table 1. The cDNA was prepared following the protocol of TaKaRa Prime Script reverse transcription kit and used for PCR analysis. The products were resolved in 1.5% agarose gel in 1X Tris EDTA (TAE) containing ethidium bromide (Sambrook and Russell, 2001).

Results

The *Xanthosoma* leaf symptoms observed were yellowing of leaves, mosaic and curling (Figure 1). The possible presence of mixed infection of these samples was verified through PCR and results found some positive samples for phytoplasmas, CMV and PVY. For phytoplasmas a clear 1,500 bp band was observed in gel electrophoresis (Figure 2). Similarly the expected 328 bp and 593 bp bands were observed for CMV and PVY respectively (Figure 3). The PCR analysis results found that the samples collected for the study gives positive result for phytoplasma presence (weak or strong bands), while only two samples give positive result for the presence of CMV and PVY. Among four of the samples tested only one resulted infected only by phytoplasmas (Figures 2 and 3). The PCR analysis results confirmed that there is mixed infection in some of the symptomatic *Xanthosoma* samples.

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Table1. Primer pair used for the PCR analysis to detect the CMV and PVY presence.

Primer name	Sequence	Product size	Virus
CMV S1-F	5´-GCCACCAAAAATAGACCG-3´	328 bp	CMV
CMV S2-R	5´-ATCTGCTGGCGTGGATTTCT-3´	328 bp	CMV
MJ1-F	5'-TGGTHTGGTGYATHGARA AYGG-3'	593 bp	PVY
MJ2-R	5' TGCTGCKGCYTTCATYTG-3'	593 bp	PVY

Figure 1. Symptomatic *Xanthosoma* leaf samples showing yellowing, curling and mosaic.
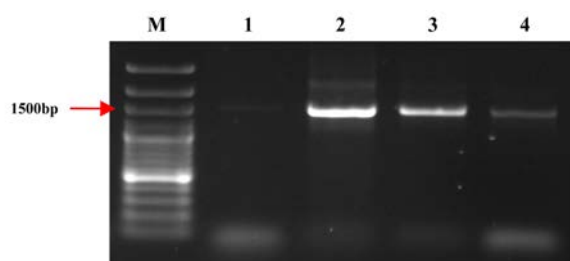
Figure 2. Gel electrophoresis results of PCR amplification from *Xanthosoma* collected samples amplified with primers P1/P6 for phytoplasma detection. M, 100 bp marker; 1-4, samples.
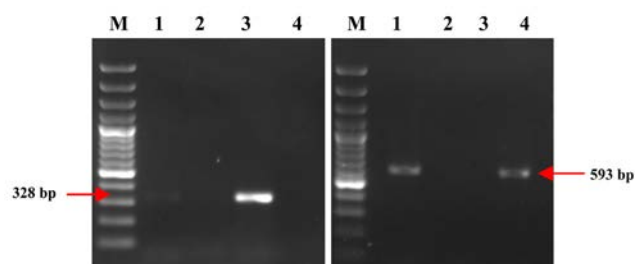
Figure 3. Gel electrophoresis results of RT-PCR on *Xanthosoma* collected samples amplified with primers for virus detection. M, 100 bp marker; CMV-328 bp (left), PVY-593 bp (right).

Discussion

Mixed infection of phytoplasmas with CMV and PVY in *Xanthosoma* plants was detectd through PCR analysis. This is the first report of phytoplasma mixed infection with CMV and PVY together with the first phytoplasma detection in *Xanthosoma* in India. Identification of insect vectors for phytoplasma transmission and phytoplasma identification are in progress. It must be pointed out that this species is a tuberous and shows not strong symptoms also when is multiply infected by different pathogens as in this case, therefore it represents a quite relevant possible source of infections for the crops cultivated in the proximity.

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Mixed infection

Coconut lethal yellowing disease and the *Oryctes monoceros* beetle: a joint venture against coconut production in Mozambique

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Abstract

The coconut palm, an important cash and subsistence crop, is widely grown in the coastal regions of the tropics, including Mozambique. However, outbreak of an invasive and emerging coconut lethal yellowing disease (CLYD) is now a major challenge for the coconut industry and livelihood of millions of Mozambican. The observed successions of CLYD epidemics in Mozambique, suggest that other factors than plant-insect-phytoplasma triangle might be associated with the current disease trends. This study investigated the impact of *Oryctes monoceros* beetle on the CLYD epidemic in Mozambique, using questionnaire survey and direct observation in the fields. The results revealed that there was a significant and negative correlation between beetle and CLYD incidence. This negative correlation may be explained by beetle movement from adult CLYD-infected palms to younger palms which pose a great challenge to the replacement of palms lost due to CLYD. Management strategies must therefore stress the role of beetle larvae in palm trunks killed by CLYD and the threat of *Oryces* beetles to the replacement of death palm with new plantation.

Keywords: coconut palm, Mozambique, coconut lethal yellowing phytoplasma, *Oryctes rhinoceros*

Introduction

Outbreaks of coconut lethal yellowing disease (CLYD) have caused successions of epidemics and losses of millions of palms (*Cocos nucifera* L.) threatening the industry and the livelihood of over three million people in Mozambique. The current CLYD epidemic status is concurrent with a severe *Oryctes rhinoceros* (*Coleoptera*, Dynastidae) infestation (Figure 1). Damage and crop loss of coconut palms by *O. rhinoceros* is only caused by the adult beetle, burrowing into the crown of the palm and feeds internally on the soft developing fronds before they have unfolded. Light attacks may have no discernible effect, but heavy attacks can kill the palm. Intermediate attacks weaken the palm and reduce the nut production.

The *O. monoceros* is one of the most dangerous pests of coconut palm and oil palm plantation in Côte d'Ivoire and throughout West Africa, causing up to 40% damage. The beetle larvae develop in decaying dead palm wood (Kouassi *et al.*, 2012). The final stage symptom of CLYD result in rotting of the stem apical tissues (heart) and death of the palm crown (McCoy, 1976; Yankey *et al.*, 2018). The palms trunks left over after the palm deaths due to CLYD, can host the eggs and larvae stages of *O. rhinoceros*. The current epidemic status of CLYD under co-infestation with *O. monoceros* beetle in Mozambique was therefore investigated.
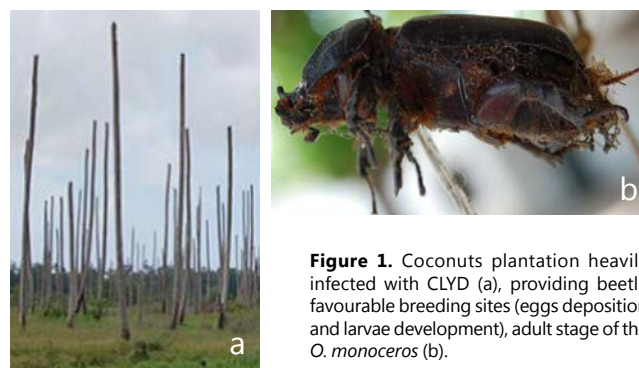
Figure 1. Coconuts plantation heavily infected with CLYD (a), providing beetle favourable breeding sites (eggs deposition and larvae development), adult stage of the *O. monoceros* (b).

Materials and Methods

The methodology included field work for information gathering and data analysis. Field work consisted of two main activities: (a) a questionnaire survey to the households (Hhs) and (b) a direct observation on palm plantations to estimate

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the incidence and severity of CLYD and the presence and numerosity of *O. rhinoceros*. All coconut trees were counted in this study, including the dead and felled ones, which gave a more realistic estimate of the CLYD infection level and its relationship with the beetle infestation. The percentage of CLYD infection and *O. rhinoceros* beetle infestation (PI) was estimated using the following formula:

$$PI = P\left(\frac{NI}{NT}\right) \times 100 \qquad (1)$$

Where PI represents the percentage of infection, NI represents the number of coconut trees infected in the plantation and NT represents the total number of coconut trees in the plantation. Disease severity for each plant was evaluated based on the following CLYD rating scale (Yankey *et al.*, 2018): 0 – healthy or asymptomatic plants; 1 – premature fall of fruits (1A) and/or one or more necrotic inflorescences (1B); 2 – discoloration of older leaves (basal leaves) (2A) and/or yellowing of the basal and middle leaves (2B); 3 – all leaves are yellow, from the base to the top; formation of a skirt (a group of dry leaves hanging vertically); 4 – death of the spear (immature) leaf, apical death (4A) and/or dead palm plant (telephone poles) (4B). The CLYD severity (S) was estimated using the following equation:

$$S = \frac{\sum (i \times Ni)}{N} \qquad (2)$$

Where S represents the severity, i represents the value of the scale, Ni represents the number of plants with class i symptoms, and N represents the total number of observed plants.

Results

The average CLYD incidence (PI) in the study area was 15%, ranging from 2% to 69% of coconut trees infected per farm (Table 1). From the total of 534 inspected farms 61.4% presented a PI of up to 10%, 32% beyond 10 up to 50% and 7% with PI beyond 50%. The disease severity was generally low, falling in the first 3 disease rating scale, namely 0 (no symptoms), 1 - premature fall of fruits (1A) and/or one or more necrotic inflorescences (1B) and 2 - discoloration of the old leaves (2A) and/or yellowing of the basal and mid leaves (2B). The relationships between *O. monoceros* and CLYD incidence and severity were investigated and the results indicated that the higher is the disease incidence or severity the lower is the percentage of *O. monoceros* infestation (Table 2).

Table 1. CLYD infection and severity levels by class of infection.

Districts	Avarage	Classes of CLYD incidence (%)		
		[0;10]	[10;50]	[50;100]
Average incidence	14.9 (n=534)	2.3 (n=333)	28.3 (n=161)	68.7 (n=40)
Average severity	0.51 (n=534)	0.10 (n=333)	0.99 (n=161)	2.11 (n=40)
Average severity		61.4 (n=305)	31.7 (n=155)	6.9 (n=39)

Table 2. Correlation between percentage of infection by *O. monoceros* and CLYD severity and incidence.

Variables		Number of observations	Correlation coefficient (r)	p value
O. monoceros incidence	CLYD severity	534	-0.2342	0.0000
	CLYD incidence	534	-0.2017	0.0000

Discussion

The increasing CLYD prevalence in Mozambique, may indicate that the control strategy used is not efficient. Furthermore, the negative correlation between *O. monoceros* and disease intensity it is line with Kouassi *et al.*, (2012) stating that beetle damage is generally caused by adults making feeding galleries in the apical section of the spear leaves, while rotting of the stem apical tissues (heart) and death of the palm crown is likely to be observed under CLYD infected coconut tree. As a result the *O. monoceros* beetle will migrate from CLYD infected tree to the still healthy or younger plants. The fact that *O. monoceros* prefer young coconut tree (Kouassi *et al.*, 2012) is a great constraint factor for the current CLYD management strategy in Mozambique, which rely on replanting tolerant varieties, since this new plantation are being heavily affected by *O. monoceros*. Another constraint factor is that the *O. monoceros* larvae thrive in dead palm trunks (Kouassi *et al.*, 2012) left after CLYD infections have killed the trees. These facts explain the negative correlation between CLYD and *O. monoceros* incidence and calls for a holistic approach on the current and future large scale replanting program, taking in consideration both the CLYD and *O. monoceros* beetle pathosystem.

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Mixed infection

Ultrastructural analysis of spiroplasmas detected in palm species infected with the lethal yellowing phytoplasma from Yucatan and Baja California Sur, Mexico

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Abstract

Lethal yellowing (LY) disease was reported from the Mexican states of Baja California Sur and Yucatan, and the presence of 16SrIV group phytoplasmas was fully confirmed by molecular detection assays. Using scanning electron microscopy technique some spiroplasma-like structures along with phytoplasmas were observed in the phloem of four analyzed palm species. Ultrastructural analysis revealed the similarity in morphology of these structures with reported plant-pathogenic spiroplasmas. The possible relationship between spiroplasmas, 16SrIV phytoplasmas and the LY disease is discussed.

Keywords: spiroplasma, palms, SEM, mixed infection, Mexico

Introduction

Palm lethal yellowing (LY) is a destructive emergent disease of palms, associated with phytoplasma presence. It is included in the EPPO List of Euromediterranean quarantine pests (EPPO, 2018). The LY disease initially was reported only in coconut palm for its disastrous impact on the coconut production (Nutman and Roberts, 1955). Currently the disease is distributed worldwide and associated with different phytoplasmas according with the different geographic infected areas (Gurr et al., 2016). In Mexico LY was reported from eight Mexican states in different palm and wild species, and the detected phytoplasmas were classified in six ribosomal subgroups from 16SrIV-A to -F (Narvaez et al., 2006; Cordova et al., 2017; Poghosyan et al., 2019). The aim of this work is to provide the results of ultrastructural analysis of spiroplasma-like structures in palms from Yucatan and Baja California Sur in Mexico, reporting the possible association of LY disease with phytoplasmas and spiroplasmas.

Materials and Methods

Samples from leaves, rachis, inflorescences and root tissues were collected from four palm species: *Cocos nucifera* and *Pritchardia pacifica* in Merida, Yucatan and *Brahea brandegeei* and *Washingtonia robusta* in Baja California Sur (BCS), analyzed with scanning electron microscopy (SEM) (Hitachi S-3000N) and molecular techniques. Samples for SEM analysis were prepared by the improved protocol of Lebsky and Poghosyan (2007). DNA was extracted by the method described from Tapia-Tussel et al. (2005), nested PCR was performed as reported (Gundersen and Lee, 1996). The final amplicons of about 1.2 kb were cloned in pGRM-T-easy vector, sequenced at Genewiz, NJ, USA and dnaseq.ucdavis.edu, USA then analyzed in BLASTn database and *i*PhyClassifier (Zhao et al., 2009).

Results

Analysis of symptomatic and some asymptomatic samples of palm species revealed in phloem tissue the presence of phytoplasma-like bodies, variable in size - 400 to 1,500 nm and concentration (Lebsky et al., 2012; Poghosyan et al., 2019). Samples from rachis and roots revealed in the phloem tissue some unknown structures, morphologically very similar to spiroplasma cells. In all examined palm species spiroplasma-like structures were observed with helical, tubular and pleomorphic forms (Figure 1), similar to the spiroplasmas growing *in vitro* and observed with SEM (Ammar et al., 2004). The tapered end (tip structure) of spiroplasmas was noted, and invagination on plasma membrane of host cell were visualized in some SEM images (Figure 1A). Helical forms were not clearly seen, but could be distinguished (Figure 1B). The presence of elongated non helical structures varying from 2,000 to 3,000 nm was also observed.

Table 1. Operational taxonomic units (OTUs) corresponding to *Mollicutes*. Data obtained from metagenomic analysis of DNA extraction from roots of two palm species from BCS. Sequencing technology: Ion Torrent PGM; amplification of V3 region of 16S rDNA; metagenomic analysis with GreenGenesDataBase. R2 and RB1-samples from *B. brandegeei*, RW1-2 and W 2- from *W. robusta*.

Sample							Reads
R2	Bacteria	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae		1
RB1	Bacteria	Tenericutes	Mollicutes	Enteroplasmatales	Spiroplasmataceae		1
RW1	Bacteria	Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmaceae	'*Ca*. Phytoplasma'	1
RW2	Bacteria	Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmaceae	'*Ca*. Phytoplasma'	1
W2	Bacteria	Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmaceae	'*Ca*. Phytoplasma'	1
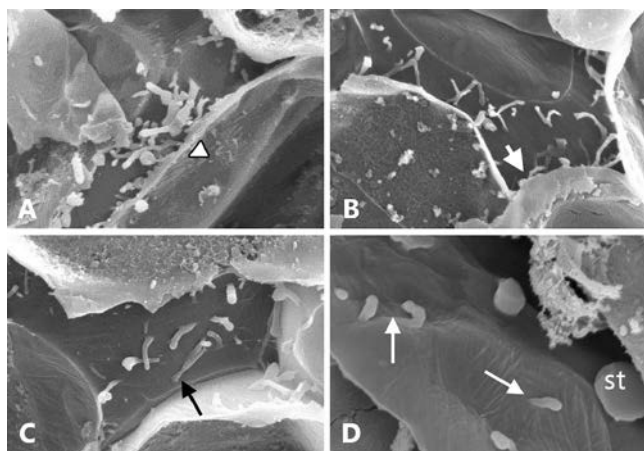
Figure 1. SEM images of spiroplasmas in palm species: *Cocos nucifera* (A, B); *Pritchardia pacifica* (C); *Brahea brandegeei* (D). Arrows-tip structures (B, C, D); arrow heads: helical form (B); connection to plasma membrane (A); st, starch grain.

The presence of phytoplasmas in all analysed samples from Yucatan and BCS was confirmed by molecular techniques. The results of molecular analysis were published by Cordova *et al.* (2017) and Poghosyan *et al.* (2019). Data presented in Table 1 are from a metagenomic analysis of the microbial community of roots of two palm species and could be a confirmation of the ultrastructural observations.

Discussion

Spiroplasmas are *in vitro* cultured *Mollicutes*, three species have been identified as plant pathogenic: *Spiroplasma citri, S. kunkelii* and *S. phoenicium* (Cisak *et al.*, 2015; Cacciola *et al.*, 2017). They are pleomorphic as phytoplasmas, and not only or always helical in sieve tubes; in some cases they are also spherical allowing possible misinterpretation of ultrastructural features. The cell morphology of spiroplasmas in transmission electron microscopy (TEM) analysis depends from their preparation technique, but the TEM images of negatively stained culture preparation (Ammar *et al.*, 2004) and the SEM images from phloem tissue presented here are very similar. The results of ultrastructure analysis of spiroplasma-like structures in phloem tissue of palm species using SEM was supported by OTUs metagenomic data for *Mollicutes*, proving the spiroplasma presence in the analyzed palm samples. This result could be linked with the possibility of spiroplasma interaction in palm having LY diseases and indicating the presence of possible mixed infection of phytoplasmas and spiroplasmas.

The work will be continued to achieve substantial molecular and microbiological proofs of spiroplasma presence.

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Management

The chemistry of multitrophic interactions in phytoplasma disease systems and advances in control of psyllid vectors with semiochemicals

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Abstract

Phytoplasmas associated with the three most important diseases on fruit crops, '*Candidatus* Phytoplasma mali' (apple proliferation), '*Ca.* P. pyri' (pear decline), and '*Ca.* P. prunorum' (European stone fruit yellows, ESFY), have been investigated for the chemistry of their interactions in a multitrophic context. The chemically mediated ecological interactions of the phytoplasmas with insect vectors (psyllids), their (alternate) host plants, pollinators and insect antagonists (entomopathogenic fungi) were studied. Attractive and repellent compounds were identified and tested in laboratory studies and field surveys. Additionally, a new entomopathogenic fungus was isolated from psyllid host. The identified new chemical compounds, blends and antagonists are used for the development of control strategies employing the complete spectrum of available methods and materials. Traps and dispensers, microencapsulated volatiles, and also nanofibers are used for the development of appropriate formulations for field applications of semiochemicals for phytoplasma vector control.

Keywords: Phytoplasma vectors, attractants, repellents, push-and-pull strategy, microencapsulation, nanotechnology, entomopathogenic fungus, biological control

Introduction

The phytoplasmas belonging to the apple proliferation group are the economically most important fruit tree phytoplasmas in the temperate and Mediterranean regions of Europe. The three diseases apple proliferation, pear decline and European stone fruit yellows (ESFY) cause crop losses in European fruit growing regions of about 600 million US $ each year. Phloem feeding jumping plant lice or psyllids (Hemiptera: Psylloidea) from the genus *Cacopsylla* were identified as vectors, and each disease has at least one specific psyllid vector (Jarausch *et al.*, 2019). Many insect vector species show an alteration between reproduction host plant (fruit crop) and overwintering host plant (conifers). They use plant volatiles for locating food plants and oviposition sites (Gross, 2016). Additionally, it was shown that phytoplasmas can change the odor of infected plants and manipulate the behavior of their vectoring insects (Mayer *et al.*, 2008).

At the Applied Chemical Ecology Lab at JKI in Dossenheim, Germany five research projects on all three fruit crop-pathogen-vector systems are ongoing to carry out studies on the effects of primary (phloem sap content) and secondary plant compounds [volatile organic compounds (VOCs) of leaves and flowers] on vector behavior and the influence of phytoplasma infections on host plant chemistry and insect response. Based on the results achieved the design of chemically lured traps for monitoring and mass trapping as well as complementary systems for the release of the insect insect repellents were designed. Further, strategies attract-and-kill as well as push-pull-kill for vector and disease control are under development. Classical trap/dispenser designs were produced together with very innovative microencapsulated volatiles (attractants and repellents) and research on high-end nanomaterials and –technologies are in progress. Moreover recently a new entomopathogenic fungus (*Pandora* sp.; *Entomophthorales*) from sporulating cadavers of *Cacopsylla* (Jensen *et al.*, 2018) was isolated and investigated for its potential as a biological control agent of psyllids in fruit orchards. The combination of these different technologies will help to open a door to a sustainable and environmentally friendly next-generation pest control.

Materials and Methods

For the identification of behaviour-manipulating compounds (attractants, repellents), classical and advanced chemical ecology methods were used. For headspace collection a self-developed 6-channel headspace sampling device (Rid *et al.*, 2016, Gross *et al.*, 2019) and a microchamber (Markes, Rodgau, Germany) were used. For analysis of volatile organic compounds, a thermodesorber coupled with a gas chromatograph connected to a mass spectrometer (TD-GC-MS; PerkinElmer, Neu-Isenburg, Germany) is employed. Additionally electro-physiological methods like electro antennography (EAG), electro penetrography (EPG) and thermodesorption coupled with gas chromatography using a split outlet with both a flame ionisation detector and an electroantenno-graphical detector (TD-GC-FID/EAD) are used. For testing compounds and mixtures for their biological activity, behavioural assays (Y-shaped olfactometer, cage tests, wind tunnel assays) are used. Update statistical procedures were applied for data analysis (Gross *et al.*, 2019).
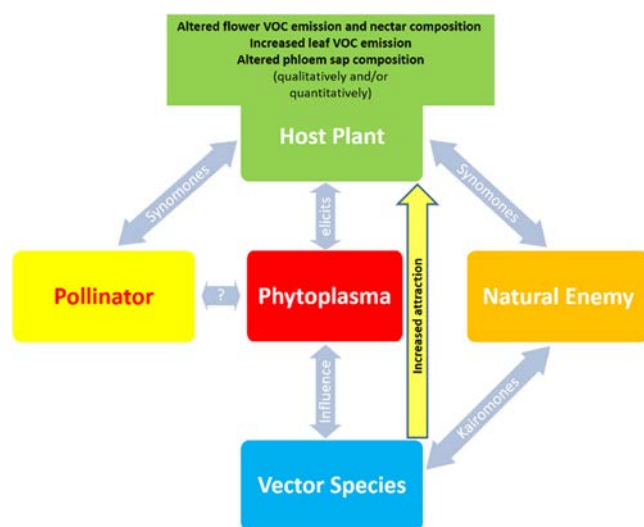
Figure 1. Chemically mediated multitrophic interactions between plant, phytoplasmas, and insect vectors, insect natural enemies (parasitoid, predator, fungi) and plant pollinators. The influence of '*Ca.* Phytoplasma' species on plant physiology elicits the increase of the production of higher amounts of volatile organic compounds (VOCs) or phloem compounds what may influence the vector behavior by increasing its attraction. Besides chemical signals directly emitted by insect infested host plant leaves (synomones) and vector odors (kairomones), the amount and composition of flower traits like volatiles and nectar compounds may influence pollinator behavior.

Results and Discussion

The influence of phytoplasmas on the emission of leaf volatile organic compounds (VOCs) and on vector insect behavior is well-known (Mayer *et al.*, 2008). However also the composition of the phloem sap is affected by phytoplasma infection and influences the behavior of the insect vectors (Figure 1). Both mechanisms affect insect behavior in plant-phytoplasma-insect interactions and the

migrating flights of some insect vector species between overwintering and reproduction host plants (Gallinger and Gross, 2018; Gallinger *et al.*, 2019). Fitness studies and feeding behavior experiments helped to unravel the reason for the alternation of host plants, which is part of the ecology of many species. Further, the effect of phytoplasma infections on flower traits was also studied and has an influence on pollinator behavior. Finally, it was isolated a new entomopathogenic fungus species (*Pandora* sp.; *Entomophthorales*) from sporulating cadavers of *Cacopsylla* (Jensen *et al.*, 2018). Fungal species belonging to this order have a narrow host range and can cause natural epidemics. The results of preliminary investigations indicate the potential of *Pandora* sp. as a biological control agent of psyllid pests in fruit orchards, as the fungus is a natural occurring pathogen on *Cacopsylla* spp. and the isolates can be grown and produce conidia *in vitro*. New formulations of the fungus combined with attractive and repellent semiochemicals (push-pull-kill strategy) are currently under development.

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Management

Optimal timing and sampling for a reliable assessment of the resistance of tomato varieties to '*Candidatus* Phytoplasma solani'

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Abstract

The difference in the resistance of two Moldovan tomato varieties to '*Candidatus* Phytoplasma solani' was determined using nested-PCR analysis on DNA extracted from individual plants by the boiling express method. Two important conclusions were drawn as a result of the study. Firstly, the period of mass fruit ripening is the most suitable period for the molecular detection of phytoplasma presence in tomato plants for assessing the resistance of varieties. Secondly, a total of 11–12 plants of each variety is sufficient for obtaining statistically significant results at this period of the tomato growth.

Keywords: '*Candidatus* Phytoplasma solani', tomato, susceptibility, molecular detection, optimal timing and sampling

Introduction

'*Candidatus* Phytoplasma solani' is a plant pathogen that affects a huge range of cultivated plants including tomato (EFSA, 2014). Phytoplasma diseases provoke considerable losses in yield as well in quality of agricultural production (Bertaccini and Duduk, 2009).

Appropriate agricultural techniques, biological control, resistant varieties, chemical treatments are considered to be the basic components of an effective integrated disease management in tomato. Chemical control is usually effective, but it has some unintended consequences such as the reduction of amount of many beneficial organisms, as well as a toxic impact on the human health and the environment (Gavrilescu and Chisti, 2005). The use of pathogen-resistant tomato varieties for controlling phytoplasma infection has a number of economic and ecological advantages. The high-quality monitoring of phytoplasmas is very important when assessing the resistance of genotypes. Molecular detection using nested-PCR assays provides the most efficient and reliable results for identifying phytoplasma presence in a single host plant (Lee *et al.*, 1994). This study focused on a comparative analysis of the resistance of two Moldavian tomato varieties to '*Ca.* P. solani'. The main approach was the molecular detection of phytoplasmas in individual plants from tomato populations (varieties) using nested-PCR analysis. The presented study had the objectives to determine which period of tomato vegetation has the maximal difference in the number of infected plants between two compared varieties and to assess the optimal number of samples (plants) for obtaining statistically significant results.

Materials and Methods

The molecular detection of '*Ca.* P. solani' presence was carried out in tomato plants cultivated in field conditions. Two Moldavian varieties of tomato, Elvira and Cerasus, were analyzed and compared. These varieties were breaded in the Institute of Genetics, Physiology and Plant Protection (Chisinau, Moldova). Twenty plants in each variety were randomly numbered at the beginning of the study. Molecular analysis of each marked plant was made in the middle each of the three months: July, August and September 2018.

The DNA for molecular detection of phytoplasmas was extracted from the fruit peduncle of each plant by the boiling express method (Guo *et al.*, 2003). This method is very fast and consists of two steps: firstly, thin sections of plant material are boiled in 10 µl 0.3 N NaOH for 5 minutes and, secondly, the mix is neutralized by adding 10 µl 0.3 N HCl. A 1 µl aliquot of obtained solution is used as a template DNA in PCR. Phytoplasma molecular detection was carried out by nested-PCR analysis with primers specific to '*Ca.* P. solani' which were designed on the chaperonin gene sequence (Zamorzaeva, 2015). The following program for amplification was used: 94°C 5 minutes; 94°C 30 seconds, 60°C 30 seconds, 72°C 30 seconds 30 cycles (direct PCR) or 35 cycles (nested PCR); 72°C 10 minutes; 4°C ∞ . The results were observed under UV light after the electrophoresis of the products of amplification (1.5% agarose gel; TBE; ethidium bromide staining).

Statistical analysis of the obtained data was carried out according to the Fisher criterion, applied to qualitative traits in limited random sampling. The selection of the optimal sampling was made using the Jackknife method (Knight, 2000).

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Table 1. P-values obtained by comparing numbers of infected plants in two tomato varieties as a function of the sampling volume (P≤0.05 is marked as grey).

Month	Number of Samples										
	20	19	18	17	16	15	14	13	12	11	10
July	0.042	0.048	0.055	0.068	0.088	0.098	0.114	0.142	0.174	0.171	0.212
August	0.001	0.002	0.003	0.004	0.007	0.010	0.016	0.021	0.031	0.045	0.052
September	0.034	0.040	0.051	0.061	0.075	0.074	0.106	0.108	0.135	0.143	0.175
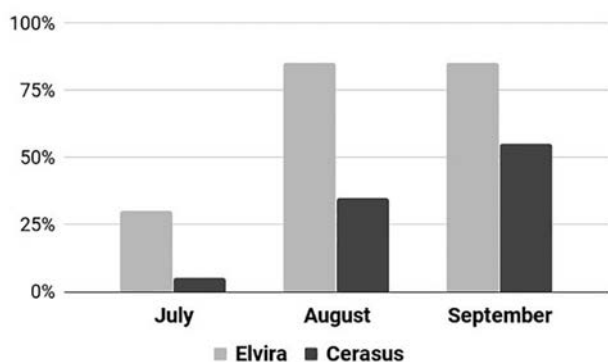
Figure 1. Distribution of '*Ca.* P. solani' in the two tomato varieties.

Results

The molecular analyses allowed to detect significant difference in the percentage of infected tomato plants between two studied varieties (Figure 1). So, the number of infected plants was larger in the variety Elvira compared with Cerasus. Results presented in Figure 1 were obtained by sampling 20 plants in each variety. An increase in the percentage of infected plants over the period of vegetation was observed for both varieties. At the same time, the distribution of phytoplasma infection in the two varieties varied in the different months. The most significant difference in the grade of infection between the two analyzed varieties was observed in August (Table 1): the difference was statistically significant in samplings of 11–14 plants (P≤ 0.05), 15–19 plants (P≤0.01), and 20 plants (P≤0.001). Conversely, statistically significant differences in June and September were obtained only in sampling 19–20 plants (P≤0.05).

Discussion

A significant difference in the susceptibility between the two Moldavian tomato varieties to '*Ca.* P. solani' was determined. It was established that the variety Elvira was more susceptible to this kind of infection than the variety Cerasus. The nature of the reduced susceptibility is not known – it may be associated with some genetic and metabolic features of the genotype, including the ability of the plants infected with phytoplasma to recover (Musetti, 2008). The susceptibility of different tomato genotypes to phytoplasma infection manifests differently throughout the growing

season. Namely, at the beginning of fruit ripening (mid-July 2018 in Moldova) the difference in susceptibility between Elvira and Ňerasus varieties to '*Ca.* P. solani' was less pronounced than at the stage of mass fruit ripening in August. The end of the vegetation season (September), during which the phytoplasma infection is widespread in both analyzed varieties in the tomato field, is less informative for assessing susceptibility. The conclusion is that the period of mass fruit ripening is the most suitable for evaluating the susceptibility of tomato varieties to phytoplasma infection. A sampling volume of 11-12 plants per variety is optimal, it is sufficient to obtain statistically significant results during this vegetation period. Molecular analysis of varieties at earlier or later phenological stages requires a larger number of samples.

Acknowledgements

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Management

Evaluation of pomological traits of pear decline-resistant rootstocks

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Abstract

Twenty pear rootstock genotypes were examined for their pear decline resistance and their pomological traits in a six years field trial. All rootstock selections were grafted with the susceptible cultivar Williams followed by graft-inoculation with a severe strain of '*Candidatus* Phytoplasma pyri' prior plantation. Seven years post inoculation all surviving trees were positive in PCR analyses. The concentration of the phytoplasmas was determined by quantitative PCR and ranged from $3x10^6$ to $6x10^7$ phytoplasma cells per gram of phloem tissue. Among the grafted rootstock genotypes clear differences in mortality, symptom expression and pomological performance were observed. Pear decline-tolerant rootstocks with promising pomological traits were selected for further evaluation.

Keywords: '*Candidatus* Phytoplasma pyri', *Pyrus*, resistance screening, phytoplasma quantification

Introduction

Pear decline (PD) is one of the most important diseases of pear (*Pyrus communis*) in Europe and North America. It is associated with the presence of '*Candidatus* Phytoplasma pyri' which is transmitted by pear psyllids. As the control of the disease is difficult, the use of resistant plants could be a promising solution. Due to the seasonal elimination of the pathogen in the scion, resistant rootstocks would be sufficient and the established cultivars could be maintained. Previous work of Seemüller *et al.* (1998, 2009) showed that PD resistance can be found in specific genotypes of various *Pyrus* taxa. In total, 39 progenies of 26 taxa were graft-inoculated and examined in a field trial for resistance and pomological traits over a period of 18 years. Significant differences in susceptibility were found not only among species, but also in the progeny of the same species. Based on this work 20 promising candidates to be used as commercial pear rootstocks were selected, multiplied *via* plant tissue culture and re-examined for their pomological performance in a field trial in a pear growing region of Northern Germany.

Materials and Methods

Twenty PD-resistant *Pyrus* genotypes identified in earlier screenings (Seemüller *et al.*, 1998; 2009) were multiplied by tissue culture. Five *ex vitro* plants per selection were grafted with the susceptible cultivar Williams and grown in pots. In a second grafting all plants were inoculated with a severe strain of '*Ca.* P. pyri'. All trees were planted in an experimental orchard near Ellerhoop (Schleswig-Holstein) and were maintained under standard growing conditions for six years. Symptoms, vigour and fruit set were recorded each year and the trunk diameter was measured in 2013, 2015 and 2018. The first evaluation of the fruit size and yield was conducted in 2015. A rating from 0 to 3 was applied each year for symptom recording and a cumulative disease index (CDI) was calculated over 6 years. Ratings were: 0.5 = mild symptoms (*e.g.* weak reddening), 1 = reddening and leaf drop, 2 = weak growth, smaller fruits, 3 = strong growth reduction, 10 = die off. Phytoplasma detection was performed in 2018 with PCR using primers fO1/rO1 (Lorenz *et al.*, 1995) and phytoplasma quantification was done with a SYBR-Green qPCR (Nikolic *et al.*, 2010). Statistical analysis was performed using the biostatistics program R (R development core team 2.9.0).

Results and Discussion

All 100 trees were successfully inoculated with '*Ca.* P. pyri'. Within the first 4 years after planting high mortality rates from 40-100% were observed for 5 rootstock/cultivar combinations (Table 1). Nine selections showed no or almost no symptoms (CDI 0-0.5), this was, however, not correlated to the phytoplasma titer.

Table 1. Susceptibility to pear decline and pomological performance of pear trees on different rootstock selections after 6 years from graft inoculation with '*Ca.* P. pyri'.

Species	Selection	'*Ca*. P. pyri' infection PCR positive/total	Mortality dead trees/total	Cumulative disease index	Phytoplasma cells/g phloem*	Trunk diameter (mm)*	Relative yield (g/cm^2)*
P. amygdaliformis	470/84s	5 / 5	0 / 5	0.4 f	1.66 x 10^7 cde	69.90 abcd	501.2 ab
P. amygdaliformis	492/84d	3 / 3	2 / 5	4.8 bcd	3.81 x 10^7 bc	71.67 abc	360.8 bcd
P. amygdaliformis	493/84j	5 / 5	0 / 5	0 f	1.62 x 10^7 cde	63.40 cdef	418.8 abcd
P. betulifolia	409/85j	5 / 5	0 / 5	0 f	3.24 x 10^7 cd	70.80 abc	272.2 cd
P. boisseriana	445/84q	5 / 5	0 / 5	0.4 f	2.97 x 10^7 cde	67.40 cde	363.2 bcd
P. bretschneideri	213/86b	5 / 5	0 / 5	2.1 cdef	1.03 x 10^7 cde	69.00 bcd	326.0 bcd
P. caucasia	485/84p	nt	5 / 5	12.2 a	nt	nt	nt
P. communis	301/83a	5 / 5	0 / 5	0.5 f	6.11 x 10^7 b	78.60 a	289.0 cd
P. cuneata	223/86p	5 / 5	0 / 5	2.3 cdef	2.04 x 10^7 cde	59.20 ef	227.4 d
P. cuneata	223/86B	4 / 4	1 / 5	4.5 bcde	8.45 x 10^6 de	63.50 cdef	368.8 abcd
P. cuneata	223/86g	4 / 4	1 / 5	7.2 b	9.00 x 10^6 de	64.75 cdef	285.8 cd
P. eleagrifolia	237/83a	2 / 2	3 / 5	7.0 b	3.38 x 10^6 e	77.50 ab	280.3 cd
P. eleagrifolia	293/83I	5 / 5	0 / 5	0.2 f	3.40 x 10^7 cd	60.80 def	362.2 bcd
P. eleagrifolia	465/84q	5 / 5	0 / 5	0.1 f	3.26 x 10^7 cd	68.80 bcd	407.6 abcd
P. gharbiana	221/86D	5 / 5	0 / 5	1.6 def	1.75 x 10^7 cde	66.60 cdef	457.6 abc
P. nivalis	474/84d	5 / 5	0 / 5	1.0 ef	2.16 x 10^7 cde	57.60 f	565.2 a
P. nivalis	459/84c	3 / 3	2 / 5	5.4 bc	1.96 x 10^7 cde	64.00 cdef	241.0 d
P. pyraster	208/86q	5 / 5	0 / 5	0.1 f	1.11 x 10^7 cde	67.40 cde	514.2 ab
P. serrulata	243/83i	5 / 5	0 / 5	0.1 f	3.74 x 10^7 bc	68.80 bcd	242.6 d
P. ussuriensis	391/85i	nt	5 / 5	12.0 a	nt	nt	nt

*Values with the same letter are not significantly different from each other at p = 0.05

Symptom expression was in most cases in agreement with a previous report of Seemüller *et al.* (2009) where *P. communis* Moscow, *P. betulifolia*, *P. amygdaliformis* (Göttingen) and *P. pyraster* have been the best selections.

As the susceptible cultivar Williams had a considerably high phytoplasma titer 6 years after the graft inoculation regardless of the rootstock genotype, it is more reasonable to regard those genotypes as tolerant, therefore preventing the symptom expression of the scion. Important differences were observed for vigour as determined by the trunk diameter after 6 years and for the relative yield determined in 2015. Unfortunately, fruit set in later years was not homogenous enough for a proper statistical analysis. A high relative yield with tolerance to pear decline was observed for *P. nivalis* 474/84d, *P. pyraster* and *P. amygdaliformis* 470/84s. The other tolerant selections were bad performing as crop production.

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Management

Plasma activated water as a possible sustainable strategy towards grapevine yellows disease management

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Abstract

Plasma activated water (PAW) possesses significant antimicrobial properties due to the synergistic effect of reactive oxygen and nitrogen species (RONS) produced by the plasma treatment. To test its efficacy as plant resistance inducer, trials were carried out in five Glera and one Chardonnay vineyards in grapevine plants preliminary tested for the presence and identity of phytoplasmas. The PAW treatments were performed for two years injecting into the plant vascular tissues 10-20 ml of PAW or sterile distilled water (SDW) as control in a total of 130 plants (105 treated with PAW and 25 with SDW). A relevant number of PAW treated symptomatic plants showed a reduction of the symptoms, an higher number of the bunches and a higher berry weight per plant, compared to the SDW treated. Moreover the molecular analyses after the two year-treatment show a decrease of phytoplasma infected plant number treated with PAW (35.5%) compared with the SDW treated ones (12,5%).

Keywords: grapevine yellows disease, phytoplasma, control

Introduction

The plasma activated water (PAW) produced using distilled water with atmospheric pressure plasmas possesses antimicrobial efficacy due to the synergistic effect of reactive oxygen and nitrogen species (RONS) accumulated in the solution after the treatment (Laurita *et al.*, 2015). These reactive species might also have an important role in plant defence responses, involving both hypersensitive reaction and systemic acquired resistance; thus plant treatment with PAW could represent an innovative alternative in the control of plant diseases due to phytoplasmas (Zambon *et al.*, 2018a; Perez *et al.*, 2019). Phytoplasmas are insect-transmitted plant pathogenic prokaryotes, associated with severe diseases in agronomic important crops (Bertaccini *et al.*, 2014). Management of these diseases has mainly focused on insect vector control and on infected plant rouging. There is a strong need for effective and environmentally friendly phytoplasma control strategies, therefore the possibility to use PAW as sustainable and effective method to manage phytoplasma-associated diseases was evaluated. In this study, the efficacy of PAW was tested in vineyards on phytoplasma infected grapevine plants showing symptoms associated to the grapevine yellows disease. This is among the most economically relevant infectious diseases affecting vineyards in the European continent, able to reduce up to 100% the wine production. The hypothesis behind the experiments was that the reactive species such as H_2O_2, NO_3^-, NO_2^- contained in the PAW acid solution (pH 2.5), once in contact with the plant tissues, can act as elicitors activating plant secondary metabolism with a production of phytoalexins and other compounds involved in biotic or abiotic stress responses. The study was aimed to verify PAW's ability on eliciting defensive responses in grapevines in the field, with a reduction of the phytoplasma-presence and an enhancement of the plant fitness.

Materials and Methods

Sterile distilled deionized water (SDW) was exposed for 10 minutes to a nanosecond pulsed dielectric barrier discharge, operated with a peak voltage of 19 kV, a pulse repetition frequency of 1 kHz in ambient air. The plasma treatment induced the production of nitrates and hydrogen peroxides, and a decrease of the pH (Table 1).

Table 1. Chemical composition of PAW.

Solution	H_2O_2 [mM]	NO_3^- [mM]	NO_2^- [mM]	pH
PAW	0.2	2.4	0	2.5

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Experimental trials using this PAW were carried out for two years in five Glera and one Chardonnay vineyards in grapevine plants preliminary tested to verify the presence and identity of phytoplasmas using both, symptomatic and asymptomatic plants. Experiments were performed injecting at three times (April, June and July) 10-20 ml of PAW or sterile distilled water (SDW) as control on each selected phytoplasma-infected and phytoplasma-free plant for a total of 130 plants (105 treated with PAW and 25 with SDW). The remaining untreated plants in each vineyard were used as a control to verify the disease behaviour in the time. Injections were carried out at different day times to evaluate the grapevine PAW absorbance (Figure 1).

Figure 1. A) PAW treatment by injection into the grapevine vascular system and B) longitudinal trunk section of treated plant in which a blue dye was added to the PAW.

Total nucleic acids were extracted from the PAW, SDW and untreated plants using a phenol-chloroform method (Prince *et al.*, 1993). Nested-PCR/RFLP analyses were then carried out to identify the phytoplasmas in the grapevine plants (Zambon *et al.*, 2018b). Moreover to evaluate the effect of PAW treatments on quantitative and qualitative production parameters, an agronomic test was performed on 4 vineyards. At the harvesting time the number of bunches and the weight of 100 berries per plant were measured. Real acidity, total acidity and Babo degree chemical analyses were then determined on the grapevine must obtained from the 100 berries collected from PAW and SDW treated plants respectively.

Results and Discussion

A relevant number of PAW treated symptomatic plants showed a reduction of symptoms and an increased performance of the weaker plants (Figure 2); no modification in the symptom presence was observed in the SDW and the non treated plants. Molecular analyses carried out on samples from the PAW treated plants showed a 35.5% reduction of phytoplasma positive plants, while a 12.5% reduction was detected in the SDW-treated grapevines. No phytotoxicity was observed in the plants treated with PAW. These latter grapevine plants showed also a higher number of bunches and a higher berry weight per plant, while the chemical analyses on the grapevine must didn't show differences. These are promising results that are confirmed by transcriptional analyses (Y. Zambon *et al.*, unpublished) and are subjected to further evaluation for a larger scale field application.

Figure 2. Grapevine phytoplasma-infected plant before (April) and after 3 PAW treatments (July).

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Management

Methyl jasmonate triggers metabolic responses and gene expression on *Vitis vinifera* cultivar Loureiro infected with "flavescence dorée"

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Abstract

"Flavescence dorée" (FD) is a grapevine epidemic quarantine phytoplasma disease associated with high yield losses. This study aimed to evaluate the effect of methyl jasmonate (MeJA) on the induction of defence mechanisms of grapevines cultivar Loureiro against FD, comparing defence metabolites production (proline and saponins) and gene expression patterns in healthy and infected grapevine plants. The MeJA treatments significantly increased saponin and proline synthesis only in infected plants. Moreover, they induced gene expression of rubisco activase and pathogen-related proteins (PBSP, CHITC4c and PIN) quantified 6 hours after the MeJA application.

Keywords: "flavescence dorée", gene expression, grapevine, methyl jasmonate, proline, saponin

Introduction

"Flavescence dorée" (FD) is a grapevine quarantine disease widespread in Europe. During the last years some studies have been developed to control its impact and spread (Margaria and Palmano, 2011). Elicitors are becoming important tools in disease management, nevertheless studies testing their potential role to mitigate FD symptoms are scarce (Oliveira *et al.*, 2019). This study aims to understand the effect of methyl jasmonate on the synthesis of defence metabolites and gene expression activation in FD-infected grapevines.

Materials and Methods

Field treatments

The study was carried out between June (1 week before flowering) and August (veraison) 2016 at Fafe, Portugal, in a twenty-year-old vineyard cultivar Loureiro where FD was detected in 2009. A selection of 16 healthy and 16 infected grapevines was divided in four groups: control plants (untreated) and plants treated with 0, 12.5 and 25 mM of methyl jasmonate (MeJA) dissolved in 2.5% ethanol, applied before flowering and repeated at veraison.

Metabolites quantification

Saponins and proline were extracted and quantified in leaves collected in September (12 weeks after MeJA application), according to Patel *et al.* (2012) and Bates *et al.* (1973), respectively.

Gene expression

Leaf samples were collected before treatments (T0) and 6 hours after MeJA application (T1). RNA extraction was performed with a RNeasy Plant Mini Kit (QIAGEN GmbH), according to Le Provost *et al.* (2007) and RNA yield and quality were measured using a nano-photometer. Synthesis of complementary DNA was performed using the iScrip cDNA Kit in a thermal cycler. RT-qPCR was performed using the NZY Taq 2x Green Master Mix Kit with l denaturation of 120 seconds at 95°C followed by 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 60 seconds at 72°C; the final extension was of 5 seconds at 72°C. 18S rDNA and ubiquitin were used as housekeeping genes to normalize the Ct values. A total of 12 target genes were selected encoding: (i) plant PR proteins (thaumatin I – ThauI, thaumatin II – ThauII, osmotin-like protein – Osm, PBSP – plant basic secretory protein, CHIT4c – acidic class IV chitinase, PIN – inhibitors of serine protease, PGIP – polygalacturonase-inhibiting protein and GLU – β-1,3-glucanase), (ii) enzymes involved in primary metabolism (RubAct – rubisco activase AAA+), (iii) protein degradation (Protα5 – proteasome á5 subunit) and (iv) phenylpropanoid biosynthesis (STS – stilbene synthase and PAL – phenylalanine ammonia lyase) (Belhadj *et al.*, 2006; 2008; Margaria and Palmano, 2011).

Results and Discussion

Untreated plants, when infected with FD showed 54% lower

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saponins' concentration compared with healthy plants (Figure 1). Interestingly, when FD plants were elicited with 12.5 and 25 mM MeJA the saponin concentration increased by 3.1-fold and 2-fold, respectively, as compared with untreated FD plants. Proline concentration was only significantly higher (2-fold) in infected plants treated with 25 mM MeJA.
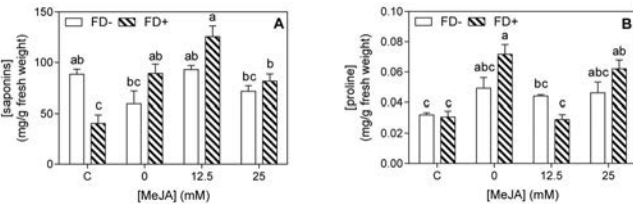
Figure 1. Saponins (A) and proline (B) concentration in healthy (FD-) and "flavescence dorée" infected (FD+) grapevine cultivar Loureiro untreated (C) and treated with 0 (solvent), 12.5 and 25 mM MeJA measured 12 weeks after the elicitor application. Data are means ± SEM of 4 biological replicates. Different letters indicate significant differences ($P < 0.05$) according to two-way ANOVA.
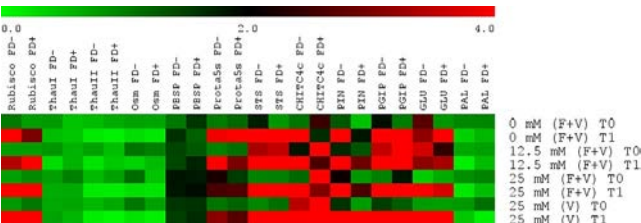
Figure 2. Heat map obtained by RTqPCR of leaf samples from healthy (FD-) and infected (FD+) grapevines cultivar Loureiro before (T0) and 6 hours after (T1) MeJA application performed at flowering and at veraison (F+V) or only at veraison (V).

This study showed that both MeJA concentrations upregulated *Rubisco*, *Protα5s*, *STS*, *CHITC4c*, *PIN* and *PGIP* genes in the healthy grapevines (Figure 2). In diseased plants a single MeJA application at veraison lead to upregulation of *Rubisco*, *PIN*, *PGIP* and *GLU* genes in FD plants, whereas a double application of this elicitor additionally induced an overexpression of *CHITC4c* gene 6 hours after MeJA treatment. An under expression of *ThauI*, *ThauII*, *Osm* and *PAL* genes was found when applying 25 mM MeJA in FD plants (single or double application). The elicitation with both MeJA concentrations in FD infected grapevines highly enhanced the saponins concentration, whereas the proline concentration was only significantly increased at 25 mM MeJA. Interestingly, in healthy plants no effects were found on these metabolites' concentration in response to MeJA. It is however important to stress out that part of the MeJA elicitation effect on the described metabolic responses seems to be related to the solvent *per se*, since its application (0 MeJA treatment) in FD plants induced a significant increase of the concentration of saponins and proline. Therefore, more studies are needed to clarify these responses. The *Rubisco* gene was downregulated in untreated FD infected plants as compared to healthy in agreement with a previous study reporting FD repression of photosynthetic

genes (Margaria and Palmano, 2011). In contrast with the present work, other authors have shown an increase in expression of *ThauI*, *ThauII* and *Osm* genes in grapevines infected with FD (Margaria and Palmano, 2011) and "bois noir" (Albertazzi *et al.*, 2009), but their study did not evaluate the gene expression pattern following the MeJA treatments. Here, MeJA concentrations upregulated *CHITC4c*, *PIN* and *PGIP* genes in FD infected plants, which in turn is in accordance with earlier studies conducted in healthy grapevines that demonstrated a higher expression of *STS*, *CHIT4c*, *PIN* and *PGIP* genes following MeJA application (Belhadj *et al.*, 2006; Martinez-Esteso *et al.*, 2009). This study contributes to a better understanding of the defence mechanisms triggered by the MeJA application in FD-infected grapevines under field condition.

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Management

Does salicylic acid alleviate the impacts on growth, development and productivity of "flavescence dorée" in Portuguese "Vinhos Verdes" grapevines?

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Abstract

"Flavescence dorée" (FD) is a quarantine disease of great concern to the stability and sustainability of the wine industry, due to drastic harvest losses and death of infected plants. Previous studies have suggested that salicylic acid (SA) may improve the plant resistance against grapevine diseases, but no studies have been carried out for FD. The effect of 25 mM SA applied at the flowering stage to healthy and FD infected grapevines cultivar Loureiro was evaluated in a field trial. The evaluation of development and productivity (percentage of budburst, fertility index, time to veraison, and chlorophyll content) and grape quality parameters (degree Brix and titrable acidity) was carried out. Fertility index and chlorophyll content were significantly lower in infected plants and SA partly reduced the FD symptoms, since at veraison these plants did not show significant differences when compared to healthy plants for those parameters. However the productivity was significantly lower in the infected plants, with no significant effect of SA application. The berry quality was no significantly different comparing healthy and infected plants or SA-treated and untreated plants.

Keywords: berry quality, fertility index, salicylic acid, yield

Introduction

"Flavescence dorée" (FD) is a quarantine disease transmitted by insects (*Scaphoideus titanus* and *Orientus ishidae*) and by the commercialization of infected vegetative propagation material. The current management strategies for FD control are costly, difficult to implement and have a high environmental impact (Oliveira *et al.*, 2019). Elicitors such as salicylic acid (SA) could be exploited since they activate multiple plant defence systems against several pathogens (Dodds and Rathjen, 2010). Other studies with an SA analogue (benzothiadiazole) showed in grapevine plants induced resistance towards *Plasmopara viticola, Erisyphe necator* (Dufour *et al.*, 2013) and *Botrytis cinerea* (Iriti *et al.*, 2005), reducing the disease symptomatology. This study evaluated the potential of SA on the mitigation of the negative effects of FD on grapevine growth, development and productivity.

Materials and Methods

Field treatments

The study was carried out between April (budburst) and September (harvest) 2015 in Fafe, Portugal, in a twenty-year-

old vineyard of cultivar Loureiro. A total of 16 grapevine plants were selected: 8 healthy and 8 FD-infected. SA was diluted in Milli-Q water and applied to the plants at the flowering stage at 0 mM (control) and 25 mM (n=4).

Biometric measurements

Phenological stages were registered biweekly until flowering and then weekly according to the Baggiolini (1952) scale. The buds left on the shoots after the winter pruning were used to calculate the percentage of budburst and fertility index (Alonso *et al.*, 2007), according to the formula:

$$\% \text{ of budburst} = \frac{\text{no. of sprouted buds}}{\text{pruning load}} \times 100 \qquad \text{Fertility index} = \frac{\text{no. of bunches}}{\text{pruning load}}$$

Chlorophyll content was assessed weekly using a SPAD meter on five leaves randomly selected from each plant. Leaf area was determined using the method of Lopes and Pinto (2005) and both assays were performed in triplicate.

Productivity

The number of bunches and the average bunch weight were determined using five berries per bunch collected randomly

Table 1. Growth and development parameters evaluated at veraison in healthy (FD⁻) and "flavescence dorée" infected (FD⁺) grapevines cultivar Loureiro. Data are means ± SEM, identical letters indicate statistically identical values.

Plant	[SA] (mM)	Time to veraison (days)*	Budburst (%)	Fertility index	Chlorophyll content (SPAD values)	Leaf area increase (%)**
FD⁻	0	201.5 ± 1.3 [b]	81.0 ± 3.2 [a]	1.05 ± 0.07 [a]	38.1 ± 1.7 [a]	52.9 ± 7.2 [a]
	25	203.0 ± 1.2 [b]	79.4 ± 2.7 [a]	0.85 ± 0.08 [ab]	37.3 ± 0.9 [a]	112.8 ± 32.3 [a]
FD⁺	0	225.3 ± 1.5 [a]	78.2 ± 2.7 [a]	0.65 ± 0.06 [b]	24.4 ± 0.5 [b]	86.7 ± 0.7 [a]
	25	225.8 ± 1.1 [a]	67.8 ± 4.6 [a]	0.79 ± 0.04 [ab]	30.5 ± 3.1 [ab]	136.7 ± 4.4 [a]

*days of the year (1 January = day 1)   **relative increase in leaf area from flowering to veraison

Table 2. Productivity in healthy (FD-) and FD infected (FD+) grapevines cultivar Loureiro at the harvest stage. Data are means ± SEM, identical letters indicate statistically identical values.

Plant	[SA] (mM)	Productivity (kg/plant)	No of bunches	Average bunch weight (g)	TSS (ºBrix)	TA (g tartaric acid/L)
FD⁻	0	23.8 ± 1.2 [a]	52.8 ± 6.6 [a]	480.0 ± 64.3 [a]	16.9 ± 0.7 [a]	8.9 ± 0.2 [a]
	25	23.3 ± 2.0 [a]	43.8 ± 6.7 [ab]	565.2 ± 60.1 [a]	16.7 ± 0.9 [a]	7.7 ± 0.3 [a]
FD⁺	0	8.3 ± 0.3 [b]	17.3 ± 1.0 [b]	488.5 ± 45.9 [a]	16.6 ± 0.2 [a]	9.0 ± 0.5 [a]
	25	6.0 ± 0.7 [b]	19.0 ± 0.7 [ab]	313.9 ± 25.5 [a]	16.8 ± 0.3 [a]	8.8 ± 0.8 [a]

in six bunches. The total soluble solids (ºBrix) and titrable acidity were measured and statistical analysis was carried out with two-way ANOVA.

Results

Phenological stages evaluation revealed that FD infected plants showed a significant ($P < 0.05$) delay in development, resulting in 23 extra days in the time to veraison compared to healthy plants and the 25 mM SA application at flowering had no effect on this parameter (Table 1). However, both groups of plants did not show differences in the budburst rate. Fertility index and chlorophyll content were significantly lower, 39% and 36% respectively, in infected plants however at veraison these plants did not differ significantly from the healthy ones on these parameters (Table 1). The FD infected plants had a 65% lower yield due to a significant reduction on the number of bunches, while the average bunch weight was not affected (Table 2). No significant differences were observed between healthy and infected or between SA-treated and untreated plants in the berry quality.

Discussion

The FD presence is associated with unripen cane wood (Caudwell, 1957), leading to a delay in reaching veraison as observed in the present study. In spite of the positive effect of SA application on growth and development parameters in FD infected plants, it had no significant effect on grapevine productivity. This result is likely due to the lower pruning load left on FD plants reflecting their lower vigour. This is the first study on SA application to grapevines infected with FD and it is concluded that this elicitor seems to have a beneficial effect on plant growth and development, but only for a restricted number of parameters. This result opens doors for the development of more effective and environmentally friendly tools for phytoplasma disease control. However, more studies should be carried out in order

to further verify if diverse elicitor concentrations' or time of application would induce a better plant responses.

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Management

Preliminary results of auxin and brassinosteroid application on 'Candidatus Phytoplasma prunorum' infected plants

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Abstract

In this study the effect of foliar application of brassinosteroid (24-epibrassinolide, EBR) and auxin (indole-3-butyric acid, IBA) was tested on 'Candidatus Phytoplasma prunorum' (CP) presence and symptom manifestation in CP infected stone fruit trees. Two CP inoculum sources, apricot cultivars Poyer and Hargrand and two rootstocks, peach GF-305 and apricot M-VA-2, were used. Based on the results, phytohormones did not affect the concentration of CP in the treated plants. Leafroll symptom manifestation was increased after IBA treatment in almost all cases, while inconsistent results were obtained for the chlorosis symptoms in cultivars, whereas rootstock chlorosis symptoms decreased after EBR and IBA treatments. However, in most of the cases, the results were not significantly different.

Keywords: 'Candidatus Phytoplasma prunorum', auxin, brassinosteroid, quantitative PCR, stone fruit species

Introduction

The most common and severe fruit tree phytoplasma of stone fruits is 'Candidatus Phytoplasma prunorum' (CP) which belongs to the 16SrX group. CP is the agent associated with European stone fruit yellows (ESFY) disease. It can cause considerable economic losses by inducing a decrease of size, quality and yield of fruit and tree death. The severity of this disease is increased by the fact that there is no effective direct protection against it. The current trend in plant protection is focused on testing chemical substances extracted from plants against various pathogens. Carvacrol, eugenol, esculentin, terpineol, α-pinene were used in *in vitro* conditions for suppression of phytoplasmas (Aldaghi *et al.*, 2008), but without an effect. Phytohormones play an important role in plant defence mechanisms and the main phytohormones connected with interactions of plants with pathogens are salicylic and jasmonic acids, ethylene, auxins and brassinosteroids. In *in vitro* studies the application of exogenous auxin to the medium suppressed and caused the disappearance of 'Ca. P. asteris' and 'Ca. P. pruni' symptoms in *Catharanthus roseus* plants (Leljak-Levanic *et al.*, 2010; Curkovic-Perica, 2008). Brassinosteroids increase the resistance against fungal, bacterial and viral pathogens in various plant species (Krishna, 2003). Moreover, foliar application of epibrassinolide decreased the concentration of 'Candidatus Liberibacter asiaticus' in infected citrus plants (Canales *et al.*, 2016). In this study, the effect of foliar application of brassinosteroid (24-epibrassinolide, EBR) and auxin (indole-3-butyric acid, IBA) was tested on presence and symptom manifestation of CP in infected stone fruit trees.

Materials and Methods

Two apricot cultivars, Poyer and Hargrand, expressing strong and mild symptoms respectively, and two rootstocks, peach GF-305 and apricot M-VA-2, were used for the study. Combinations of both cultivars and rootstocks were chip budded in the year prior to the test and grown in containers under insect proof nets. From the beginning of July until the end of August the plants were sprayed every 2 weeks (5 applications) on leaves with IBA (1 g/l), EBR (1x10^{-7} g/l) or water (control). For each application, 5 to 10 plants were used for each cultivar-rootstock combination. Before (June) and after (September) the application of phytohormones DNA was extracted from leaves of rootstock and cultivar of each tested plant and subjected to CP quantification by quantitative PCR (Christensen *et al.*, 2004). Leafroll and chlorosis, symptoms of CP, were evaluated separately at the end of vegetative period using a chart: 0 - no symptoms, 1 – weak symptoms, 2 – mild symptoms, 3 - strong symptoms. The symptom manifestation, CP concentration and its increase before and after the treatments were the parameters for testing the effect of phytohormones. Data were statistically evaluated by ANOVA with subsequent grouping by Duncan test (P>0.05) in software Statistica 12 (Statsoft).

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Table 2. Average values of symptom manifestation and CP increment before and after treatments with phytohormones. Values are shown as mean ± standard error. Indexes [a], [ab] and [b] refer to the homogenous grouping based on the Duncan test (p>0.05).

Combination	Application	Leafroll	Chlorosis	CP increment before and after treatment (x times)	Leafroll	Chlorosis	CP increment before and after treatment (x times)
Hargrand / GF-305	IBA	1.78 ± 0.15[b]	0.22 ± 0.15[a]	8.25 ± 5.30	1.60 ± 0.27[b]	0.10 ± 0.10[a]	42.16 ± 25.89
	EBR	0.78 ± 0.15[a]	0.33 ± 0.17[ab]	8.37 ± 4.64	0.11 ± 0.11[a]	0.11 ± 0.11[a]	13.63 ± 6.91
	Control	1.40 ± 0.24[b]	1.00 ± 0.45[b]	7.90 ± 3.97	0.44 ± 0.24[a]	0.78 ± 0.28[b]	6.61 ± 1.72
Poyer / GF-305	IBA	1.90 ± 0.23	1.4 ± 0.27	167.30 ± 97.23	1.00 ± 0.23	0.10 ± 0.10[a]	796.70 ± 602.22
	EBR	2.00 ± 0.00	1.14 ± 0.40	227.03 ± 103.63	0.80 ± 0.29	0.73 ± 0.27[ab]	118.29 ± 43.23
	Control	2.00 ± 0.00	1.00 ± 0.31	76.55 ± 33.81	0.91 ± 0.25	1.00 ± 0.30[b]	197.00 ± 125.37
Hargrand / M-VA-2	IBA	2.00 ± 0.00[b]	0.00 ± 0.00[a]	5.01 ± 2.21	0.75 ± 0.48	0.00	14.51 ± 6.87
	EBR	0.40 ± 0.24[a]	1.00 ± 0.00[b]	16.70 ± 7.33	0.20 ± 0.20	0.00	73.14 ± 34.62
	Control	0.33 ± 0.33[a]	0.33 ± 0.33[a]	2.41 ± 0.67	0.29 ± 0.18	0.00	17.60 ± 13.01
Poyer / M-VA-2	IBA	0.50 ± 0.29	0.00	323.64 ± 135.70	0.67 ± 0.21	0.00	3000.08 ± 2519.92
	EBR	0.20 ± 0.20	0.20 ± 0.20	184.73 ± 155.68	0.17 ± 0.17	0.33 ± 0.21	2595.19 ± 2201.46
	Control	0.00	0.00	13.12 ± 5.88	0.00	0.50 ± 0.50	71.03 ± 17.48

Results

The phytohormone treatments did not affect the CP concentration in the plants. Differences in CP increment before and after phytohormone application were not significantly different among the application variants, however, the increase of Poyer CP concentration was higher than of the Hargrand CP (Table 1).

IBA treatment affected the leafroll manifestation, where on leaves of Hargrand cultivar grafted on M-VA-2 and GF-305 significantly stronger symptom manifestation was observed after IBA treatment compared to EBR, control, and EBR, respectively (Table 1). For the rootstock leaves, the leafroll manifestation was, in general, higher in plants treated with IBA compared to other treatments, where GF-305 rootstocks budded with Hargrand had significantly higher leafroll manifestation than of the EBR and control variants (Table 1). Inconsistent results were obtained from chlorosis evaluation of cultivar leaves. Leaves of Hargrand cultivar budded on M-VA-2 had significantly higher chlorosis manifestation after treatment with EBR compared to the control and IBA, whereas significantly higher chlorosis manifestation on leaves of Hargrand on GF-305 was observed on control plants compared to the IBA and EBR (Table 1). For the rootstock leaves, the chlorosis symptoms were always higher in control plants compared to EBR and IBA, except for the M-VA-2 budded with Hargrand, where no symptoms were observed (Table 1). Rootstock leaves of control plants of GF-305 budded with Hragrand and Poyer had significantly higher chlorosis manifestation than of the IBA, EBR and IBA, respectively.

Discussion

According to the preliminary results collected after one year, foliar application of IBA and EBR did not decrease the concentration of CP in the tissues of rootstock and cultivars. This conclusion is in contradiction with the results of Canales *et al.* (2016) where the foliar application of brassinosteroids decreased the concentration of '*Candidatus* Liberibacter asiaticus' in infected citrus plants. However, despite the same localization in phloem tissues of '*Ca*. Liberibacter' and '*Ca*.

Phytoplasma' species their different reaction to various chemical compounds present in plant tissues can not be predicted.

The treatment with IBA increased the leafroll symptom manifestation, which is not in accordance to the results of Leljak-Levanic *et al.* (2010) and Curkovic-Perica (2008), who observed a decrease of the symptoms of '*Ca*. P. asteris' and '*Ca*. P. pruni' infected *C. roseus* plants in *in vitro* conditions. Partial agreement with decreased symptom manifestation was observed in this study for the rootstocks, where both IBA and EBR decreased the chlorosis. However, inconsistent results were obtained for the cultivar chlorosis presence. More years of study are needed to reliably conclude the effect of EBR and IBA on CP infected plants in *in vivo* conditions, however, it appears that these phytohormones could have an influence on symptom manifestation rather than on CP concentration.

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A simple and reliable method to assess the effects of antimicrobial compounds on phytoplasmas

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Abstract

The only active compounds able to control phytoplasmas are the antibiotic oxytetracyclines and the direct and fast screening of new antimicrobials compounds on media is very complicated due to the difficulty to culture phytoplasmas. A simple and reliable screening method to evaluate the effects of antimicrobial compounds on phytoplasmas by an *ex-vivo* approach was developed. By the use of scanning electron microscopy in parallel with molecular tools the direct activity of tetracyclines on phytoplasmas was verified.

Keywords: phytoplasmas, control, antimicrobials, scanning electron microscopy, ddRT-PCR

Introduction

Phytoplasma diseases control is mainly attempted by chemical treatments targeting insect vectors. Based on the scientific literature, the only active compounds able to control phytoplasmas are the oxytetracyclines (Ishiie *et al*., 1967; Singh *et al*., 2007). Many strategies have been tried to reduce the use of insecticides to control phytoplasmas vectors. A promising research is based on the use of acetic acid bacteria *Asaia* as a biocontrol agent of "flavescence dorée" (FD) phytoplasma. These symbionts would be able to act during the FD-vector acquisition phase, because of their ability to invade almost all the organs of the insect vectors tested up to now (Favia *et al*., 2008; Gonella *et al*., 2018). Limited attempts have been made however to evaluate and screen antimicrobials compounds for their efficacy against phytoplasmas. In this study a simple and reliable screening method to evaluate the effects of antimicrobials directly on phytoplasmas by an *ex-vivo* approach was developed.

Materials and Methods

Periwinkle plants [*Catharanthus roseus* (L.) G. Don] infected by the "flavescence dorée" phytoplasma were propagated by grafting on healthy plants under insect proof and environmental controlled conditions. The first experiment was conducted on 30 cuttings collected from infected plants. Three treatments were used: tap water as negative control, 1% of the "FI Plus" compound (NDG Natural Development Group Srl) and 150 mg/l of tetracyclines as a positive control. Ten cuttings were left to feed for 48 hours on each treatment with the help of a magnetic stirrer. Three stalk sections were collected from each cutting 3 cm above the feeding end and prepared for scanning electron microscopy (SEM) observation. After SEM observation total nucleic acids were extracted from each section and analysed by droplet digital RT-PCR (ddRT-PCR) (Figure 1), using a QX200 droplet digital PCR System (Bio-Rad), with the aim to quantify phytoplasmas DNA and RNA in each section by specific primers and TaqMan probe (Ratti *et al*., 2019).
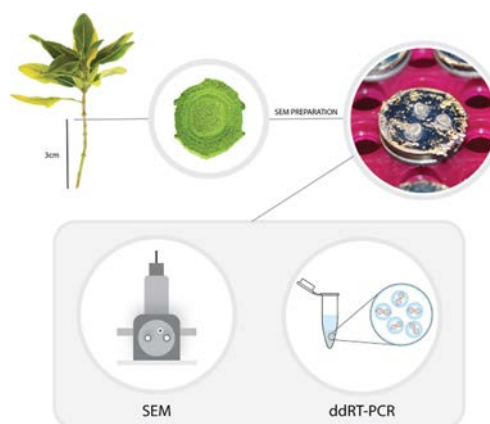
Figure 1. Processing of periwinkle cuttings infected by "flavescence dorée" phytoplasma exposed to treatments.

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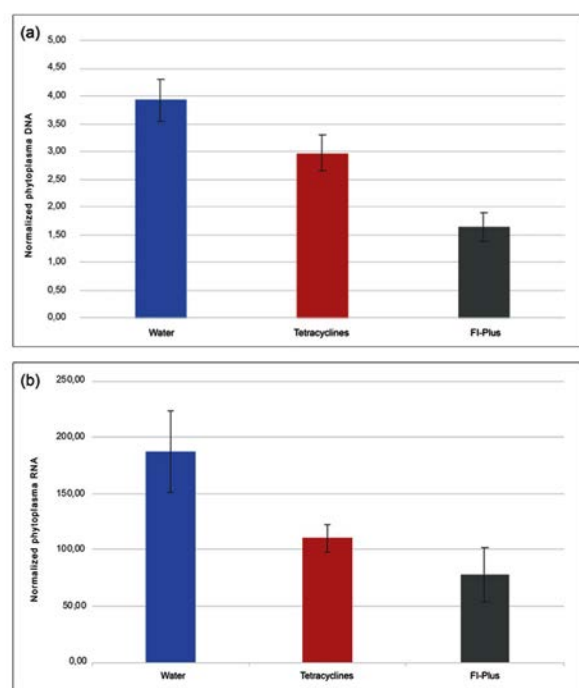
Figure 2. Phytoplasma DNA (a) and RNA (b) quantification in sections from infected periwinkle shoots exposed to water, tetracyclines and "FI-Plus" treatments. ddRT-PCR quantification has been normalized using plant DNA content of each section.

Results

The SEM observation allowed to differentiate the phytoplasma cells according to the variation of their morphological characteristics using a scale of three morphology types. The morphology type 1 was observed in the 94% of the samples treated with water and in the 84% of the samples treated with tetracyclines. Phytoplasma cells maintained their typical shape, size, and concentration in untreated samples. Morphology type 1 was not observed in samples treated with "FI-Plus". The morphology type 3 was found similar in the 99% of samples treated with "FI-Plus" where phytoplasma cells appeared smaller, with lower concentration in comparison with control ones. The morphology type 2 is the intermediate one, where in a single plant cell both the type 1 and type 3 morphologies were observed. Type 2 morphology was observed in 6%, 16% and 1% of the samples treated with water, tetracyclines and "FI-Plus", respectively.

The results of ddRT-PCR analyses allowed to quantify DNA and RNA of phytoplasmas (Minguzzi *et al.*, 2016).

DNA concentration in the samples treated with tetracyclines was significantly lower than the one detected in the samples treated with water and higher than the one treated with "FI Plus". On the other hand RNA concentration was not significantly different between samples treated with tetracyclines and "FI-Plus" but it was significantly lower in the samples treated with water (Figure 2).

Discussion

The development of an *ex vivo* test to evaluate the efficacy of antimicrobials and antibiotics for the growth and development of phytoplasma cells using SEM was achieved. Additional experiments were performed changing the treatments conditions (concentration and time of exposure) which revealed that both tetracyclines and "FI-Plus" are able to reduce the phytoplasma concentration and changing their morphology. The results obtained suggest that the protocol developed could be used to investigate the activity of antimicrobial compounds against phytoplasmas. The assay results should be particularly useful for a preliminary screening of potential antimicrobials before the efficacy verification in field trials.

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Management

Influence of meteorological parameters on leafhopper vector population and sesame phyllody disease incidence in Assam, India

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Abstract

Sesame phyllody disease incidence was recorded as 19.47% and 21.10% and highest leafhopper vector (*Hishimonus phycitis*) populations as 5.4 and 6.2 insects per 5 sweeps in November during 2015 and 2016, respectively. Disease incidence was highly negatively correlated with maximum, minimum and average temperature, day temperature, night temperature, diurnal variation and heat sum pertaining to the previous week and fortnightly interval. Strong positive correlation was observed between leafhopper population and the sesame phyllody disease incidence. The disease could be predicted correctly up to 86% with vector population 14 days before disease and up to 98-99% by addition of maximum, minimum, average or day temperature to the equation.

Keywords: sesame phyllody, disease incidence, leafhoppers

Introduction

Sesame phyllody is a very serious disease occurring worldwide where this plant species is grown; it was first reported from Burma (Mc Gibbon, 1924) and later in India (Kashiram, 1930) where the first molecular detection indicated its association with 16SrI group '*Candidatus* Phytoplasma asteris' (Khan *et al.*, 2007). The leafhopper *Orosius albicintus* was reported as the vector of sesame phyllody phytoplasma (Khan *et al.*, 2007) and later Nabi *et al.* (2015) reported *Hishimonus phycitis* (Dist.) as a second natural vector. The abiotic factors always play a major role in the appearance and spread of the insect pests and diseases in crops. Once the effect can be ascertained it helps in formulating efficient management strategies. Therefore, the present study was undertaken to know the effect of some meteorological parameters in the incidence of sesame phyllody disease in Assam, India.

Materials and Methods

The study was conducted in sesame grown in the "rabi" season (variety Kaliabor) during 2015 and 2016 in Biswanath College of Agriculture, Assam, India. The observations on disease incidence and the leafhopper vector *H. phycitis* population was recorded at weekly interval starting from 20 days after the germination of the crop. Five plots of 5 m² sizes were selected randomly in each field and total number

of plants showing sesame phyllody symptoms within that area were counted. The percentage of disease incidence was calculated with the formula of Sridhar *et al.* (2013).

$$\text{Disease incidence (\%)} = \frac{\text{Total number of infected plants}}{\text{Total number of plants examined}} \times 100$$

Leafhoppers were captured by sweeping net, collected separately in plastic bags, brought to laboratory and the average numbers of the population were recorded. The meteorological parameters like daily rainfall, maximum and minimum temperature, and wind speed were collected from the Department of Agrometeorology, College of Agriculture, Biswanath Chariali. Meteorological data derived indices like day temperature (DT), night temperature (NT), diurnal variation (DV) and heat sum (HS) were calculated as per Venkataraman and Krishnan (1992). Correlation, simple and multiple regression analysis were done to establish the influence of the leafhopper vector and meteorological parameters on the disease incidence.

Results

The disease incidence started in the month of October in both years of the study and the highest incidence was observed in November (47 week) as 19.47% in 2015 and 21.10% in 2016. Disease symptoms observed were phyllody, flower virescence and proliferation, fasciation, witches' broom and

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splitting of the capsule (Figure 1). The highest leafhopper population was observed in the fourth week of October (45 week) as 5.4 and 6.2 insects per 5 sweeps of net in 2015 and 2016 respectively; after that the population started decreasing. Similar findings were reported by Mathur and Verma (1973) and Nabi *et al.* (2015). Correlation studies of pooled data revealed a highly negative correlation of the disease incidence with maximum, minimum and average temperature, DT, NT, DV and HS and highly positive correlation with the leafhopper population pertaining to the previous weeks at 14 days intervals (Table 1). Likewise the leafhopper population was also negatively correlated with total rainfall, maximum, minimum and average temperature, DT, NT and HS pertaining to the previous weeks at 14 days intervals (Table 1). Similar results were also reported by Cagirgan *et al.* (2013). Simple and multiple linear regression analysis (pooled data) for prediction of sesame phyllody disease revealed that it could be predicted correctly up to the 86% with vector population 2 weeks before the appearance of the symptoms. The prediction was enhanced to 98% by the addition to the equation of maximum temperature and up to 99% of minimum, average or day temperature (Figure 2). It is observed that the temperatures with a maximum of 24-26°C, minimum of 18-20°C, an average of 11-12°C, a day around 29-30°C and night of 19-20°C are the most favourable for the disease development.

Figure 1. (A) Phyllody, (B) flower virescence and (C) proliferation, (D) fasciation, (E) witches' broom and (F) splitting of the capsule.
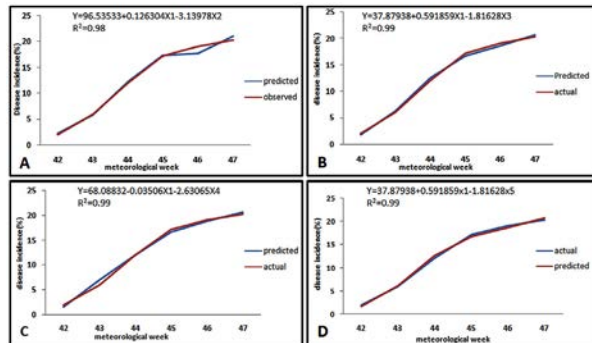
Figure 2. Observed and predicted sesame phyllody disease incidence in relation to (X$_1$) *H. phycitis* population 14 days before recording the disease in the week before its appearance with (A) maximum temperature (X$_2$), (B) minimum temperature (X$_3$), (C) average temperature (X$_4$), (D) day temperature (X$_5$).

Table1. Correlation of sesame phyllody disease incidence, leafhopper population and meteorological parameters (pooled data of 2015 and 2016).

Parameters	Disease incidence (%)		Leafhopper population (5 sweeps)	
	7 days	14 days	7 days	14 days
Rainfall	0.16	-0.38	0.14	-0.42
Temperature maximum	-0.99**	-0.98**	-0.93**	-0.95**
Temperature minimum	-0.96**	-0.99**	-0.85**	-0.97**
Temperature average	-0.98**	-0.99**	-0.91**	-0.97**
DT (day temperature)	-0.99**	-0.99**	-0.92**	-0.96**
NT (night temperature)	-0.97**	-0.92**	-0.93**	-0.93**
DV (day temperature)	-0.63*	-0.89**	-0.02	-0.12
HS (heat sum)	-0.98**	-0.99**	-0.92**	-0.97**
WS (wind speed)	-0.28	-0.19	-0.41*	-0.10
Leafhopper population	0.93**	0.96**	--	--

*Significant at 5% level   **Significant at 1% level

Discussion

The study reveals that the prevailing meteorological parameters and their variability significantly influenced the sesame phyllody disease incidence and *H. phycitis* population build up. Meteorological parameters like precipitation, temperature, and relative humidity are known to affect reproduction, development, behaviour, and population dynamics which might influence the incidence and spread of the disease. The regression equation developed can be used for the prediction of the disease and formulating efficient management strategies.

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Management

Identification and management of 16SrII-D phytoplasmas in cluster bean and sesame crops in the Haryana province of India

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Abstract

Little leaf, phyllody and witches' broom symptoms with incidence of 8% to 20% were recorded in cluster bean, sesame and a weed (*Phyllanthus niruri*) in farmer's fields at Ranila and Begoa villages of Bhiwani district, Haryana, India during September-October 2017-2018. The phytoplasma presence was confirmed in symptomatic samples by phytoplasma-specific universal primer pairs nested PCR assays (P1/P7 and R16F2n/R2). *Empoasca motti* feeding in symptomatic cluster bean and sesame fields was also found positive for phytoplasma presence with the same nested PCR assays. Comparison of 16S rDNA sequence and virtual RFLP analysis of R16F2n/R16R2 primed sequences of cluster bean little leaf, sesame phyllody, *P. niruri* witches' broom and leaf hopper phytoplasmas indicated the presence of a '*Ca*. P. aurantifolia'-related strain classified in subgroup 16SrII-D. Spray of imidacloprid and thiamethoxam effectively controlled the leaf hopper population and reduced the incidence of phytoplasma disease in the affected cluster bean and sesame fields.

Keywords: *Cyamopsis teragonoloba*, *Sesamum indicum*, *Empoasca motti*, '*Candidatus* Phytoplasma aurantifolia'

Introduction

Guar or cluster bean (*Cyamopsis tetragonoloba* L.), Fabaceae, is grown on commercial scale as vegetable and for its guar gum. India accounts for 90% of world's cluster bean production and is the major exporter of guar gum to the world market with 494,101.2 Mt, worth approximately 60 million US dollars (Anonymous, 2018). Major factor that limits the cluster bean productivity is its extreme susceptibility to bacterial blight and *Alternaria* leaf spot diseases. Sesame (*Sesamum indicum* L.) is an important oil-yielding crop of India, which is grown in an area of 1.9 Mha. Among the major sesame biotic constraints, the phyllody associated with the presence of 16SrI, 16SrII, 16SrVI and 16SrIX phytoplasmas is responsible for about 34% of yield losses (Rao *et al.*, 2015).

Materials and Methods

A survey was made in cluster bean and sesame fields in Ranila and Begoa villages of Bhiwani district, Haryana in 2017-2018 for phytoplasma disease incidence. The pathogens associated with sesame phyllody (SP) and cluster bean little leaf (CBLL) disease were studied. An attempt was also made to control the diseases by periodical spray of insecticides in the symptomatic cluster bean and sesame fields. The predominant leaf hopper (LH) species feeding in cluster bean and sesame fields and weed species showing typical phytoplasma symptoms were also collected for phytoplasma indexing. DNA was extracted from leaf tissues of symptomatic and non-symptomatic cluster bean, sesame, weed and the whole body of ten individual's leaf hopper species by a CTAB method (Ahrens and Seemüller, 1992). The DNA from sugarcane infected with the grassy shoot phytoplasma was used as positive control. The DNA extracted from the non-symptomatic plants and leaf hoppers collected from disease free fields were used as negative controls. The PCR reactions were performed with phytoplasma universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by nested primer pairs R16F2n/R16R2 (Gundersen and Lee, 1996). The amplicons of ~1.25 kb obtained from nested PCR assays were gel purified using Wizard^R SV Gel extraction kit (Promega, USA) and directly sequenced in both directions. The sequences of PCR products were assembled using DNA Base V.4, aligned using ClustalW software and used to construct a phylogenetic tree with the neighbour joining method in MEGA 7.0. *In silico* RFLP subgroup identification was carried out using the *i*PhyClassifier online tool (Zhao *et al.*, 2009).

The vector control experiment was conducted during the Kharif season (June to October) 2017 at Ranila and Begoa villages in a randomized block design (RBD) (5 x 5 m) with 2 treatments and three replications. Two concentrations of imidacloprid (1 ml/10 l and 2 ml/10 l) and thiamethoxam (1 g/10 l and 2 g/10 l) were sprayed. The first spraying was

done after appearance of symptoms (45 days after sowing) and subsequently at 15 days intervals. Observation of phytoplasma disease incidence was recorded in insecticide sprayed and non-sprayed fields at both locations.

Results and Discussion

Little leaf and stunting in cluster bean (Figure 1a), phyllody in sesame (Figure 1b) and witches' broom in *Phyllanthus niruri* (Figure 1c) were observed with incidences of 8-20%. The most numerous leafhopper species feeding in symptomatic cluster bean and sesame fields were identified as *Empoasca motti* (Sohi and Dworakowska), *Cofana subvirescences* (Stal) and *C. unimaculata* (Signoret). Symptomatic cluster bean, sesame and weed species and the positive control sample yielded approximately 1.8 and 1.25 kb amplicons in direct and nested PCR assays respectively. However, amplification of 1.25 kb was obtained only with the leafhopper *E. motti*. No amplification was detected in asymptomatic plants and *E. motti* collected from non-symptomatic fields. BLASTn and phylogenetic analysis of the *16S rRNA* partial gene sequences of phytoplasmas from cluster bean (GenBank, acc. nos. 308634 and 307836), sesame (acc. nos. 307834 and 307835), *P. niruri* (acc. nos. 314467 and 314468) and *E. motti* (acc. nos. 307883 and 308633) from both locations revealed 99% sequence identity with 'Candidatus Phytoplasma aurantifolia' strains. The virtual RFLP analysis of the R16F2n/R2 region using 17 restriction enzymes revealed that all the amplicon sequences produced RFLP restriction profiles identical to the one of '*Ca*. P. aurantifolia' (acc. no. Y10097) belonging to 16SrII-D subgroup.

Figure 1. a) Little leaf and stunting in cluster bean; b) phyllody and witches' broom in sesame and c) witches' broom in *P. niruri*.
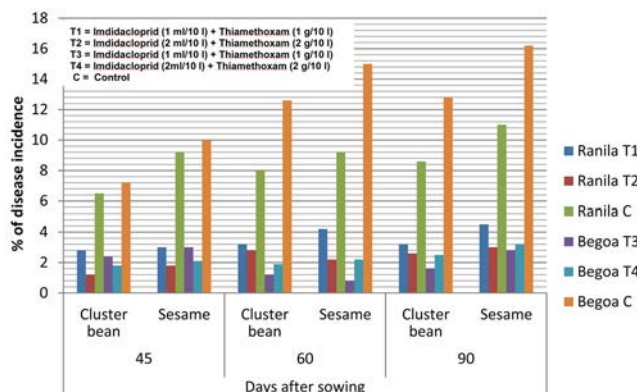
Figure 2. Phytoplasma disease incidence in cluster bean and sesame crops in Ranila and Begoa after foliar spray of insecticides (imidacloprid + thiamethoxam).

Spraying imidacloprid and thiamethoxam significantly reduced the leafhopper population and the phytoplasma disease incidence at both locations (Figure 2). The minimum disease incidence in both the tested crops was recorded after the second foliar sprays of imidacloprid (2 ml/10 l) followed by thiamethoxam (2 g/10 l).

In India phytoplasmas infect many important crop species causing considerable losses in different areas of the country (Rao *et al.,* 2017). In the present study cluster bean is reported for the first time infected with 16SrII-D phytoplasmas. *Hishimonus phycitis* is the vector 16SrII phytoplasmas associated with sesame phyllody (Nabi *et al.,* 2015) and *E. motti* is for the first reported as 16SrII-D phytoplasma infected and is therefore a potential vector of the studied diseases. Moreover the presence of 16SrII-D strain in *P. niruri* indicated its reservoir role for crops. The management of the leafhoppers in the infected cluster bean and sesame fields by spraying insecticides was successful and may be recommended after further evaluation of its impact on the cultivation and environmental health.

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Repellent strategy to avoid transmission of '*Candidatus* Phytoplasma mali' by its vector *Cacopsylla picta*

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Abstract

Cacopsylla picta overwinters on conifers and remigrates in the early spring to apple. These remigrants are highly infectious and readily transmit '*Candidatus* Phytoplasma mali', the agent associated with apple proliferation, to apple. Insecticide treatments before and during blossom are not everywhere applicable and shall be avoided for environmental reasons and in addition, they rarely prevent the transmission of the pathogen. Trials to establish a repellent strategy against *C. picta* which reduces the probe feeding and transmission of the phytoplasma were carried out. It was hypothised that the volatiles emitted by conifers have a repellent effect during the remigration phase of the psyllids. Hot water and EtOH extracts of spruce were sprayed on small *ex vitro* apple plants. Remigrant *C. picta* could chose in a double choice cage experiment conducted in the greenhouse between treated and non-treated plants. Conifer extracts significantly reduced the presence of the psyllids population on the treated plants. Oviposition and larval development was also recorded less on treated plants. However, this effect was not sufficient to reduce the number of transmission events of the phytoplasma and improvement of the technique is needed for possible field application.

Keywords: apple proliferation, conifer extract, phytoplasma transmission

Introduction

Apple proliferation is an economically important disease in several major apple growing regions of Europe. It is associated with the presence of '*Candidatus* Phytoplasma mali' which is efficiently spread mainly by *Cacopsylla picta* (Jarausch *et al.*, 2011; 2019). Up to now, the spread of the disease and its economic impact can only be limited by repeated insecticide treatments, however these are not allowed in every region especially during the blossom. *C. picta* is an univoltine species with an obligate alternation between reproduction host plant (apple) and overwintering host plant (conifers) (Jarausch and Jarausch, 2014). Overwintered adults remigrate to apple in the early spring before or during blossom (Jarausch *et al.*, 2011; Mayer *et al.*, 2011). Volatile signals are used during this migration (Mayer *et al.*, 2011). Remigrant *C. picta* are highly infectious and can transmit the phytoplasma very efficiently to test plants (Jarausch *et al.*, 2011). Insecticides which do not prevent probe feeding and have no immediate effect on the insects do not limit the phytoplasma transmission, therefore efficient repellent strategies are needed. Ideally, they can also reduce or even avoid undesirable insecticide treatments. Univoltine psyllid species which overwinter on conifers, like *C. picta* and *C. pruni*, start remigration to their Rosaceae host plants when photoperiod and climatic conditions are suitable. It is thought, that during this host plant change conifers become repellent. This effect has been demonstrated by Ermacora *et al.* (2012) who showed a repellent effect of conifer extracts on *C. pruni*, the vector of European stone fruit yellows. In this work this strategy was applied to *C. picta* and similar trials were conducted under controlled conditions in the greenhouse.

Materials and Methods

Branches of spruce (*Picea abies*) trees at overwintering sites of psyllids near Neustadt/W. were cut in the late winter. Needles were collected from the branches and cut into small pieces. Three extraction methods were used with 50 g of needles each: hot water (60°C) to extract water soluble substances, 50% EtOH to extract liposoluble substances and 100% EtOH to extract remaining substances. Extractions were done in a column with 30 minutes flow time each. Twenty ml of each extract were sprayed with a fine dosage spray flask dripping wet on 4-6 weeks old *ex vitro* plants of *Malus* x *domestica* cultivar Golden Delicious.

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Table 1. Effects of different conifer extracts on presence, oviposition and '*Ca*. P. mali' transmission to *ex vitro* apple plants.

Treatment with conifer extracts	Psyllid presence on plant (psyllids per day)		Oviposition (N° plants with larval development)		'Ca. P. mali' transmission infected plants/total	
	treated	non-treated	treated	non-treated	treated	non-treated
50% EtOH extract	0.58*	2.25	0.25	0.47	3 / 9	0 / 10
100% EtOH extract	1.00*	2.00	0.00	0.00	1 / 10	1 / 10
Hot water extract	1.27*	3.00	0.17	0.22	1 / 10	2 / 10

* statistically significant at p = 0.05

Figure 1. The double choice cage experiment.

Double choice cage experiments were conducted in the greenhouse with field captured remigrants of *C. picta* just after the psyllid arrival in the orchards. Each cage (80x40x40 cm) consisted of 3-4 treated and 3-4 non-treated plants at each side (Figure 1). Five psyllids per cage were released in the centre and their presence on the plants was recorded for 7 days always in the late afternoon when the insects were settled on the plant.

Each experiment was repeated three times and surviving psyllids were removed from the plants and oviposition and larval development was recorded. In autumn, total DNA was extracted from each plant as well as from recollected psyllids and '*Ca*. P. mali' was detected by PCR as described in Jarausch *et al.* (2011). Statistical analysis was performed using the biostatistics program R (R development core team 2.9.0).

Results

All three conifer extracts caused no phytotoxic effects on the leaves of the young *ex vitro* apple plants. The presence of *C. picta* on treated plants was significantly reduced compared to non-treated plants (Table 1). Oviposition and subsequent larval development did not take place on every plant but it was lower on treated plants – although not statistically significant. Thus, a repellent effect on *C. picta* could be seen with all three conifer extracts. The most effective was the 50% EtOH extract by reducing the psyllid population on the plant by 75%.

The remigrants of *C. picta* have been caught in old highly apple proliferation-infected orchards. Almost all individuals were recollected from the different trials and tested for '*Ca*. P. mali' presence. In total, 18% of the psyllids were

infected and had the potential to transmit the phytoplasma. Analysis in autumn revealed that 13.5% of the plants became infected (Table 1). There was however no statistical difference between treated and non-treated plants.

Discussion

The result of the trial demonstrate the repellent effect of conifer extracts on *C. picta* remigrants. This might be even more prominent under non-caged conditions in the field. The data obtained are very similar to the results of Ermacora *et al.* (2012) who tested conifer extracts against *C. pruni* in an insect-proof tunnel. *C. picta* however is a very efficient vector of '*Ca*. P. mali' (Jarausch *et al.*, 2011) thus, the repellent effect was not sufficient to prevent phytoplasma transmission. The transmission efficiency was in the range of previous results (Jarausch *et al.*, 2011) and occurred even in plants where no oviposition happened. Therefore, further repellent components have to be added to successfully prevent probe feeding and phytoplasma transmission by *C. picta*.

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Management

Isolation of fungal endophytes for the biocontrol of phytoplasmas associated with fruit trees

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Abstract

Endophytic fungi were isolated from apricot and peach trees which had recovered from European stone fruit yellows disease. To test for their phytoplasma antagonistic activity a plant tissue culture system based on apple proliferation-infected apple plants was used. Six selected fungi were inoculated and their effect on plant growth and phytoplasma concentration was measured. One fungus, *Epicoccum nigrum*, significantly reduced apple proliferation symptoms and phytoplasma concentration both in *in vitro* and in *ex vitro* plants.

Keywords: '*Candidatus* Phytoplasma mali', apple proliferation, European stone fruit yellows, *in vitro* screening

Introduction

Apple proliferation (AP) and European stone fruit yellows (ESFY) are important diseases of European pome and stone fruits. They are associated with the presence of two closely related phytoplasmas: '*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma prunorum'. Recent studies indicated that bacterial and fungal endophytes might play an important role in the recovery of perennial plants from phytoplasma infection (Bianco *et al.*, 2013). Different endophytic bacterial community compositions have been found in symptomatic, recovered and healthy apple plants (Bulgari *et al.*, 2012). However, a direct test system for their phytoplasma antagonistic activity was missing. Here the use of a well established plant tissue culture system (Bisognin *et al.*, 2008) is reported to evaluate the biocontrol potential of selected fungal endophytes.

Materials and Methods

Fungal endophytes were isolated from freshly cut shoots from apricot and peach trees in an experimental orchard at Neustadt/W. where recovery from ESFY has been recorded for several years. The shoots were surface sterilised with 70% EtOH and 5% $NaCl_2O_2$ and pieces were put on 50% PDA plates (Potato Dextrose Agar with 200 mg/l streptomycin and 100 mg/l penicillin). Pure fungal cultures were further maintained on HMG (10 g/l malt extract, 10 g/l glucose, 4 g/l yeast extract) agar plates. Molecular identification was done by sequence analysis of ITS4/ITS5 PCR products (White *et al.*, 1990). Fresh hyphae of each fungus were inoculated with a microscalpell into micropropagated plants of *Malus* x *domestica* cultivar Golden Delicious healthy or '*Ca*. P. mali'-infected. Ten replications per fungus and plant culture were made. Phytotoxicity, fungus outgrowth as well as plant growth parameters like shoot height, leaf size and number of axillary shoots were recorded and compared to non-inoculated control plants after 2 months subculture. Specific primers were selected in the ITS sequence of each fungus to monitor the inoculation success by PCR detection. For *Aureobasidium* sp. and *Epicoccum* sp. published primers Apu F2/R2 and Eni F1/R1 were used (Martini *et al.*, 2009). The fungal endophytes which were able to colonise the apple were then inoculated as described above into one year old *ex vitro* plants of *M.* x *domestica* cultivar Golden Delicious healthy or '*Ca*. P. mali'-infected. Inoculation success and phytoplasma titer were analysed 3 months post inoculation in autumn.

Table 1. Fungal endophytes isolated from ESFY-recovered trees and results of *in vitro* inoculation experiments in *Malus* x *domestica*.

Code	Fungus species	Plant of origin	Inoculation success
P2	*Aureobasidium* sp.	*Prunus persica*	phytotoxic
P3	*Alternaria* sp.	*P. persica*	no growth
P4	*Epicoccum* sp.	*P. persica*	endophytic growth
P5	*Sordaria* sp.	*Prunus armeniaca*	no growth
P6	*Peniophora* sp.	*P. armeniaca*	endophytic growth
P7	*Ascochyta* sp.	*P. armeniaca*	no growth

Table 2. Effect of fungal endophytes on growth and phytoplasma titer in '*Ca.* P. mali'-infected apple plants *in vitro*.

Fungus	N° axillary shoots*	shoot height (cm)*	leaf size (cm)*	Cells of phytoplasmas per plant*
Epicoccum sp. P4	5.00 ab	2.25 a	1.25 ab	2.29 b
Peniophora sp. P6	5.22 ab	1.97 ab	1.08 ab	9.29 a
'*Ca.* P. mali'-infected control	6.50 a	1.6 b	1.03 b	12.50 a
healthy control	4.20 b	2.15 a	1.35 a	

* values with the same letter are not different at p = 0.05

Results and Discussion

The concentration of '*Ca.* P. mali' in AP-infected fungus-inoculated and control plants was measured by quantitative PCR according to Jarausch *et al.* (2004). The data were normalised to the concentration of a single copy gene of apple (Liebenberg, 2013). Statistical analysis was performed using the biostatistics program R (R development core team 2.9.0).

Results and Discussion

Fungal endophytes were isolated by standard methods from ESFY-recovered apricot and peach trees. Eighteen isolates were obtained. Eleven different species could be identified by ITS sequence analysis out of which six were selected to test for their efficacy to control the fruit tree phytoplasmas (Table 1). For this, a well established test system to screen for AP resistance *in vitro* was used (Bisognin *et al.*, 2008). Fungal hyphae were inoculated into '*Ca.* P. mali'-infected *in vitro* plants of *Malus* x *domestica* cultivar Golden Delicious. Phytoplasma-free plants were inoculated as controls. Successful endophyte inoculation was checked by molecular detection with specific PCR primers. However, endophytic fungal growth without harm to the *in vitro* plant was only obtained for fungal isolates P4 and P6 (Table 1). The analysis of the growth parameters revealed that isolate P4 significantly reduced phytoplasma specific symptoms like stunting, small-sized leaves and axillary shoot proliferation (Table 2). The phytoplasma titer in inoculated and control plants was measured by qPCR. Fungus P4-inoculated plants had a significantly reduced concentration of '*Ca.* P. mali' (Table 2). To validate these results, fungus P4 was inoculated in greenhouse into '*Ca.* P. mali'-infected *ex vitro* plants. Molecular analyses 3 months post inoculation confirmed a successful plant colonization and again a significant 50% reduction of the phytoplasma titer compared to the non-inoculated control plants.

The P4 is an isolate of *Epicoccum nigrum*, a fungus which already has been shown to reduce symptoms of apple proliferation and the titer of the phytoplasma in inoculated *Catharanthus roseus* plants (Musetti *et al.*, 2011). In this species it induces ultrastructural modifications which might enhance the plant defence system. However, *E. nigrum* is a ubiquitous fungus and is known to produce antibiotics (Elmer *et al.*, 2001) which might explain the effects on '*Ca.* P. mali'

observed in this study. This is the first successful application of the apple plant tissue system to study the phytoplasma antagonistic activities of endophytic fungi. Further research is needed for a practical application in the field.

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Management

"Bois noir" incidence reduction by grafting recovered grapevine shoots

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Abstract

It was evaluated if grafting of recovered material on symptomatic and symptomless grapevines can have curative and preventive effects against the "bois noir" phytoplasma disease. Symptom observation and molecular detection of '*Candidatus* Phytoplasma solani' were conducted on grafted and ungrafted grapevines. The percentage of symptomatic and infected plants was significantly reduced in grafted symptomatic grapevine, indicating that grafting of recovered shoots may have a curative effect.

Keywords: grapevine yellows, '*Candidatus* Phytoplasma solani', recovery, phytoplasma control

Introduction

"Bois noir" (BN), a grapevine yellows (GY) disease associated with the presence of '*Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013), is a major limiting factor for grapevine production in the European countries. The complexity of '*Ca.* P. solani' biological cycle, including different insect vectors and reservoir plants, renders difficult to design efficient control strategies. To limit long distance dissemination and in field spread it is advisable to use propagation materials treated through thermotherapy (Belli *et al.*, 2010). Insecticides applied to the grapevine canopy did not influence the presence of the main insect vector of this phytoplasma *Hyalesthes obsoletus* Signoret and *Reptalus panzer* Löw; thus, the management of the plants, both host for the insects and source of the phytoplasma, is crucial for the BN control. Other BN control strategies are based on removing symptomatic parts by pruning (Riedle-Bauer *et al.*, 2010), trunk cutting above the engagement point (Credi *et al.*, 2011) and treatment by resistance inducers (Romanazzi *et al.*, 2013). These last strategies could act by promoting the spontaneous recovery of infected grapevines, leading to the remission of symptoms along with the elimination of the pathogen, at least from the canopy. Recent studies evidenced the activation of the jasmonate signalling pathway in recovered plants, suggesting its potential importance in preventing '*Ca.* P. solani' infection (Paolacci *et al.*, 2017). In this study, grapevine recovered material, grafted on symptomatic and symptomless grapevines, was used to determine its possible curative and preventive effects against '*Ca.* P. solani' presence in grapevine.

Figure 1. Grafting preparation (left) and development of grafted shoots (right).

Materials and Methods

Shoots for grafting were collected in February 2016 in a Chardonnay vineyard in Erbusco (Brescia) from recovered grapevines, showing symptom remission and phytoplasma absence tested by a '*Ca.* P. solani'-specific PCR assay (Fabre *et al.*, 2011) since four years. Experimental activities, conducted in 2016 in a symptomatic Chardonnay vineyard in Gussago (Brescia), were: i) in May, grafting (Figure 1) recovered grapevine shoots in 50 symptomatic (for curative effect) and in 50 symptomless (for preventive effect) grapevines; 50 symptomatic and 50 symptomless ungrafted grapevines were used as controls; ii) during the vegetative season, symptom observation on grafted and ungrafted plants; iii) in September,

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collection of leaf samples from grafted and ungrafted plants for molecular analyses performed to detect the presence of 'Ca. P. solani' after total nucleic acid extraction; nested-PCR amplification with 'Ca. P. solani' specific *stamp* gene (Fabre *et al.*, 2011). The percentage of symptomatic and 'Ca. P. solani'-infected grapevines was compared by T-student test (p < 0.05) and one-way ANOVA followed by post-hoc Tukey test (p < 0.05), carried out using the software SPSS version 22 (IBM).

Results

Ninety-two percent of the plants grafted with recovered grapevine shoots sprouted (Table 1). The percentage of symptomatic and 'Ca. P. solani'-infected plants was significantly lower (T-student, p = 0.000) in grafted grapevines; no significant differences were found comparing the percentage of symptomatic and 'Ca. P. solani'-infected plants in grafted and ungrafted grapevines (T-student, p = 0.069). The percentage of symptomatic and 'Ca. P. solani'-infected plants in the ungrafted symptomatic grapevines was significantly higher compared to grafted symptomatic, grafted and ungrafted symptomless grapevines (one-way ANOVA, p = 0.000) (Figure 2).

Table 1. Symptom presence and 'Ca. P. solani' detection in the analyzed grapevines.

	Number of plants			
	Sprouted	Symptom.	Collected	Infected
Grafted symptomatic	49	10 (20%)	45	9 (20%)
Ungrafted symptomatic	49	37 (76%)	41	28 (68%)
Grafted symptomless	43	1 (2%)	40	5 (12%)
Ungrafted symptomless	50	7 (14%)	43	10 (23%)
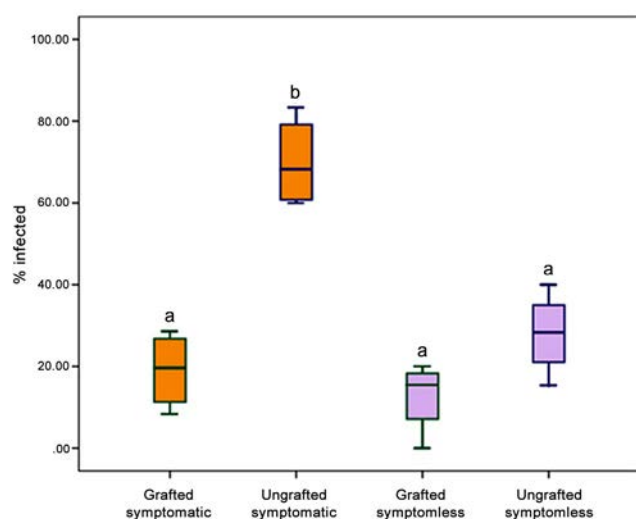
Figure 2. Statistical analysis of differences in infection percentage in grafted and ungrafted grapevines.

Discussion

In the last years, the interest in grapevine yellows control strategies promoting the recovery of infected grapevines and leading to the remission of symptoms along with the elimination of the pathogen (at least from the canopy), is increasing. Preliminary results obtained in the present study indicated that the grafting of recovered shoots may have a curative effect, probably inducing recovery of the diseased grapevines. Such effect is comparable with that observed in previous studies (Riedle-Bauer *et al.*, 2010; Credi *et al.*, 2011; Romanazzi *et al.*, 2013). In order to verify the effectiveness of recovery induction in the grafted plants, monitoring of grapevine yellows symptoms and 'Ca. P. solani' presence in the grafted and ungrafted plants will be performed for at least further two years following previous reports (Osler *et al.*, 1993). Moreover a cost benefit analysis is necessary to evaluate the profitability of grafting symptomatic plants before suggesting the possible adoption of this technique in the grapevine yellows management.

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Management

Phytoplasma recovery phenotype – a case study for aster yellows in South Africa

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Abstract

Aster yellows phytoplasma (AY) is associated with grapevine yellows disease in South Africa that has been observed in local vineyards since 2006. The disease rapidly spread through its natural insect vector and is responsible for significant yield losses in affected production areas. The lack of effective control measures prompted producers to adopt alternative measures, such as inducing the so-called recovery phenotype in infected grapevines. In South African vineyards, this is done by coppicing, *i.e.* cutting symptomatic plants just above the graft union, and allowing regrowth of a single shoot. These shoots typically display the recovery phenotype – a complete remission in symptom expression, as well as testing negative in nested-PCR assays. In this study, the recovery phenotype was monitored in a production vineyard over three growing seasons, by PCR-screening of individual infected and uninfected, coppiced and non-coppiced plants for the presence of AY. A severe drought that occurred during the latter part of 2016 until 2018 influenced the results dramatically.

Keywords: grapevine yellows disease, phytoplasma detection, grapevine coppicing

Introduction

Grapevine yellows (GY) disease was noticed for the first time in South Africa in 2006, and the associated agent in local vineyards, the aster yellows phytoplasma (AY), formally identified by 2010 (Engelbrecht *et al*., 2010). Since then, multiple studies followed to understand the etiology and epidemiology of this fast-spreading disease. The local insect vector was identified to be the leafhopper *Mgenia fuscovaria* (Kruger *et al*., 2011). Although initially discovered in the Olifants river wine producing region, the disease spread within a few seasons to more regions (Carstens, 2014). To date, no direct control measures exist for phytoplasma diseases, resulting in the use of disease prevention strategies like insect vector control using insecticides and rogueing of symptomatic plants.

Alternative strategies of inducing physical (partial uprooting) or chemical (H_2O_2) stresses in plants have been employed to induce a remission of symptom expression (recovery phenotype) in phytoplasma-infected grapevine plants (Musetti *et al*., 2007; Romanazzi and Murolo, 2008). In South Africa, producers induce this remission by coppicing, *i.e.* cutting symptomatic plants just above the graft union, and allowing regrowth of a single shoot. To our knowledge, no long-term studies have been undertaken to investigate the sustainability of this recovery phenotype (RP). In this study, the recovery phenotype was monitored in a production vineyard by recording symptom expression and by PCR-screening of individual infected and uninfected, coppiced and non-coppiced plants for the presence of AY, over three growing seasons.

Materials and methods

An experimental vineyard (cultivar Colombar) in the Vredendal region of the Western Cape, South Africa was selected for this study, based on the visual assessment of typical GY symptomatology. During February 2016 (late summer), symptomatic and asymptomatic plants were sampled and the AY presence was confirmed using three consecutive PCRs (nested PCR), using P1/P7, R16F2n/R16R2 and R16(I)F1/R16(I)R1 primer sets (Deng and Hiruki, 1991; Schneider *et al*., 1995; Gundersen and Lee, 1996; Lee *et al*., 1994) and resolved by agarose gel electrophoresis. For this, total DNA was extracted from phloem tissues of cane material of experimental plants using a CTAB method (Angelini *et al*., 2001) and the purity and concentration of the DNA confirmed with a Nanodrop® ND-2000 spectrophotometer. Fragments obtained from the nested PCRs were gel-purified and subjected to Sanger sequencing to confirm the phytoplasma identity. Based on the results of these diagnostic assays, 40 AY-positive and 40 AY-negative plants were selected, of which 20 plants each were coppiced just above the graft union, during the late winter of 2016.

Subsequent monitoring by PCR screening using the same nested PCR procedure was performed bi-annually (November 2016, February 2017, November 2017, February 2018, November 2018 and February 2019) during the next three growing seasons.

Results

Expected fragment sizes of 1,792 bp, 1,244 bp and 1,100 bp respectively for the three PCR assays were initially confirmed to be AY by sequencing. For subsequent screenings, only the PCR products of the final nested reaction were subjected to electrophoresis. Results for the PCR screenings over three growing seasons are presented in Figure 1. At the outset of the project, all the 20 selected symptomatic plants tested positive for AY. Approximately 50 asymptomatic plants had to be screened before 20 AY-negative plants could be identified. By the time of the second round of diagnostics in November 2016, the numbers of AY-positive plants dropped to five for the coppiced plants and two for the uncoppiced plants. By February 2017, these numbers dropped to four and zero, respectively. During the two screens for the 2017/18 growing season, no AY-positive plants could be detected. After the drought was broken during the winter of 2018, numbers of AY-positive plants steadily increased again with four and five AY-positive plants in the coppiced and uncoppiced plants, respectively. This trend continued into 2019 with five positive plants in each category for the February 2019 screening. All coppiced and uncoppiced plants that tested AY-negative at the beginning of the project maintained their AY-negative status throughout the three growing seasons, except for the last screening in February 2019, when seven and five plants tested AY-positive in the coppiced and uncoppiced groups, respectively.
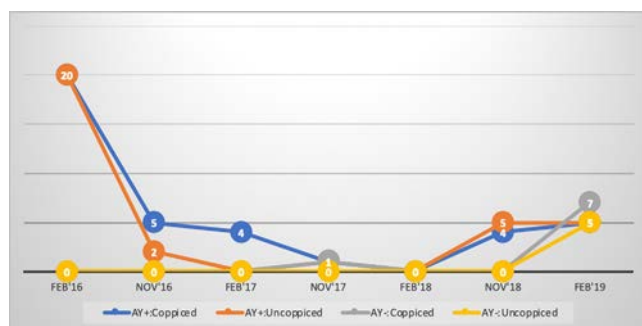
Figure 1. Results of PCR screening of AY-infected and uninfected plants over three growing seasons, comparing coppiced and uncoppiced plants.

Discussion

The recovery phenotype has been observed in a number of grapevine-phytoplasma pathosystems. In South Africa, GY disease reached such economical proportions that producers spontaneously started a practice of coppicing symptomatic grapevines, allowing a single shoot to regrow. The initial results indicated that, apart from a remission in symptom expression, the levels of the pathogen drop to undetectable concentration, even by triple-nested PCR assays. The aim of this study was to investigate the sustainability of this RP over a period of three growing seasons. Unfortunately, very early into the project the Western Cape province of South Africa, where grapevines are grown, experienced one of the worst droughts in decades. This drought therefore seems to have severely impacted the results in that AY levels in all experimental plants, both coppiced and uncoppiced, dropped drastically. In addition to the PCR assays, the monitoring of symptom expression in the experimental plants correlated very well with the PCR results. It is speculated that this general reduction in pathogen concentration was a result of a natural RP-induction caused by the drought in all experimental plants. The obvious follow-up will be to keep monitoring these plants now that the drought has been broken during the rainfall season of 2018. Preliminary diagnostic results (Nov 2018 and February 2019) suggested that the AY concentrations in plants of all categories are increasing.

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Recovery phenomena in Japanese plum trees grafted with apricot that stably recovered from European stone fruit yellows

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Abstract

European stone fruit yellows (ESFY) is the most destructive phytoplasmas disease of apricot and Japanese plum in Europe. All conventional preventive defence strategies have been ineffective in the past, however it has been demonstrated that individual plants can recover from the disease, behaving as completely tolerant to ESFY. The status of tolerance seems transmissible by grafting, so investigations about the possibility to transmit this status from visually recovered apricot to Japanese plums in the field, under high ESFY-infection pressure were carried out.

Keywords: Japanese plum, recovery, graft transmission

Introduction

'*Candidatus* Phytoplasma prunorum' is associated with the quarantine phytoplasma European stone fruit yellows (ESFY). The ESFY disease generally induces typical symptoms such as early bud break, plum leptonecrosis and leaf rolling of apricot (*Prunus armeniaca*), Japanese plum (*Prunus salicina*) and other cultivated *Prunus* species in Europe (Poggi Pollini *et al.*, 2012). To date, all approaches applied to phytoplasma infected plants did not reduce the incidence of these epidemic diseases (Osler *et al.*, 2014). A great relevance has been recently attributed to the recovery, a long-time known phenomenon consisting in the spontaneous remission of symptoms in diseased plants. Despite the physiological basis of this recovery is not completely understood spontaneous and stabile recovery can occur in a low numbers of ESFY-diseased apricot plants (Osler *et al.*, 2014, Poggi Pollini *et al.*, 2012). The long-lasting recovered plants behave in the fields as tolerant to ESFY and this acquired tolerance resulted transmissible by grafting, with a high efficiency to successive generations of apricot (Osler *et al.*, 2014). The aim of this work was to investigate the possibility to transmit this status of acquired tolerance from recovered apricot plants to Japanese plum plants and to verify how long this status can be maintained under natural conditions.

Materials and Methods

Apricot plants 12 years-old naturally recovered form ESFY symptoms and detected in 2015 in the province of Trento, were employed together with plants, located in Friuli Venezia Giulia kindly provided by the University of Udine, with acquired tolerance (Osler *et al.*, 2014) These plants did not show disease symptoms but were positive for the presence of the '*Ca*. P. prunorum' by RT-qPCR detection (Minguzzi *et al.*, 2016, Ratti *et al.*, 2019). Two-years old Japanese plum of several cultivars grafted on myrobalan 29C rootstocks, were grafted by chip-budding with tolerant apricot plants (4 plants per cultivar plus one ungrafted control) (Table 1). Three months later a second chip-budding was performed with ESFY-infected material collected form symptomatic Japanese plum cultivar Santa Rosa. The plants were visually inspected since June 2017, at least three times a year to monitor the presence of the typical symptoms. All the plants were tested annually using of 1 g of phloem from woody shoots of the plum trees, placed in extraction bags (Bioreba) using reported procedures (Minguzzi *et al.*, 2016, Ratti *et al.*, 2019).

Results

The results of field observations showed that the grafting combinations can be divided into 2 groups. Many Japanese plums (4 groups out of 7) showed off season growth, severe

Table 1. Results of grafting tests on Japanese plums.

Japanese plums grafted (cultivars)	Mother plants (apricots)	Symptomatic plants*	ESFY-positive plants*
Black Sunrise	Bulida SP 3/25	4/4	4/4
Friar	Reale di Imola n° 15	4/4	4/4
Larry Ann	Harcot n° 11	4/4	4/4
Larry Ann	Harcot n° 5	1/4	4/4
Obilnaja	Bergeron B**	0/4	4/4
Ozark Premier	Reale di Imola n° 8	4/4	4/4
TC Sun	Bergeron TO**	0/4	4/4

* Control plants were symptomatic and positive in RTq-PCR in all thesis.   **Kindly provided by Fondazione Edmund Mach, Italy

foliar symptoms, fruit deformation, progressive decline and sometimes total die-back in the last three years (2017-2019). On the contrary in the remaining 3 combinations most of the plants (Bergeron B/Obilnaja, Bergeron TO/ TC Sun and Harcot n°5/Larry Ann) did never shown symptoms (Table 1). Moreover, in the last group no undersized fruits were noticed and a good production was achieved.

Specific amplification was obtained from positive controls as well as from all grafted plants. No phytoplasmas were detected in any of the healthy plants used as controls.

Discussion

The potential epidemic threat posed by '*Ca*. P. prunorum' in apricot and Japanese plum orchards is confirmed by the dramatic increase in the number of infected trees, especially when highly susceptible cultivar, like most of the available stone fruit cultivars, are grown under high European stone fruit yellows infection pressure (Poggi Pollini *et al.*, 2012). Like other phytoplasmas, direct protection of the trees from '*Ca*. P. prunorum' cannot be achieved by chemical control measures. Moreover, all preventive measures employed to prevent ESFY spread as sanitary selection, use of clean propagation material, removal of the infected plants and vector control with insecticide treatments have not reduced the progression of the disease in Italy (Poggi Pollini *et al.*, 2007). The results presented showed that recovery can occur in ESFY-diseased Japanese plums grafted with apricots with acquired tolerance. Similar results were observed earlier (Osler *et al.*, 2014) and the present findings may have a

significance after future studies to confirm that the acquired tolerance would be maintained in the time.

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'Candidatus Phytoplasma' and 'Candidatus Liberibacter' species detection in citrus

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Abstract

"Huanglongbing" (HLB) is one of the most devastating citrus diseases in the world. Citrus samples with symptoms referable to HLB were collected in different Caribbean Countries to verify the possible association of 'Candidatus Phytoplasma' and HLB. In Cuba, 140 samples out of 142, resulted positive for HLB and 32 were positive also for phytoplasmas. In Jamaica, out of 6 samples, 5 were positive to HLB and 1 was positive to phytoplasmas. In Guadeloupe, 17 samples were positive for HLB and 7 for phytoplasmas. These results indicate that the symptomatology is not discriminating between the two pathogens and the mixed infection is not an uncommon event.

Keywords: phloematic prokaryotes, molecular detection, mixed infection, "huanglongbing", phytoplasmas

Introduction

"Huanglongbing" (HLB) (Figure 1) is one of the most devastating citrus diseases in the world detected in Cuba at the end of 2006 (Luis *et al.*, 2009), in Jamaica in 2009 (Brown *et al.*, 2011) and in Guadeloupe in 2012 (Cellier *et al.*, 2014). Citrus samples with symptoms of HLB were collected in different regions of these countries to verify the presence of 'Candidatus Phytoplasma' species in association with HLB.

Figure 1. Symptoms of asymmetric mottle in the different citrus species collected in Cuba: 1) sour orange, 2) grapefruit, 3) sweet orange, 4) volkameriana, 5) Persian lime, 6) trifoliate.

Materials and Methods

A total of 142 citrus samples were collected in Cuba, 6 in Jamaica, and 26 in Guadeloupe. DNA extraction was performed from 1 g of leaf midribs using both a CTAB and a phenol-chloroform based methods. PCR amplification for 'Ca. L. asiaticus' was performed with primers OA1/OI2 and OI1/OI2c (Jagoueix *et al.*, 1996). For the 'Ca. Phytoplasma' species, universal primers R16mF2/R16mR1 (Gundersen and Lee,

1996) and U5/U3 (Lorenz *et al.*, 1995) were employed in nested-PCR; further nested-PCR with primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and 16SrIV specific primers 16S503f/LY16Sr (Harrison *et al.*, 1999) was also performed. 'Ca. L. asiaticus' was identified by amplicon sequencing while RFLP analyses and/or sequencing was employed for the phytoplasma identification.

Results and Discussion

In Cuba, 140 samples resulted positive for 'Ca. L. asiaticus' and 32 were positive also for phytoplasmas belonging to 16SrI, 16SrIV, 16SrVII, 16SrXI, and 16SrXII ribosomal groups. In Jamaica, out of 6 samples, 5 were positive to HLB and one was positive to phytoplasmas enclosed in the 16SrI+16SrXII group. In Guadeloupe, 17 samples were positive for 'Ca. L. asiaticus' and 7 for phytoplasmas of 16SrI, 16SrIII, 16SrVI and 16SrXII groups. The results of this work indicate the prevalent presence of the phytoplasmas in mixed infection with HLB (Table 1). Phytoplasmas were also found in samples from four symptomatic citrus plants without 'Ca. L. asiaticus' detection confirming that the symptomatology cannot discriminate between the two pathogens. The analysis seems also to indicate that 'Ca. L. asiaticus' has a higher titre than phytoplasmas since the latter were always detected by nested-PCR assays. The phytoplasma presence in citrus represents however a source of infection for other crops and increases the difficult to prevent their spreading to the surrounding crops.

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Table 1. Results of the survey for the detection of '*Ca*. Liberibacter' and '*Ca*. Phytoplasma' in citrus species; in bold single infections.

Sample	Location	Phytoplasma positive/ total tested	Phytoplasma ribosomal groups	HLB positive/ total tested
Cuba				
Persian lime	Ceballos/Ciego de Avila	8/21	16SrIV (7), 16SrXII (1)	21/21
Orange Valencia	Ceballos/Ciego de Avila	11/30	16SrIV	30/30
Tangerine	Ceballos/Ciego de Avila	0/1	-	1/1
Grapefruit	Ceballos/Ciego de Avila	1/12	16SrIV	12/12
Persian lime	Sola/Camagüey	2/12	16SrIV	12/12
Orange Valencia	Sola/Camagüey	0/2	-	2/2
Grapefruit	Sola/Camagüey	0/2	-	2/2
Persian lime	Jagüey/Matanzas	1/16	16SrIV+16SrXII	15/16
Orange	Jagüey/Matanzas	6/30	16SrI (1),16SrXI (1),**16SrXII (1)**,16SrIV (1) 16SrXII+16SrIV (2)	29/30
Tangerine	Jagüey/Matanzas	1/2	16SrXII+16SrXI	2/2
Grapefruit	Jagüey/Matanzas	1/2	16SrIV	2/2
Lemon	Jagüey/Matanzas	0/3	-	3/3
Orange	Arimao/Cienfuegos	0/2	-	2/2
Grapefruit	Arimao/Cienfuegos	0/6	-	6/6
Mexican lime	La Habana	1/1	**16SrVII**	0/1
Guadeloupe				
Tangelo Nova	Nord Vieux-Habitants	1/1	**16SrIII**	0/1
Tangelo Jackson	Nord Vieux-Habitants	1/2	16SrI	1/2
Orange Navel	Nord Vieux-Habitants	1/1	16SrI	1/1
Mandarine Tample	Nord Vieux-Habitants	1/2	16SrI	2/2
Orange Valencia Late	Nord Vieux-Habitants	1/2	**16SrVI**	1/2
Orange Maltaise	Nord Vieux-Habitants	0/1	-	0/1
MandarineFalglo	Nord Vieux-Habitants	0/1	-	1/1
Tangelo Triumph	Nord Vieux-Habitants	0/1	-	1/1
Tangor Ellendale	Nord Vieux-Habitants	0/1	-	1/1
Orange Navelate	Nord Vieux-Habitants	0/1	-	1/1
Orange Fisher Navel	Nord Vieux-Habitants	0/2	-	1/2
Mexican lime	Trois-Rivières	0/1	-	1/1
Tahiti lime	Trois-Rivières	0/1	-	1/1
Orange Valencia Rod Red	Trois-Rivières	0/1	-	0/1
Orange Navelina	Trois-Rivières	0/1	-	0/1
Mandarine Creole	Trois-Rivières	0/1	-	1/1
Tahiti lime	Trois-Rivières	2/4	16SrVII, 16SrXII	4/4
Flhor Am G1 4X	CIRAD Capesterre	0/2	-	0/2
Jamaica				
Citrus company	Bay Brook	0/5	-	4/5
Citrus Montego Bay	Montego Bay	1/1	16SrI+16SrXII	1/1

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New contribution about the epidemiology of grapevine yellows-associated phytoplasmas in Chile

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Abstract

Three 16SrIII-J phytoplasma-positive vineyards were visited each 15 days from September 2017 to August 2018 to capture insects using entomological sweep nets and yellow sticky traps. Additionally weeds showing typical symptoms associated with the presence of phytoplasmas were also collected. The phytoplasma 16SrIII-J was detected for the first time in the leafhoppers *Amplicephalus ornatus*, *A. pallidus* and *Bergallia* sp., and in the weed *Brassica campestris*. Another phytoplasma classified as 16SrXI, was detected in one leafhopper belonging to the genus *Paratanus*. The identification of the 16SrXI ribosomal subgroup and the species of *Bergallia* sp. and *Paratanus* sp. are in progress, as well as the transmission trials with these two insects plus *A. pallidus* and *A. ornatus*.

Keywords: insects vector, GY, nested-PCR, RFLP, sequencing, weeds

Introduction

In Chile, grapevine yellows (GY) are associated with phytoplasmas belonging to diverse ribosomal subgroups as 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'-related), 16SrIII-J ('*Ca*. P. pruni'-related), 16SrV-A ('*Ca*. P. ulmi'-related), 16SrVII-A ('*Ca*. P. fraxini'-related), and 16SrXII-A ('*Ca*. P. solani'-related or "stolbur") (Gajardo *et al*., 2009; Fiore *et al*., 2015). The phytoplasma 16SrIII-J is the prevalent in the vineyards located in the central zone of the country, and so far it has been reported in various crops and spontaneous plant species in Chile (Quiroga *et al*., 2017). Several alternative host plants, present in or closer to some infected vineyards, have been found infected by phytoplasmas, and two insect species, *Paratanus exitiousus* (Beamer) and *Bergallia valdiviana* Berg 1881, have been proved as vector of the phytoplasma 16SrIII-J (Longone *et al*., 2011; Quiroga *et al*., 2019). This work describes new potential phytoplasma insect vectors, as well as a new reservoir plant species of the phytoplasma 16SrIII-J in Chilean vineyards.

Materials and Methods

From September 2017 to August 2018, three 16SrIII-J phytoplasma-positive vineyards were visited each 15 days to capture insects using entomological nets and yellow sticky traps. The vineyards planted with cultivar Pinot Noir are located in Casablanca (Valparaiso region), with Carménère in San Javier (Maule region) and with Cabernet Sauvignon in Marchigue (O'Higgins region). In these vineyards, weeds with typical symptoms associated with the phytoplasma presence, were also collected. The insects were sorted out by their external morphological features and analysed in batches of 5 individuals for phytoplasma presence. Male specimens were preserved in ethanol 70% and send to the University of Turin, Italy, to be identified by their genitalia. Weeds were identified and 150 mg of plant samples were separated and frozen for phytoplasma detection. The extraction of nucleic acids was carried out according to a silica capture method (Rott and Jelkmann, 2001). The PCR amplification was carried out using 20 ng/µl of nucleic acid; direct PCR with primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and nested PCR with R16F2n/R2 primers on the 16SrRNA gene (Gundersen and Lee, 1996) were performed following Schaff *et al*. (1992). Amplicons from nested PCRs were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania). Putative recombinant clones were analysed by colony PCR and

Table 1. Phytoplasmas detected in weed and insect species.

Vineyard locality	Insect vectors	Phytoplasma ribosomal group	Spontaneous plants	Spontaneous plants
Casablanca	*Amplicephalus ornatus*	16SrIII-J	*Brassica campestris*	16SrIII-J
Casablanca	*Amplicephalus pallidus*	16SrIII-J	*Brassica campestris*	16SrIII-J
Casablanca	*Bergallia* sp.	16SrIII-J	*Brassica campestris*	16SrIII-J
Marchigue	*Paratanus* sp.	16SrXI	No plants collected	-
San Javier	*Paratanus exitiosus*	16SrIII-J	*Brassica campestris*	16SrIII-J
San Javier	*Amplicephalus curtulus*	16SrIII-J	*Brassica campestris*	16SrIII-J

selected fragments were sequenced in both directions in MacrogenUSA Corp (Rockville, MD, USA). The sequences were then aligned with those of strains deposited in GenBank using BLAST engine for local alignment (version Blast N 2.2.12). The 16SrIII-J phytoplasma identification was performed using *in silico* restriction fragment length polymorphism (RFLP) analysis with *Hha*I, *BstU*I and *Rsa*I restriction enzymes.

Results

The 16SrIII-J phytoplasma was detected in two samples of *Brassica campestris*, from San Javier and Casablanca, which showed yellowing, dwarfism and corky leaves. The analyzed insects were 450. *Amplicephalus ornatus* Linnavuori, *Amplicephalus pallidus* Linnavuori and *Bergallia* sp. from Casablanca, were positive to the phytoplasma 16SrIII-J. *A. curtulus* and *P. exitiosus* captured in San Javier were also positive to the same phytoplasma. In one group of five individuals belonging to the genus *Paratanus* from Marchigue, was detected a phytoplasma classified as 16SrXI (Table 1). The nucleotide sequences of the phytoplasma 16SrIII-J showed a close correlation (99.8%) with the strain Ch10 (GenBank accession number, acc. no., AF147706), corresponding to chayote witches' broom phytoplasmas (16SrIII-J) from Brazil. The fragment obtained from the *Paratanus* sp., (1,155 bp) showed 99.5% of nucleotide identity with strains of sugarcane grassy shoot phytoplasma from India (acc. no. MG744609).

Discussion

Based on these results, it seems that new insect species within the *taxon* Auchenorrhyncha could be involved in the transmission of phytoplasmas in the Chilean vineyards. Furthermore, *B. campestris* is an herbaceous species that begins its life cycle after the first autumn rain and stays green until the end of the spring. This means that it may play a relevant role as reservoir of the phytoplasma 16SrIII-J. The identification of a 16SrXI phytoplasma in *Paratanus* sp., is the first report in the Americas. It is important to emphasize that the 16SrXI phytoplasma finding in Chile was never reported, thus an intense sampling of vegetal material, grapevines and weeds, is currently taking place in the O'Higgins region where it was detected.. The identification of the species of *Bergallia* sp. and *Paratanus* sp. are in progress, as well as the transmission trials with these two insects along with *A. pallidus* and *A. ornatus*.

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'Candidatus Phytoplasma' species detection in coconuts in Cuba

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Abstract

Coconut lethal yellowing (LY) is the single most important disease presently affecting the coconut production worldwide. Symptomatic and asymptomatic coconut plants were sampled in some selected areas to verify the identity of phytoplasmas associated with LY in Cuba. Diverse phytoplasma ribosomal groups were detected in the samples from symptomatic palms such as 16SrXII, 16SrVII, and 16SrI. In several other palms 16SrIV-A subgroup phytoplasmas were identified, in the *groEL* gene the only positive plant from Pilón resulted diverse from the others and identical to the 16SrIV-A strains detected in Jamaica LY infected coconut palms. This is the first record of occurrence of 16SrI, -VII and -XII groups in coconut palms in Cuba.

Keywords: phytoplasmas, coconut lethal yellowing, molecular detection, genetic diversity

Introduction

Among the more serious phytoplasma disease are lethal yellowing of palms (Eziashi and Omamor, 2010). The phytoplasmas associated with palm species are classified in 16SrI, -IV, -XI, -XIV, -XXII and -XXXII groups (Harrison *et al.*, 2014). In Cuba, coconut is used as ornamental plant and some small commercial plantations are present in Baracoa (Guantánamo province), Niquero y Pilón (Granma province) and some municipalities from the central region (Cueto, 1986). In previous report the Cuban lethal yellowing (LY)-associated phytoplasmas were classified in the 16SrIV group (Llauger*et al.*, 2002). Nowadays there is a national program to recover the Cuban coconut industry and the present study was undertaken to verify the identity of phytoplasmas associated with LY to support the further development of strategies to more effectively monitor and manage the disease.

Materials and Methods

Symptomatic (Figure 1) and symptomless coconut samples were collected from the most active LY *foci* (Figure 2 and Table 1). DNA extraction was performed from 1 g of trunk boring, using CTAB and/or phenol-chloroform based methods. Positive controls from LY infected palms 16SrIV-A from Mexico and Jamaica and 16SrIV-D from Mexico were used. PCR amplification was performed on 16S rRNA gene with universal primers P1/P7 (Deng and Hiruki, 1991;

Schneider *et al.*, 1995), followed by U5/U3 (Lorenz *et al.*, 1995) and 16SrIV group specific primers 16S503f/LY16Sr (Harrison *et al.*, 1999) in nested-PCR and RFLP and/or sequencing. Moreover, the samples positive for 16SrIV were amplified on the *groEL* gene with primers *gro*ELF1/R1 in direct and *gro*ELF2/R2 (Myrie *et al.*, 2011) in nested PCR. RFLP analyses with *Tru*1I restriction enzyme on U5/U3 amplicons, and with *Alu*I on 16S503f/LY16Sr amplicons, was performed. *Hinf*I enzyme was employed to digest *gro*ELF2/R2 amplicons.

Figure 1. Symptomatology observed in the coconut trees: yellow leaves in horizontal position and necrotic inflorescences.

Figure 2. Locations in Cuba where plants were sampled.

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Table 1. Results of the survey for '*Ca.* Phytoplasma' detection.

Sample	Location/ Province	Phytoplasma ribosomal group
Co1*	Playas del Este, La Habana	-
Co2	Playas del Este, La Habana	-
C1	Moronta, Camagüey	-
C2	Moronta, Camagüey	-
C3	Moronta, Camagüey	-
C4	Moronta, Camagüey	16SrVII
C5	La Gloria, Camagüey	-
C6	La Gloria, Camagüey	16SrIV, 16SrXII
C7	Sola, Camagüey	16SrIV
C8	St. Lucia, Camagüey	16SrIV
C9	St. Lucia, Camagüey	16SrI
C10	St. Lucia, Camagüey	16SrXII
C11	St. Lucia, Camagüey	-
C12	St. Lucia, Camagüey	-
C13	Mayabeque	16SrIV
C168	Pilón Granma	16SrIV

* no symptom

Results and Discussion

Seven out of 16 trunk borings were positive for phytoplasmas (Table 1) and the sequencing confirmed the identity of the detected phytoplasmas (Figure 3).
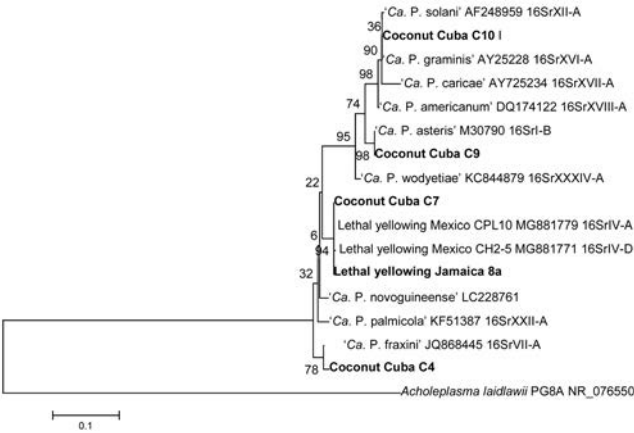
Figure 3. Phylogenetic tree with Neighbour-Joining method in MEGA6 showing in bold the phytoplasmas detected in palms in Cuba and Jamaica.
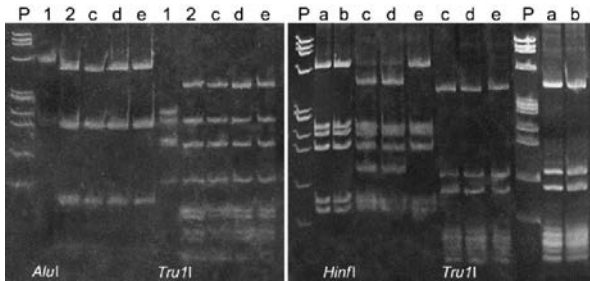
Figure 4. RFLP of amplicons obtained from coconut samples with the enzymes listed at the bottom. Left, primers 16S503f/LY16Sr; right, primers groELF2/R2. Samples from Cuba: c, C7, d, C13 and e, C168; samples from Mexico 1, 16SrIV-D and 2, 16SrIV-A; samples from Jamaica a and b; P, marker phiX174 DNA digested with *Hae*III.

These results indicate the presence of different phytoplasma ribosomal groups in palms. The 16SrIV strains detected were enclosed in the subgroup –A, while the RFLP on *groe*EL gene (not amplifying the 16SrIV-D subgroup) showed that the phytoplasmas in the sample C168 is identical to the strains from Jamaica (Figure 4). In agreement with previous studies the predominant group in cuban coconut palms was the 16SrIV (Llauger *et al.*, 2002); however strains in 16SrI, -VII and -XII groups were also detected. These results indicated for the first time the occurrence of diverse phytoplasma groups in coconut palms in Cuba as reported in other coconut palms infected areas (Contaldo *et al.*, 2019).

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Distribution of lethal yellowing and associated phytoplasma strains in Jamaica, Mexico and other countries in the region

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Abstract

The relentless spread of the fatal disease lethal yellowing (LY) throughout the coconut growing areas is having a serious impact on many vulnerable communities. Phytoplasmas from the 16SrIV group are the associated agents. These obligated phytopathogenic mollicutes systemically colonize phloem tissues inducing numerous biochemical and physiological changes leading to symptom development and ultimate death of coconut palms. Large numbers of coconut trees succumbed to the disease over the past three decades. There are different subgroups of the 16SrIV phytoplasmas affecting palms in the Americas. It is important for these to be properly identified along with the epidemiology of the disease. The effective molecular tools used to identify these subgroups show variations in some conserved genomic regions. In the Americas the subgroups have been identified, the current distribution of LY in Jamaica and Mexico and the 16SrIV subgroups occurring in these countries and in other Caribbean countries are described.

Keywords: phytoplasmas, coconut lethal yellowing, molecular detection, genetic diversity

Introduction

The relentless spread of the fatal disease lethal yellowing (LY) throughout the coconut growing areas is having a serious impact on many vulnerable communities. Phytoplasmas from the 16SrIV group are the agents associated with this disease in the Americas and Caribbean areas. These obligated phytopathogenic mollicutes systemically colonize the phloem tissues inducing numerous biochemical and physiological changes leading to ultimate death of coconut palms. There are different subgroups of the 16SrIV groups affecting palms in the Americas. It is important for these to be properly identified along with the epidemiology of the disease.

Materials and Methods

Samples were collected from coconuts trees displaying LY symptoms and visually healthy trees in Mexico and in some of the islands of the Caribbean area (Figures 1 and 2). DNA extraction was performed from 50 g of trunk boring, using a CTAB extraction protocol (Doyle and Doyle, 1990) PCR amplification was performed on 16S rRNA gene with universal primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) followed by 16SrIV group specific primers 16S503f/ LY16Sr (Harrison et al., 1999) in nested PCR assays. Samples were also amplified by groELF1/R1 primers followed by groELF2/R2 (Myrie et al., 2011) in nested-PCR. Positive samples were subjected to RFLP and/or sequencing. The subgroup identification was obtained on 16S rDNA sequences of appropriate size (about 1,200 bp in the 16S ribosomal gene) with the iPhyClassifier (Zhao et al., 2009).

Figure 1. Symptomatology observed in the coconut trees: yellow leaves in horizontal position and necrotic inflorescences.

Figure 2. Distribution of LY disease and some of the places where the samples were collected.

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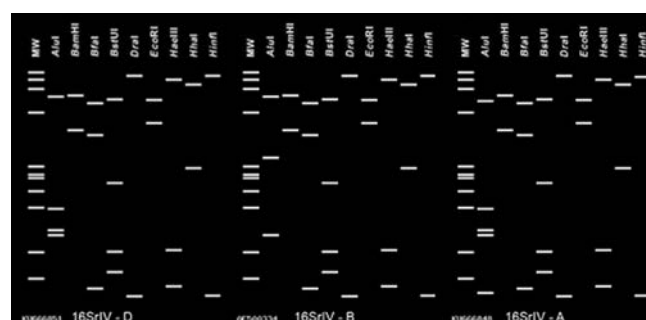
Figure 3. Virtual RFLP of amplicons obtained from coconut samples with the enzymes left to right *AluI, BamHI, BfaI, BstUI, DraI, EcoRI*, HaeIII, *HhaI* and *HinfI*. Amplicon size was 1,249 bp, Ribosomal subgroups are listed at the bottom.

Results and Discussion

Studies in Jamaica on the diversity of phytoplasmas associated with LY disease revealed that only 16SrIV-A subgroup is affecting the coconut palms. No variations were found in the 16S rRNA and the *groEL* genes in Jamaica. The RFLP characterization on 16S rDNA showed distinct difference with *Alu*I enzyme of the 16SrIV-A and -D strain subgroups (Figure 3). It seems that under favourable conditions all the LY pathotypes can move aggressively through the fields and this can be mitigated by the management strategies which have proven to be effective. Studies on the diversity of phytoplasmas associated to LY or LY-type diseases (LYDs) of palm and non-palm species in Mexico, have shown the occurrence of subgroups 16SrIV-A, -B and –D (Harrison *et al.,* 2002). LY disease in Antigua, St. Maarten, St, Kitts and Nevis is associated with the 16SrIV-A subgroup (Myrie *et al.,* 2006, 2012, 2014). Subgroup 16SrIV-A is associated with *Cocos nucifera* and other palm species (Cordova *et al.,* 2000, 2017) while subgroup 16SrIV-B has been found only in *C. nucifera.* Subgroup 16SrIV-D was identified in *C. nucifera*, other palm species and also the non-palm species *Carludovica palmata* (Vázquez-Euán *et al.,* 2011; Narvaez *et al.,* 2006, 2016, 2017). Studies have shown that there is a wide geographic distribution of 16SrIV phytoplasmas in the Caribbean *i.e.* Jamaica, St. Maarten/St. Martin, Antigua (Myrie *et al.,* 2014) and Barbuda (Myrie *et al.,* 2014), St. Kitts and Nevis (Myrie *et al.,* 2006, 2012) and in Mexico (Campeche, Yucatan), Gulf of Mexico (Tabasco, Veracruz), Centre (Guanajuato) and South Pacific (Guerrero, Oaxaca).

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Genetic differentiation of the 16SrXXII-B phytoplasmas in Ghana based on the *leucyl tRNA synthetase* gene

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Abstract

The Cape St. Paul wilt disease is a lethal yellowing type disease of coconut in Ghana. It is associate with the presence of a phytoplasma belonging to the 16SrXXII-B subgroup also referred to as '*Candidatus* Phytoplasma palmicola'-related strain. A *leucyl tRNA* gene based assay was used to discriminate the two strains of the phytoplasma detected in Ghana since sequences from the 16S rRNA gene were unable to reveal these genetic differences in the phytoplasmas.

Keywords: phytoplasma, CSPWD, Ghana, *leucyl tRNA synthetase* gene

Introduction

The coconut palm is indispensable to the economies of the coastal belt of Ghana. The crop is, however, threatened by a lethal yellowing type disease, locally referred to as Cape St. Paul wilt disease (CSPWD). The disease was first discovered in the South eastern part of the country (Volta Region) in 1932. It subsequently spread to the Western and Central Regions of Ghana in 1964 and 1981 respectively (Ofori and Nkansah-Poku, 1997). CSPWD associated with the presence of phytoplasmas belonging to the 16SrXXII-B subgroup recently officially named '*Candidatus* Phytoplasma palmicola'-related strain (Harrison *et al.*, 2014). This subgroup also includes the strain that is associated with the Cote d'Ivoire lethal yellowing disease in the neighbouring Cote d'Ivoire (Arocha *et al.*, 2014). While the disease is still active in the Western and Central Regions of Ghana, it has been observed to be less aggressive in the Volta Region (Nkansah-Poku *et al.*, 2009). Using a limited number of samples (14 samples from the three coconut growing regions) and based on a ribosomal protein gene, a geographical differentiation of the 16SrXXII-B phytoplasma in Ghana was suggested (Pilet *et al.*, 2011). In this study, a ribosomal (16S rRNA) and non-ribosomal (leucyl tRNA) based assays was used to verify the diversity of the 16SrXXII-B phytoplasmas by performing a more extensive sampling across the coconut belt in Ghana.

Materials and Methods

Coconut trunk borings collected from symptomless and CSPWD infected palms in the Western (43); Central (47) and Volta Region (18) of Ghana were the sources of phytoplasma DNA. The samples were collected from palms at all stages of the disease. Three palms in a disease-free area in the Central Region were used as negative controls. DNA extraction was done with a modified Daire *et al.* (1997) protocol using CTAB buffer. Nested PCR targeting the 16S rRNA was carried out using primers P1 (Deng and Hiruki, 1991) and P7 (Smart *et al.*, 1996) followed by G813f (Tymon *et al.*, 1998) and GAKSR (Dollet *et al.*, 2006). The non ribosomal *leucyl tRNA synthethase* gene was amplified using primers described by Abeysinghe *et al.* (2016). All positive PCR samples were sequenced and the sequences compared to NCBI GenBank sequences using the BLAST algorithm. The sequences were aligned and sequence variations investigated using Bioedit version 7. Phylogenetic and molecular evolutionary analyses were performed with MEGA version 7.

Results

Samples from all CSPWD symptomatic palms gave positive results in nested PCR (Table 1). The results from the two assays were consistent with each other. Two samples from symptomless palms also gave positive results. Sequences from the 16S rRNA gene were found to be 100% identical to each other across the sampling locations in the three regions. However, sequencing the *leucyl tRNA synthetase* gene showed that the samples from the Volta Region were distinct from those from the Central and Western Regions with 2 nucleotide differences across the 952 bp length of the sequence (Figure 1).

Table 1. Nested PCR assay of coconut trunk boring samples. DS1-DS4 represents disease stages 1-4; SL: symptomless palm; H: healthy palm. PCR positive/number of samples analysed.

Locations	DS1	DS2	DS3	DS4	SL	H
Western	6/6	13/13	14/14	10/10		
Central	6/6	10/10	13/13	13/13	1/5	0/3
Volta	2/2	3/3	9/9	1/1	1/3	0/3
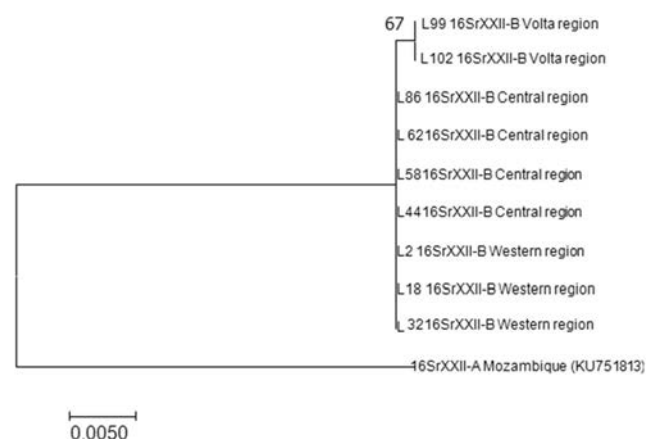
Figure 1. Phylogenetic tree based on the *leucyl tRNA synthetase* gene showing that the phytoplasmas found in the Volta region are distinct from those in the Western and Central Regions (only a few representative samples have been used to draw the tree).

Discussion

The identification of two SNPs in the *leucyl tRNA synthetase* gene of the CSPWD phytoplasma (16SrXXII-B) confirms the geographic differentiation observed by Pilet *et al.* (2011). The distinction between the phytoplasma in the Volta region on one hand, and the Western and Central regions on the other hand may explain the differences in the virulence of the pathogen in the geographic areas as observed by Nkansah-Poku *et al.* (2009). The absence of historical samples or data makes it difficult to determine whether the two strains have evolved independently from each other, or that the original strain that started the disease has mutated or was selected. The identification of two strains of the phytoplasma has implications for resistance breeding experiments: a variety resistant to one strain may succumb to the other. Breeding trials must therefore take this fact into account.

Acknowlegements

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Potential insect vectors and alternative host plants of phytoplasmas in the Fynbos and Succulent Karoo biomes in South Africa

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Abstract

Potential insect vectors and alternative host plants of the phytoplasmas associated with grapevine yellows (GY) were surveyed in the Fynbos and Succulent Karoo biomes in the Western Cape, South Africa. Aster yellows phytoplasma (16SrI-B), which has been reported infecting grapevine in three regions in the Western Cape, was identified in a plant species belonging to the Aizoaceae. Other phytoplasmas were detected from species belonging to the Brassicaceae, Montiniaceae, Proteaceae and Zygophyllaceae and in a few insect specimens. The information will be used to confirm the insect vector status and the role of the plant species identified as alternative host plants in controlled transmission experiments.

Keywords: aster yellows phytoplasma, grapevine yellows, natural vegetation, epidemiology

Introduction

Grapevine yellows (GY), *e.g.* "bois noir", "flavescence doreé" and aster yellows, are serious diseases of grapevine (*Vitis vinifera*, Vitaceae) threatening production and international trade. Associated with these insect-transmitted diseases are a number of phloem-limited bacteria ('*Candidatus* Phytoplasma'). The grapevine production in South Africa is currently threatened by aster yellows (AY) phytoplasma, '*Candidatus* Phytoplasma asteris' subgroup 16SrI-B (Engelbrecht *et al.*, 2010). AY has been reported in grapevine from countries in Africa, Europe, North and South America. Surveys were carried out in the Fynbos and Succulent Karoo biomes in the Western Cape in order to identify potential insect vectors and alternative host plants of these phytoplasmas.

Materials and Methods

Three surveys were carried out in September (spring) in 2017, and January (summer) and August (winter) in 2018 in the natural vegetation at nine sites (Figure 1). Insects were collected with vacuum sampling from 20 randomly selected plant samples per species at the specific sites. Branches with leaves from the same plant species were collected. Insects were preserved in 95% ethanol. Insects and plants were identified to species level when possible. Insects were identified based on morphological characteristics and plants based on morphological characteristics and sequencing. Both potential phloem-feeding insect vectors in the Hemiptera and plant samples were tested for the presence of phytoplasmas.

DNA from intact insects was extracted using a non-destructive TNES buffer method adapted from J. Peccoud and N. Sauvion (INRA Montpellier, France) based on Sambrook and Russell (2001). DNA from plant material was extracted using a 3% CTAB extraction buffer method (Doyle and Doyle, 1990). The extracted insect and plant DNA was used as template for nested PCR of a segment of the elongation factor Tu (*tuf*) gene using the primer pairs Tuf340a/Tuf890a and Tuf400a//Tuf835a (Makarova *et al.*, 2012), and of a segment of the 16S rRNA gene with the primer pairs P1/P7 (Deng and Hiruki, 1991, Schneider *et al.*, 1995), R16F2n/R2 (Gundersen and Lee, 1996) and R16(I)F1/R1 (Lee *et al.*, 1994). RFLP analysis was performed with *Tru*I

restriction enzyme on R16F2n/R2 amplicons, and with *Hha*I on R16(I)F1/R1 amplicons. Direct sequencing of the amplified products using the primers indicated above was also carried out. The psbA-trnH intergenic spacer region and the *rbcL* gene were used for barcoding for plant species identification as described (Roberts and Pietersen, 2017).
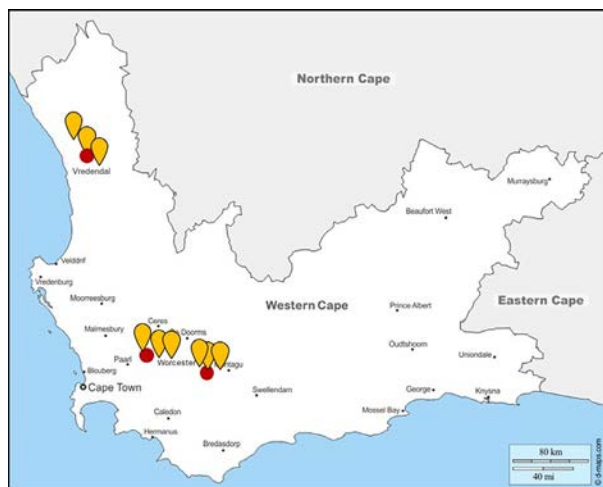
Figure 1. Sampling sites in the Western Cape in South Africa (red circle: areas where aster yellows phytoplasma has been detected; orange markers: sampling sites).

Results

The survey resulted in more than 1,200 insect specimens collected from 989 plant samples from 19 families and 42 species. Only two plant samples from *Mesembryanthemum crystallinum* (Aizoaceae), plant native to South Africa, tested positive for AY with both 16S rDNA RFLP analysis and sequencing analysis on the *tuf* gene amplicons placing the phytoplasma in the 16SrI-B subgroup. This plant species also occurs in other parts of Africa, Australasia, Asia, the Americas and Europe. Other phytoplasmas were detected in species belonging to the Brassicaceae, Montiniaceae, Proteaceae and Zygophyllaceae.

The majority of insects collected belong to the leafhopper family Cicadellidae, comprising more than 50 species in the Deltocephalinae (32 species), Agallinae, Coelidiinae, Typhlocybinae and Ulopinae. Common genera in Deltocephalinae included *Aconurella, Balcutha, Bonaspeia, Caffrolix, Cicadulina, Curculifier, Discolopeus, Exitianus, Hadroca, Maiestas, Orosius, Pravistylus* and *Renosteria*. Fulgoroidea, the planthopper group, included species in Cerocopidae, Cixiidae, Delphacidae, Issidae, Meenoplidae and Tropiduchidae. As yet unidentified phytoplasma-positive insect species were collected from the wild clove bush, *Montinia caryophyllacea* (Montiniaceae), and *Sorocephalu spinifolius* (Proteaceae). Five insect samples tested positive for phytoplasmas with primers for the *tuf* gene, but only two were positive using the 16S ribosomal gene primers. These latter phytoplasmas were identified as belonging to the 16SrII and 16SrIII groups.

Discussion

Phytoplasma infection of Proteaceae has been reported from South Africa (Wieczorek and Wright, 2003) and the 16SrII group phytoplasmas were sporadically detected in grapevine in this country (Botti and Bertaccini, 2006). The identification of phytoplasmas in potential insect vectors and plant species occurring in natural vegetation suggest that they could potentially serve as alternative vectors and reservoirs, respectively, in the vicinity of the vineyards. Further studies with controlled transmission experiments are required to confirm the insect vector status and the role of the plant species identified as alternative host plants.

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Genome-informed design of LAMP assays for specific detection of aster yellows phytoplasmas

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Abstract

Genome sequence data is becoming more available and represents a source of potential target sequences for specific molecular tests to verify the feasibility and efficiency of genome-inform test design the attempt to identify novel specific sequences in the publicly available genomes of aster yellows phytoplasmas and use them to design primers for a spcific LAMP assays. Coupling RUCS automated analysis with PrimerExplorer V5 primer designer and applying criteria for selection of assays at each stage of the process resulted in 13 promising LAMP assays targeting four newly identified regions specific to aster yellows phytoplasmas.

Keywords: molecular tests, genome data, aster yellows target sequences

Introduction

Research on biology and epidemiology of phytoplasmas relies on the ability to detect them. Since most phytoplasmas are difficult to culture, serological and molecular methods could be applied to detect and characterize them. Among the promising methods for detection of phytoplasmas is LAMP (loop mediated isothermal amplification), a molecular method generating high quantities of amplified DNA from a specific DNA fragment using a set of four to six primers (Notomi *et al.*, 2000). The advantages of this method include: isothermal amplification, speed and high resilience to various inhibitors present in the samples. These characteristics in turn allow detection of pathogenic bacteria, including phytoplasmas, on-site in the field with a minimal sample preparation (Kogovsek *et al.*, 2015; 2017). However, DNA sequences most commonly used for detection of phytoplasmas are often not suitable targets for LAMP which requires 4-6 primers with specific characteristics. To expand the range of potential target sequences of LAMP an exploitation of available genomic data of various phytoplasmas was performed to identify potential novel sequences specific to the phytoplasma subgroups of interest. As a test case attempt to identify and design LAMP assays against aster yellows phytoplasmas were done.

Materials and Methods

Two publicly available genomes of aster yellows phytoplasma (complete genome of aster yellows witches' broom phytoplasma AYWB, Accession number CP000061.1; draft genome of New Jersey aster yellows phytoplasma strain NJAY, Accession number MAPF01000000) were selected as target. The specific sequences were identified through comparison of the target genomes with a set of 70 non-target including 15 phytoplasmas genomes (5 closed) and 55 genomes of mycoplasma (22 closed). An automated analysis using previously described and freely available pipelines, namely "find differential primers" (Pritchard *et al.*, 2012) and RUCS (Thomsen *et al.*, 2017), was used to identify target unique sequences of aster yellows phytoplasmas. In the second step, the specific sequences were filtered based on pre-defined characteristics required for the LAMP design and used to design LAMP primer sets using PrimerExplorer V5 (http://primerexplorer.jp /lampv5e/index.html) and LAMP Designer (http://www.optigene.co.uk/lamp-designer/). The proposed primers were further inspected *in silico* for the most relevant properties related to their expected performance including specificity assessment using BLASTn, leading to a selection of the most promising LAMP assays.

Results

Of the two pipelines used to identify unique sequences in the aster yellows genomes, the RUCS pipeline was found to be more suitable. Compared to the pipeline "find differential primers" it was easier to install and use, and required no adaptation (artificial closing) of draft genomes before

analysis. In addition, it is web based, and identifies unique sequences of various sizes. Applying RUCS to the selected 72 genomes resulted in identification of 5,139 sequences unique for the aster yellows genome. The majority of these sequences (97%) was 199 nucleotides long or less. In the range most suitable for the LAMP design *i.e.* 300-600 nucleotides, 137 sequences were identified. The unique sequences of all lengths were distributed throughout the aster yellows genome. In total, 150 of the longest unique sequences were further examined for their specificity using BLAST program. Sequences with similarities to partially sequenced phytoplasma genomes, common bacterial species or phytoplasma hosts were excluded. Out of the remaining 57 specific sequences, 10 longest sequences with mostly unknown function were used for the LAMP primer design (Table 1).

Table 1. Sequences unique for aster yellows phytoplasmas as identified with RUCS and the number of the accepted LAMP assays.

Sequence	Position[a]	Length (nucleotides)	Average % GC	LAMP assays[b]
Seq1	402,284	1,946	27%	0
Seq2	44,205	1,981	26%	2
Seq3	667,186	1,972	23%	6
Seq4	404,906	1,581	22%	0
Seq5	46,673	1,241	19%	0
Seq6	266,394	1,083	21%	0
Seq7	692,620	1,080	20%	0
Seq8	112,041	1,045	22%	0
Seq9	397,407	985	26%	5
Seq10	255,325	932	29%	0

[a]start position of sequence in the complete genome of onion aster yellows phytoplasma.

[b]total number of LAMP assays which passed the quality control analysis.

Both softwares applied for designing the LAMP primer sets were found to be easy to use, adaptable and gave comparable results in the preliminary test. However, PrimerExplorer V5 was selected for final design because it is freely available. When necessary, the stringency of the primer design parameters was modified to allow primer design. Default parameters were modified for AT rich sequences with length of F2/B2 and F3/B3 primer pair increased to 15-25 bp and GC percentage lowered to 25-65%. The proposed LAMP assays were checked manually and filtered using pre-defined criteria: free energy of primer dimers, positioning in different parts of target sequences, quality of 5' primer ends and their melting temperature. Finally, assays were further checked for primer specificity using BLASTn algorithm. LAMP primers considered of sufficient quality were successfully designed on three out of the ten selected unique sequences. Altogether 13 promising LAMP assays were identified spanning three unique regions in the closed genome of the onion aster yellows.

Discussion

Molecular detection of phytoplasmas is focused on a few target genes. This presents a limitation for the design of tests for specific detection of ribosomal groups or subgroups and design of tests with very specific requirements for the target sequence as *e.g.* the LAMP. On the other hand, genomic data is becoming more available and can be tapped as a source of potential novel target sequences for test design. Here the coupled use of the RUCS web based pipeline for identification of potential novel target sequences in the publicly available genomes of aster yellows phytoplasmas and the design of specific LAMP assays using PrimerExplorer V5 LAMP primer design is reported. The approach lead to the successful identification and quality control based selection of 13 promising LAMP assays spanning three unique regions in the closed genome of the onion aster yellows phytoplasma. While the LAMP assays require experimental testing the approach seems a viable way of increasing the pool of potential target sequences in phytoplasmas. The reliability of this approach however, relies heavily on the completeness and the quality of the genome sequence data. It is expected that additional complete or draft phytoplasma genome sequences will significantly improve the accuracy of the identification of unique sequences common among defined '*Candidatus* Phytoplasma species' or ribosomal groups and subgroups.

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Preliminary evaluation of the use of an antiserum obtained from phytoplasma culture

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Abstract

The development of methods for culturing phytoplasmas in artificial media suggested the evaluation of strategies to produce antibodies against a phytoplasma target. To this purpose, an aster yellows phytoplasma isolate obtained from field collected infected grapevine was used for the production of a polyclonal antiserum. It was then tested by ELISA and IFAS methods in order to check and evaluate its suitability for phytoplasma detection in plant samples.

Keywords: Aster yellows isolate, polyclonal antiserum, DAS ELISA, IFAS

Introduction

Phytoplasma- associated diseases are affecting several plant species worldwide, with severe yield losses in economic important crops. Their isolation and growth in artificial media is not easy and usually it takes quite a long time to obtain a substantially pure culture (Contaldo *et al.*, 2019). The development of methods for culturing phytoplasmas in artificial media suggested the evaluation of possible strategies to raise antibodies against a phytoplasma target. Such possibility could indeed provide a tool to overcome the present technical limitations in preparing phytoplasma antigens for serology, such as poor antigen yield from *in vivo* purification methods (Bellardi *et al.*, 1992), and problematic specificity assessment of proteins targets produced via *in vitro* recombinant expression (Hodgetts *et al.*, 2014). The availability of an aster yellows isolate obtained from field infected grapevine was suggesting the exploitation of its use for polyclonal antiserum production for serological test such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescent antibody staining assay (IFAS), currently used for routine diagnosis of several mycoplasma species such as *Mycoplasma pneumoniae* (Dorigo-Zetsma *et al.*, 1999; Loens *et al.*, 2010).

Materials and Methods

In the early summer 2017 phytoplasma isolation trials from field collected grapevine samples were conducted as previously reported (Contaldo *et al.*, 2016). One of the phytoplasma isolates obtained, AY-3J, showed the best growing

performance and the largest identity to the aster yellows-related phytoplasma (AY) 16SrI-B strain detected in the original grapevine by routine molecular tools (Duduk *et al.*, 2013), and it was therefore selected for the antiserum production. Six aliquots of AY-3J liquid cultures were prepared at the concentration of 10^7 CFU/ml, as required for the immunization protocol. Three out of 6 liquid culture aliquots were employed for the rabbit immunization procedures. In particular, one fresh cell culture aliquot was used for the first injection, while the other two -20°C stored aliquots were used for the following injections done at 21 and 42 days after the first immunization, respectively. The antiserum was collected after 50 days and evaluated. In particular, its titration was assessed against the -20°C stored AY-3J aliquots and the rabbit pre-immune samples, both in ELISA (1: 1000) and in Western blot (1: 200 to 1: 1000). The antiserum was then purified by affinity chromatography to prepare the IgGs for the serological tests.

The AY-3J aliquots, stored at -20°C, were employed to calibrate both, the ELISA-DAS and IFAS serological assays with the produced antiserum. The standard ELISA-DAS protocol (Clark and Adams, 1977) was calibrated against the -20°C stored AY-3J and lyophilized periwinkle samples aster yellows infected (Table 1). Moreover antiserum testing by IFAS assays (Elphistone *et al.*, 1996) was used to verify its ability to detect AY phytoplasmas in fresh infected periwinkle plant tissues (strain Hyd35, 16SrI-B) from collection (Bertaccini, 2014). The -20°C stored AY-3J isolate and an healthy fresh periwinkle sample were used as reaction controls.

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Table 1. Absorbance values obtained in ELISA tests with the produced antiserum from colony.

Sample	Description	OD$_{405nm}$ 1 hr	OD$_{405nm}$ 2 hr	Results
Positive	-20° AY-3J culture	1,118	2,246	+
Negative	Fresh healthy periwinkle	17	51	-
1_AY2	AY2 infected periwinkle freeze dried	30	65	-
2_AY4	AY4 infected periwinkle freeze dried	13	33	-

Results and Discussion

ELISA tests confirmed the specificity of the produced antiserum to the AY-3J cell cultures (Table 1). No chemioluminescence reaction was however observed in the two AY infected periwinkle samples freeze dried as well as from the fresh healthy periwinkle. The results obtained by ELISA were then compared to those obtained by IFAS. The antiserum tested showed a specific binding to the AY-3J cell cultures with the observation of a number of yellow fluorescent particles under fluorescence microscope (Figure 1). Furthermore, a strong fluorescence was observed in the fresh AY-infected samples, in comparison with the healthy periwinkle plant tissues, where no fluorescence was observed in the slide (Figure 1).
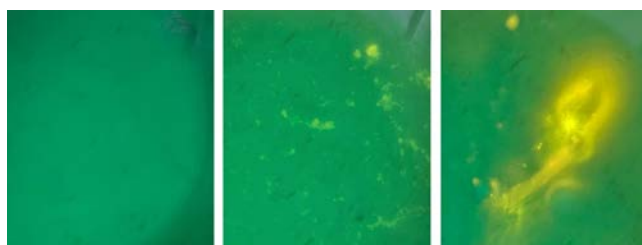
Figure 1. IFAS results under fluorescence microscope (40 X): from left healthy periwinkle, -20°C stored AY-3J isolate, and infected periwinkle plant tissues (strain Hyd35, 16SrI-B).

The work showed the preliminary application of serological methods by raising an antiserum against a phytoplasma culture. Phytoplasma isolation and growth in artificial media is still difficult and valid and trustable detection methods are needed to identify the phytoplasma colonies in both, cultures and plants. The two serological methods employed showed a good specificity to the AY-3J cultures, while the IFAS assay gave promising results also with infected plant tissues. This is a traditional laboratory technique that utilizes fluorescent dyes to identify the presence of antibodies bound to specific antigens. IFAS for the detection of *Ralstonia solanacearum* resulted slightly more reliable than ELISA, especially at low pathogen concentration, as the low numbers of cells which were visible microscopically were insufficient to generate an ELISA signal (Elphistone *et al.*, 2000). The positive results obtained should be confirmed by further assays to set up and validate the IFAS method used for detection of phytoplasmas in both cell culture and plants.

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Support provided by EPPO and Euphresco on phytoplasma diagnostics: recent activities including the integration of Q-bank

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Abstract

The European and Mediterranean Plant Protection Organization (EPPO) has established a diagnostic programme in 1998. Since that year more than hundred standards have been developed including pest specific standards and horizontal standards (e.g. on quality assurance). Activities relevant to phytoplasmas are presented. Since the 1st of May 2019, EPPO is hosting the Q-bank database, a database to support plant pest diagnostic activities. Finally, Euphresco activities and how they support plant health are presented.

Keywords: diagnostic, database, sequences, research coordination

Introduction

The European and Mediterranean Plant Protection Organization (EPPO) is an intergovernmental organization responsible for European cooperation in plant health. Founded in 1951 by 15 European countries, EPPO now has 52 members, covering almost all countries of the European, Mediterranean and Central Asian regions. One of the main roles of EPPO is to help its member countries to prevent entry or spread of dangerous pests. The Organization has therefore been given the task of identifying pests which present a risk for the region and to develop regional standards providing guidance on how to detect and identify pests. Reliable and rapid diagnostic processes are indeed essential to support inspection activities conducted by National Plant Protection Organisations Organizations (NPPOs) in the framework of their official mandate. In this context, validated and internationally accepted diagnostic protocols are of outmost importance as they support the harmonization of detection and identification procedures worldwide, and contribute to greater transparency and comparability in the diagnosis of regulated pests (Petter *et al.*, 2007, 2008).

EPPO programme on diagnostics

The EPPO diagnostic programme started in 1998 with the preparation of pest specific diagnostic protocols. Development of quality management systems and accreditation became quickly a concern for many laboratories in the EPPO region. It was noted that the International Standardisation Organization (ISO) had developed a Standard, the ISO/IEC Standard 17025 General requirement for the competence of testing and calibration laboratories, however, plant health experts considered that it would be beneficial to prepare guidance based on this standard but that is specific to plant pest diagnostic. Two EPPO Standards were prepared PM 7/84 - Basic requirements for quality management in plant pest diagnosis laboratories and PM 7/98 - Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. These two standards provide specific guidance for plant pest diagnostics, in particular on how to perform the validation of tests for different pest groups and different methods. Specific tables have been prepared for the validation of tests to detect and identify phytoplasmas. PM 7/98 - Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity is currently under revision to update it with most recently developed approaches in accreditation (*e.g.* the operation of flexible scopes). Laboratories in the EPPO region are increasingly working under quality assurance systems and this is becoming a requirement of National Plant Protection Organizations for laboratories performing official tests. In such a framework, the need for laboratories to have access to well characterised biological reference material for morphological identification and for the use, development and validation of tests has been recognized. EPPO participated in an EU FP7 project on collections (Q-collect) which resulted in the development of a white paper on collections which made recommendations currently considered in the EPPO framework.

EPPO-Q-bank database for phytoplasmas

The Q-bank database originally started as part of a Dutch project to strengthen the plant health infrastructure; it was launched in 2010. It was further developed in the framework of the EU funded project QBOL. The database on phytoplasmas was developed with the contributions of the curators Dr Assunta Bertaccini and Dr Nicoletta Contaldo from the University of Bologna (Italy) and Dr Mogens Nicolaisen from the University of Aarhus (Denmark) and sequences generated during QBOL have been deposited in the database. The phytoplasma database contains DNA sequences (barcodes) of more than 100 strains that are of relevance to phytoplasma researchers (International Phytoplasmologist Working Group, http://www.ipwgnet.org). The possibility that EPPO could host the Q-bank database has been discussed in the EPPO framework. The benefits of a database including curated sequence data (and for which curation of data will continue) and collection data have been recognized by different EPPO bodies including the Workshop of Heads of plant pest diagnostic laboratories. The added value of Q-bank compared to other sequence databases was evaluated and it was concluded that:

– the data are of high quality *e.g.* sequences linked to specimens in collections, for phytoplasmas the collection at the University of Bologna;
– a multilocus analysis tool is valuable;
– updates on taxonomy from the curators to guarantee the validity of the identifications;
– the tool is suited to the main laboratories performing official plant pest diagnostics.

Furthermore, an EPPO standard on DNA barcoding was approved in 2016 mainly based on the outcome of the QBOL and makes several references to Q-bank. The EPPO Council in September 2018 approved that the database should be transferred to EPPO. The data from the original Q-bank database has been transferred to the EPPO-Q-bank database which was launched on the 1st of May 2019. Links to the EPPO Global Database (https://gd.eppo.int) have been made including some taxonomical revisions for some pest groups. The primary aim of the database is to host sequence data as well as information on biological material in particular from where this material is available. The technical content of the database is managed by curators coordinated by the EPPO Secretariat. For phytoplasmas the above mentioned current curators continue to contribute to the maintenance and enhancement of the database.

Euphresco

In order to increase active collaboration among the organisations involved in plant health research activities at the national and regional levels, Euphresco (European Phytosanitary Research Coordination, www.euphresco.net) was established in 2006 and funded by the EU as an ERA-NET project. Euphresco has subsequently evolved into a self-sustaining international network hosted by EPPO.

The benefits of this coordination are multiple (Giovani *et al.*, 2015; Giovani, 2017). By fostering collaboration at the research level, Euphresco enables researchers to work on common problems and contributes to the adoption of common standards and practices in research activities, including those with diagnostic aims. From the research area, these common approaches can spread to more applied activities, contributing to their harmonisation. Every year, Euphresco members identify research priorities to be addressed through transnational collaboration. Many research projects have been funded with the aim of developing new tests for the detection and identification of pests, validating these tests or evaluating the proficiency of laboratories. Several projects on phytoplasmas have been carried out including: "Interlaboratory comparison and validation of detection methods for phytoplasmas of phytosanitary concern in European orchards" (FruitPhytointerlab), "Epidemiology and diagnosis of potato phytoplasmas and 'Candidatus Liberibacter solanacearum' and their contribution to risk management in potato and other crops" (PhyLib I, PhyLib II), "Epidemiological studies on reservoir hosts and potential vectors of grapevine flavescence dorée and validation of different diagnostic procedures" (Grafdepi I, Grafdepi II), "Evaluation of factors determining distribution, impact, detection and characterization of apple proliferation and other fruit tree phytoplasmoses in the European Community" (Apophyt), "Tracking vectors of bacteria and phytoplasmas threatening Europe's major crops" (Vectracrop), "Modelling the epidemiology of "flavescence dorée" in relation to its alternate host plants and vectors" (Flavid), "Study on the diversity of phytoplasmas detected in European forests" (PhyFor) and "Set up of reliable detection protocols for the specific identification of 'Candidatus Phytoplasma phoenicium'". Reports of the ended projects are available on the Euphresco website and on Zenodo.

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Quarantine

New insights into the emergence of the grapevine "flavescence dorée" epidemics in Europe

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Abstract

A survey of genetic diversity of "flavescence dorée" (FD)-related phytoplasmas in grapevines, alders and clematis as well as alder-feeding leafhoppers was conducted in France, Hungary, Germany, Italy and Serbia. Genotyping was based on the housekeeping gene *map* and on the *vmp* genes encoding surface variable membrane proteins. Transmission assays of the phytoplasmas were performed with alder and/or grapevine-feeding leafhoppers. The study demonstrated that European alders constitute an original reservoir of FD phytoplasma by hosting a high diversity of FD-related phytoplasma genotypes, also present in non viticultural areas. The alder phytoplasmas grouping in Vmp-I cluster were transmitted by the Macropsinae *O. alni*, but were not compatible with the FD phytoplasma vector on grapevine the Deltocephalinae *Scaphoideus titanus*. The alder phytoplasmas in Vmp-II and -III clusters were transmitted by the Deltocephalinae *Allygus* spp. and *Orientus ishidae*. Such pre-existing phytoplasmas were compatible with *S. titanus* transmissibility and can be responsible for the emergence of FD phytoplasma epidemics in grapevine. VmpA proteins of cluster II better adhered to *Euscelidius variegatus* and *S. titanus* insect cells and midguts than those of cluster I. Such adhesins might play a key role in the adaptation to new vectors.

Keywords: 16SrV-C and -D phytoplasmas, genetic diversity, vectorial competence, Vmp adhesins

Introduction

"Flavescence dorée" (FD) epidemics had been associated to the introduction of the leafhopper vector *Scaphoideus titanus*, when Europe imported American *Vitis* rootstocks. However, the geographical and ecological origin of this phytoplasma remained unclear despite evidences for a plant host-range not restricted to grapevine. FD-related phytoplasmas were described in *Clematis* sp. and *Alnus* sp. in the vicinity of vineyards and it was demonstrated that autochthonous Auchenorryncha feeding on these plants were able to occasionally transmit these phytoplasmas to grapevine (Maixner *et al.*, 2000; Filippin *et al.*, 2009). More recently, it was evidenced that the introduced leafhopper *Orientus ishidae* was able to transmit FD phytoplasma to grapevine

(Lessio *et al.*, 2016), but the source plants for acquisition remained to be elucidated. This study brings new insights into the ecological cycle of 16SrV-C phytoplasmas between the vineyards and their environment and into the emergence of the grapevine FD phytoplasma epidemics in Europe.

Materials and Methods

Plant and insect samples were collected in Hungary, France, Germany, Italy and Serbia in the surroundings of FD-phytoplasma infected and FD-phytoplasma free vineyards and in non-viticultural areas. Plants were *Clematis vitalba* and *Vitis vinifera* exhibiting yellows and *Alnus glutinosa* without typical symptoms. Leafhoppers *S. titanus* were collected on infected grapevine stocks and various Cicadellidae were collected on alder trees. For genetic characterization, the *map*

gene was amplified and sequenced as described in Arnaud *et al.* (2007). Primers used for the amplification and sequencing of *vmpA* and *vmpB* genes (Arricau-Bouvery *et al.*, 2018) were defined from the sequences of 16SrV selected strains. Phylogenetic reconstructions using maximum parsimony were performed by MEGA7. For the transmission experiments, alder leafhoppers were sorted by species and placed on *Vicia faba* or *A. glutinosa* shoots until death. Plants were regularly tested for symptoms and phytoplasma presence. Infected *V. faba* plants obtained after transmission were then incubated with *S. titanus* and *E. variegatus* larvae for phytoplasma acquisition followed by transmission to new broad bean plants. Adhesion assays were performed on cells of *E. variegatus* in culture and on midguts of *E. variegatus* and *S. titanus* as described (Arricau-Bouvery *et al.*, 2018). Retained fluorescent latex beads coated with different amounts of VmpA protein of FD92 and PGYA (Palatinate grapevine yellows) phytoplasma strains were counted for each experiment.

Results

The *map* sequence was obtained for 736 samples with 132 genotypes identified. Grapevines and *S. titanus* individuals from FD phytoplasma outbreaks hosted 11 genotypes which belonged to the clusters map-FD1, -FD2 and -FD3 (Arnaud *et al.*, 2007). *Clematis* was infected by 3 genotypes related to map-FD1 and -FD3. In contrast, 128 genotypes were detected in the alder trees and leafhoppers. Alders were infected at 86%, half of them with a mixture of genotypes. Interestingly, 8% of the phytoplasmas were identical with grapevine FD phytoplasma genotypes and could be detected in FD phytoplasma-free areas. Among the Cicadellidae tested, only three species resulted infected with 16SrV phytoplasmas. The Macropsinae *O. alni* was infected at 21% and transmitted 14 times to *V. faba* and *A. glutinosa*. These genotypes could not be subsequently transmitted by the Deltocephalinae *E. variegatus* and *S. titanus* when tested. The Deltocephalinae *Allygus mixtus/modestus* and *O. ishidae* were infected at 60% and 52% by FD phytoplasma genotypes, mainly M38 and M38/M50 respectively, which they transmitted to *V. faba* and *A. glutinosa*. The genotype M38 (map-FD2) was subsequently transmitted by *S. titanus* and *E. variegatus*; the M50 (map-FD1) was compatible with both. *VmpA* and *vmpB* genes had a variable number of 234 nt repeats with high sequence variability among phytoplasmas. The topology of phylogenetic trees was similar among *vmps* but strongly differed for *map* gene by discriminating 3 clusters: Vmp-I grouped all the alder and PGY phytoplasmas transmitted by the Macropsinae, while Vmp-II and -III grouped the map-FD phytoplasmas from grapevine and alder transmitted by the Deltocephalinae. Interestingly, repeated domains evolved independently in cluster -I, whereas they evolved by duplications in clusters -II and -III. Latex beads coated with higher ratio of VmpA-II showed enhanced adhesion to the epithelial cells of *E. variegatus* and were better retained in both *E. variegatus* and *S. titanus* midguts.

Discussion

This study demonstrate that FD phytoplasma is endemic in European alders. Its emergence as an epidemic pathogen for grapevine is restricted to some genetic variants pre-existing in alder. MLSA studies suggested multiple emergences from wild environment in Europe (Krstic *et al.*, 2018; Plavec *et al.*, 2019). The compatibility of the phytoplasma to *S. titanus* resulted from the adaptation of Vmps to other Deltocephalinae living on perennial wild plants. It was demonstrated that VmpA acts as an adhesin with cells of the vectors (Arricau-Bouvery *et al.*, 2018). Its organization, similar to adhesion-related proteins, allows the fast duplication of pre-adapted repeated domains. This suggests a key role of Vmps in the life-style of woody host phytoplasmas that rely on the adaptation to new insect vectors to expand their plant-host range. The presence of the polyphagous Deltocephalinae *O. ishidae* could expand the ecological cycle of FD-related phytoplasmas.

Acknowledgements

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Recent spread of the "flavescence dorée" disease in north-eastern Italy

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Abstract

Recently, in the northern grapevine-growing area of Udine province (Friuli Venezia Giulia region) an epidemic of "flavescence dorée" (FD) disease is spreading. With the aim to investigate the genetic variability of the FD phytoplasma, 72 and 5 strains identified respectively in grapevine and *Scaphoideus titanus* samples, collected in the years 2016-2018 both in the epidemic area and in previous infected areas of Friuli Venezia Giulia, were typed by nested-PCR/RFLP analyses based on 16S rRNA and *map* genes. Both analyses produced two restriction profiles among FD phytoplasmas: *map*-FD2 (16SrV-D) and *map*-FD3 (16SrV-C). Only the *map*-FD2 was identified in the insects. In grapevines, *map*-FD2 type was the prevalent (73.6%) and according to the province of origin, it was predominantly present in Udine province (95%); whereas in Pordenone province the two *map*-FD types were almost equally distributed: *map*-FD2 in 45% of grapevine samples and *map*-FD3 in 55%. *Map* gene sequence analysis performed on 20 FD phytoplasma selected strains, confirmed their assignment to *map*-FD types and showed 100% sequence identity within each *map*-type. Based on the preliminary data obtained from the investigation on the wild plants there are no elements that suggest their potential involvement as FD-phytoplasma source for the infected vineyards in the new epidemic area studied.

Introduction

Recently, in Friuli Venezia Giulia region a recurrence of "flavescence dorée" (FD) disease is experienced, in particular an epidemic is spreading in the northern grape-growing area of Udine province. With the aim to investigate the genetic diversity of FD phytoplasma strains from all over Friuli Venezia Giulia and the role of wild plants found in the vineyard agro-ecosystem compromised by FD epidemic, a preliminary survey was conducted during the vegetative seasons of the years 2016-2018 through the application of a molecular epidemiology approach.

Materials and Methods

In the late summers 2017 and 2018, leaf samples were collected from 20 symptomatic grapevines (*Vitis vinifera* L.) in different vineyards located in Udine province. Similarly, leaf samples were collected on the borders of the inspected vineyards from a total of 13 woody and shrubby plants belonging to species known as potential phytoplasma alternative host plants: *Vitex agnus castus* (1), *Corylus avellana* (2), *Salix* spp. (5), *Cornus sanguinea* (2), *Quercus* spp. (1), *Clematis vitalba* (1) and wild grapevine (1) (Casati *et al.*, 2017). Only one plant of *C. sanguinea* showed symptoms

of leaf cupping and reddening. Forty-nine individuals of *Scaphoideus titanus* were sampled with the beating tray method within the vineyards.

After total genomic DNA extraction, samples were analyzed by an EvaGreen real-time PCR protocol using phytoplasma universal primers 16S(RT)F1/16S(RT)R1 (Saccardo *et al.*, 2013) followed by a high-resolution melting (HRM) analysis (Martini *et al.*, 2015).

FD phytoplasma strains identified in plants and insects in this work (18 grapevine and 5 *S. titanus* DNA samples from Udine province collected in 2017-2018) and several others FD strains from grapevine (23 from Udine province and 31 from Pordenone province) collected in 2016 and provided by ERSA Plant Protection Service (Friuli Venezia Giulia) were typed by nested-PCR/RFLP analyses based on 16S rRNA (primers P1/P7 followed by P1A/16S-SR and RFLP analysis with *Taq*I) (Lee *et al.*, 2004) and *map* genes (primers FD9f5/MAPr1 followed by FD9f6/MAPr2 and double digestion with *Alu*I and *Taq*I) (Arnaud *et al.*, 2007).

For comparison of FD phytoplasma genetic variability, 11 grapevine DNA samples collected in 2004-2005 from Pordenone province, where an epidemic of FD was spreading at that time were also used. *Map* gene fragments amplified from 20 FD phytoplasma strains, representative of the

obtained RFLP profiles, the year of collection, the geographic origin and phytoplasma hosts, were selected for nucleotide sequence analysis.

Results

Results of real-time PCR/HRM analysis showed that 19/20 grapevine and 5/49 *S. titanus* DNA samples collected in the new epidemic area of Udine province during 2017-2018, formed a cluster with FD92 phytoplasma reference strain (Figure 1) confirming that they were infected by FD phytoplasma. Only one grapevine plant was infected by '*Ca.* P. solani' and among the wild plants only the symptomatic plant of *C. sanguinea* was infected by '*Ca.* P. fragariae'.
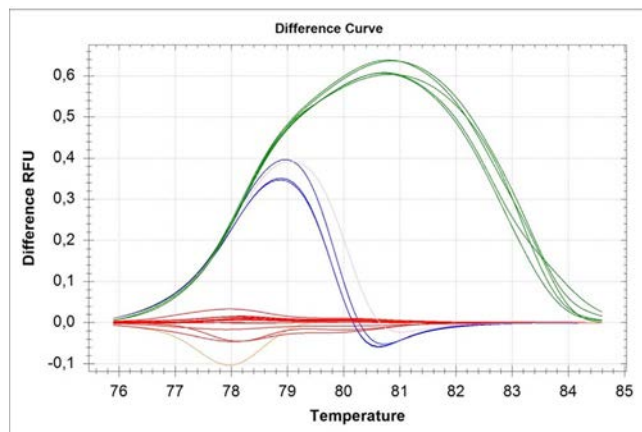
Figure 1. Difference curves from high resolution melting (HRM) analysis. In red: FD phytoplasma cluster. In orange: EY1 phytoplasma cluster. In blue: '*Candidatus* Phytoplasma solani' cluster. In pink: '*Ca.* P. fragariae' cluster. In green: non-phytoplasma cluster.
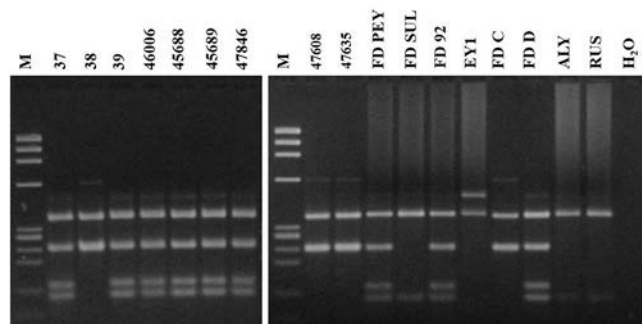
Figure 2. Analysis of *Alu*I-*Taq*I digestion on 8% polyacrylamide gels of FD9f6-MAPr2 PCR products obtained from different FD and reference strains maintained in periwinkle.

Molecular characterization on 16S rRNA and *map* genes of FD phytoplasmas showed very high correlation between the results of the two analyses, producing two restriction profiles: *map*-FD2 (16SrV-D) and *map*-FD3 (16SrV-C) (Figure 2). Among the FD phytoplasmas identified from grapevines collected in years 2016–2018 from all the region, the *map*-FD2 was prevalent (53 out of 72, 73.6%), while *map*-FD3 was identified in the remaining 26.4%. According to the province of origin,

map-FD2 type was predominantly present in Udine province with 39 out of 41 grapevines (95%); whereas in Pordenone province the two *map*-FD types were almost equally distributed: *map*-FD2 in the 45% of grapevine samples and *map*-FD3 in 55%. *Map*-FD2 was also detected in 5 individuals of *S. titanus* collected in Udine province. Among the FD strains used for comparison, *map*-FD2 was identified in 7 out of 11 DNA samples and *map*-FD3 in the remaining samples.

Sequence analysis on 20 selected FD phytoplasma strains confirmed the RFLP results. Moreover the *map*-FD2 phytoplasma strains shared 100% sequence identity among them and with strain V00-SP5 (a *map*-FD2 reference strain from France); whereas the *map*-FD3 phytoplasmas shared 100% sequence identity among them and with strain VI04-C28 (a *map*-FD3 reference strain from Veneto region, Italy).

Discussion

Real-time PCR and HRM analysis resulted in a valid method especially for the rapid and robust identification of the two most important phytoplasmas in grapevine. MLST analysis has been increasingly implemented in phytoplasma epidemiological studies (Arnaud *et al.*, 2007), and it can be used to trace the possible spread of FD phytoplasma strains. In this study this tool, still partially used, allowed to demonstrate that the *map*-FD2 type is the one prevalent in the new spreading area, and that the two *map*-FD types found in Friuli Venezia Giulia region in the last years are identical to the ones present more than 10 years ago. Further molecular analyses on highly variable genes such as *vmpA* are ongoing in order to verify this finding.

Based on the preliminary data obtained from the investigation on the wild plants, there are no elements that suggest their potential involvement as FD-phytoplasma source for the infected vineyards in the new epidemic area of Friuli Venezia Giulia.

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Quarantine

Monitoring of '*Candidatus* Phytoplasma solani' and "flavescence dorée" phytoplasma in south regions of the Russian Federation

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Abstract

The "flavescence dorée" (FD) phytoplasma is associated with a quarantine disease for Eurasian Economic Union and absent on all the territory of the Union. '*Candidatus* Phytoplasma solani' is present in some Union's countries and causes serious damages in the viticulture of the southern regions of Russia. Results of monitoring for both phytoplasmas are reported. The existing vineyards and insect vectors of phytoplasmas were studied and applying PCR technology the "bois noir" disease agent was detected in some grapevine varieties such as Cabernet, Chardonnay, Merlot and Pinot Noir. The disease insect vector was identified as the planthopper *Hyalesthes obsoletus*. The analyses for FD phytoplasma detection were negative, however specimens of its insect vector *Scaphoideus titanus* were detected, but the phytoplasma was not present in their bodies and salivary glands.

Keywords: "Bois noir" phytoplasma, "flavescence dorée" phytoplasma, monitoring, identification, distribution, insect vectors

Introduction

The south areas refer to the main regions of Russia for the cultivation of grapevines and the production of wine. One of the factors that adversely affect the efficiency of viticulture is the destabilization of the phytosanitary state of ampelocenoses because the grapevine destruction by complex of pathogens. Among the most important grapevine diseases are grouped the grapevine yellows (GY), which includes diverse phytoplasmas. The two most important and widespread phytoplasmas in viticultural regions are "flavescence dorée" (FD) and '*Candidatus* Phytoplasma solani' associated with "bois noir" (BN) disease (Dermastia *et al.*, 2017). The distribution of the grapevine yellows phytoplasmas in the world is related to the cultivation areas of vineyards and the habitat of the insect vectors.
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Materials and Methods

The studies we performed in accordance with the methodological approaches used in the domestic and international practice of viticulture and plant protection. The spread of phytoplasma of grapevines was determined by the number of symptomatic plants or part of plants, expressed as a percentage of the total number of examined trees (shoots, leaves, clusters) using the formula on Figure 1 (Borisenko *et al.*, 2015).

After visual monitoring to confirm the presence of typical symptoms and grapevine cultivar identification, PCR with primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by nested PCR with U5 / U3 (Lorenz *et al.*, 1995), and sequencing by Sanger method were used for phytoplasma detection and identification. Quantitative PCR protocol (Angelini *et al.*, 2007) and those described by the European protocol PM 7/079 (Pelletier *et al.*, 2009) were also used. For the DNA extraction an optimized CTAB-protocol (Matyashova, 2016) with a sucrose-rich grinding buffer and CTAB/chlorophorm/ phenol technology was employed.

Results and Discussion

The presence of "bois noir" was reported for the first time in Crimea in 2012. In the subsequent years, the number of identified vineyards affected by phytoplasmas increased. Symptoms of phytoplasma infection were identified visually, and then its presence in the plants was confirmed PCR analysis on Chardonnay, Aligote, Bastardo Magarach and Pinot grapevine varieties (Figures 2 and 3). Total infected area in 2018 included over 245.44 ha in the three viticultural areas of the Crimea.

$$P = \frac{n \cdot 100}{N}$$

Figure 1. P: distribution percentage (%); n: number of symptomatic plants; N: total number of examined plants.

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Figure 2. Yellowing of the leaves of yellow grapevine varieties (Chardonnay) infected by "bois noir" phytoplasma (G. Bondarenko 2018).

Figure 3. Reddish leaves of red grapevine varieties (Bastardo Magarach) infected by "bois noir" phytoplasma (N. Aleynikova 2017).

The values of the infection of grapevine varied within the following limits:
- in June, 0.5-18.6% affected plants, 1-2.2 symptomatic shoots per plant (low level of distribution with a low intensity of damage);
- in July, 2.3-52.6% affected plants, 1.2-3.9 symptomatic shoots per plant (weak and moderate level of spread with low and medium intensity of damage);
- in August, 4.8-66.8% affected plants, 1.8-7 symptomatic shoots per plant (weak and high level of distribution with low and high intensity of damage).

The dependence of the prevalence of the disease in the areas and the intensity of the symptoms in the plants from the viticulture zone and the variety was not observed. During the period of ripening of grapes and harvesting, the level of prevalence of phytoplasma symptoms on an area of 46.68 ha (19% of the total examined) was characterized as weak, for 106.18 ha (43.3%) - as moderate, for the remaining 92, 58 ha (37.7%) - as epiphytotic.

From 2012 to 2018 more than 600 samples of plant grapevine material were tested by molecular analyses in order to confirm the presence of 'Candidatus Phytoplasma solani' (BN), which resulted sparsely distributed in the Russian Federation, and to exclude the presence of the quarantine phytoplasma of the "flavescence dorée". According to the research data, the distribution of the phytoplasma infections was localized in the vineyards planted in the last 10-12 years by introducing planting material from various European countries, which became the primary source for this disease in Russia. The main insect vector of the "bois noir" in the vineyards of the European countries is *Hyalestes obsoletus* Signoret. For the Crimea, this type of insect is an aboriginal inhabitant of various plant communities, including vineyards. The vector of the quarantine pathogen "flavescence dorée" phytoplasma, the *Scaphoideus titanus* Ball also was detected. The infection presence was verified using PCR and qPCR and the results showed that the phytoplasma was not present in their bodies and salivary glands. Currently, specialists are conducting thorough testing of planting material on a number of quarantine and non-quarantine phytoplasmas in order to avoid dissemination of these phytoplasmas within the territory of Russia and of countries of the Eurasian Economic Union.

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Study of methods for detecting quarantine phytoplasma's from the apple proliferation group on the territory of Russia

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Abstract

To determine the phytosanitary state of the fruit orchards, samples were collected from plants and tested for the presence of phytoplasmas of the apple proliferation group. The samples of pear, quince and apple trees from different regions were analysed with visual inspection and molecular identification. On the collected samples, the symptoms characteristic of the diseases associated with the phytoplasmas of the apple proliferation group were observed. Only in one case positive results were obtained with the detection of the pear decline agent in southern Russia. Infected plants were uprooted and discarded to prevent the spread of the infection. A commercial quantitative PCR test was developed for the fast identification of 'Candidatus Phytoplasma mali' and 'Ca. P. pyri' in order to carry out the surveys.

Keywords: Apple proliferation, 'Candidatus Phytoplasma mali', 'Ca. P. pyri', 'Ca. P. prunorum', plant quarantine, detection, control

Introduction

According to the Ministry of Agriculture of Russia in 2017, perennial fruit and berry plantations in the country occupied 181,000 ha, of which 97,000 ha were fruit trees. In 2018, 15,200 ha of orchards were planted, including 11,000 ha of intensive type. In 2019 these volumes should be maintained and the plan is that further 15,000 ha will be planted in the autumn. Fruit crops are damaged by diseases such as apple proliferation (AP) and pear decline (PD) associated with the presence of 'Candidatus Phytoplasma mali' and 'Ca. P. pyri' respectively (Borisova and Kamenova, 2016). Both of these diseases in 2016 are included in the Quarantine List of Pests by the Eurasian Economic Union (www.vniikr.ru). However 'Ca. P. prunorum' is not included in the quarantine sheet for the territory of the Union, but is under control for the planting of imported materials of fruit crops. Prior to the inclusion of these two phytoplasmas in the quarantine list, monitoring and research by molecular diagnostic methods were not regulated by the state and were only of scientific interest.

Materials and Methods

In 2016-2018, plant samples with symptoms of phytoplasma infection were collected in the central and southern regions. For the sample preparation and the DNA extraction from plant material, a mechanical crushing using a grinding buffer containing a high concentration of sucrose was carried out.

Total DNA was extracted from the petioles and central veins of the leaves of the sampled plants. DNA extraction was performed using a method including 2-mercaptoethanol and CTAB, in an optimized modified system (Doyle and Doyle, 1990; Matyashova, 2016). DNA samples were analysed by quantitative (q)PCR with a reaction mixture including 5 µl of the 5 X Master-Mix ("Dialat", Russia); 0.75 µl primer each (Uni-F/R); 0.75 µl TaqMan probe (Uni-FAM); 14.75 µl H_2O; 3 µl of target DNA. The extracted DNA samples were also analysed by PCR with primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and U5/U3 (Lorenz et al., 1995), and sequenced by Sanger method. The reaction mixture for nested PCR included: 5 µl of the 5X Master-Mix ("Dialat", Russia); 1.5 µl of primers. The working concentration of the primers is 10 pmol/µl. The total volume of the mixture was 25 µl enclosing 14.5 µl H_2O; 2.5 µl of target DNA. The working concentration of all primers used was 10 pmol/µl. Sequence analysis was performed with BioEdit v.7.3.1.

Table 1. Sampling from non-symptomatic trees and from plants with proliferation, decline, chlorotic leaves symptoms in 2016-2018.

Fruit tree	2016	2017	2018	Total
	number of collected samples			
Pyrus communis	108	129	134	372
Malus domestica	95	114	146	355
Cydonia oblonga	22	47	61	130

Table 2. Results of testing the fruit trees in the Russian Federation with qPCR and sequencing.

Phytoplasma	orchards	old, selections and research places	positive control
'*Ca*. P. mali'	absent	absent	AP1 GenBank accession number MG748693 (Spain, 2017)
'*Ca*. P. pyri'	absent	present, one case GenBank accession number MG748692.1 (ID 4-E0003, Republic of Crimea, 2017)	"Bioreba", Switzerland
'*Ca*. P. prunorum'	absent	absent	ESFY-Ch GenBank accession number MG748694 (Czech Republic, 2017)
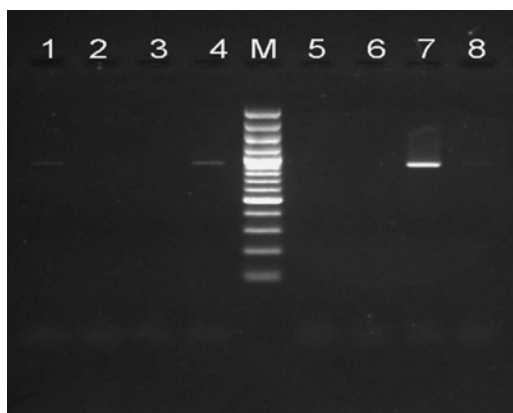
Figure 1. Electrophoresis gel picture of nested PCR amplification with primers U5/U3. Samples: 1, control AP1 (Spain); 4, E0003 (Republic of Crimea); 7, control PD ("Bioreba"); M, marker 100-3,000 bp ("Thermo Fischer Scientific", USA).

Results

During the study, 850 samples were tested (Table 1) of pears (Republic of Dagestan, Republic of Crimea, Tver and Tambov regions), apple (Moscow, Voronezh, Tula, Tver regions, Republic of Dagestan and Crimea) and quince trees (Republic of Dagestan and Crimea). Diagnostic results showed that all the commercial production orchards are free from infections of phytoplasmas of the apple proliferation group (Table 2). In 2017, a single phytoplasma infection of pear decline was detected in the Crimean old selection site of research, where scientists studied the European varieties of apple, plum, pear, peach and grapevine (Figure 1). After identifying the phytoplasma by PCR and sequencing, the specialists removed the infected tree. In 2018 the total retesting of every trees on this site showed that the whole site is free from phytoplasmas. In this thee year study sequences for 16S rDNA for strains of '*Ca*. P. mali' and '*Ca*. P. prunorum' were deposited in GenBank together with the one of the '*Ca*. P. pyri' strain from the Republic of Crimea. The chemicals from Russian companies used for PCR, as well as an optimized method for extracting DNA from phytoplasmas can be used in phytosanitary diagnostics. In 2018 a qPCR test was also developed and applied for a fast ready-to-use detection of the quarantine '*Ca*. P. mali' and '*Ca*. P. pyri'.

Discussion

Phytoplasmas of the apple proliferation group are widely distributed throughout the European Union, and also due to their high severity are included in A2 quarantine list of the EPPO (www.eppo.int/ACTIVITIES/plant_quarantine/A2_list). Previously there were no reports on the presence of phytoplasmas of the AP group on the territory of Russia. This research indicate that modern industrial orchards are free from specific '*Ca*. Phytoplasma' species infections. However with an increase in the import of propagation materials to the territory of Russia, the risk of the entry of dangerous pathogens increases. Thorough monitoring is necessary to regulate the phytosanitary condition of fruit plantations of pome crops. To identify '*Ca*. P. mali' and '*Ca*. P. pyri' in quarantine laboratories, experts use a complex of methods. When phytoplasmas and other pathogens are detected, the quarantine service takes measures to destroy the infected trees and to annual survey and analyse the total area.

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'*Candidatus* Phytoplasma phoenicium' and its phytosanitary status definition for the Russian Federation

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Abstract

In the recent years an increased attention has been paid to the development of the agriculture in the Russian Federation. Huge areas of orchards are being planned. The importation of planting material increases the risk of introducing quarantine and dangerous organisms. To determine the phytosanitary status of the new dangerous microorganism '*Candidatus* Phytoplasma phoenicium', its pest risk analysis was conducted for the territory of the Russian Federation.

Keywords: Almond witches' broom phytoplasma, pest risk analysis, quarantine, stone fruit

Introduction

In accordance with the State Program for the Development of Russian Agriculture 2013-2020 (approved by the Government of the Russian Federation in 2012), annual laying of horticulture crops and small-fruit crops at the level of 6,400 ha is provided. To accomplish this task, a program has been developed for the plantation of fruit and berry nurseries in the Russian Federation, according to which the annual production of seedlings of pome fruit crops must be at least 9 million, and stone fruit seedlings at least 3.7 million.

In 2016, about 2 millions of stone fruit seedlings were produced in the Russian Federation. The leaders in the production of seedlings are the Central, South and North Caucasus federal districts. The production capacity of Russian nurseries at the present time as a whole, provides for the needs of retail trade in seedlings for the population, the repair of existing orchards and the re-laying of old orchards with a service life that has ended. However, this capacity is not enough to implement large-scale regional programs for laying intensive fruit culture that are implemented in the Republic of Adygea, the Krasnodar Krai, the Chechen Republic, the Stavropol Krai and the Kabardino-Balkar Republic, which necessitates the import of large quantities of planting materials (Table 1).

Results and Discussion

The almond witches' broom phytoplasma – '*Candidatus* Phytoplasma phoenicium' belongs to the *Mollicutes* class (Acholeplasmataceae family) and is currently considered one of the most dangerous phytoplasma diseases of stone fruit crops (Abou-Jawdah *et al.*, 2002; Verdin *et al.*, 2003). Some experts suggest that '*Ca.* P. phoenicium' may be more harmful for the production of stone fruit than a lots of other phytoplasmas and viruses. Currently, official protocols about detection and identification of '*Ca.* P. phoenicium' were applied only from Lebanon and Iran. However, there is a high uncertainty of information about the pathogen distribution in a number of other countries.

Table 1. Import of fruit tree seedlings to the Russian Federation in 2014-2017.

No	Importing country	2014-2017	
		number	%
1	Italy	18,623,703	40.2
2	Poland	7,027,091	15.2
3	Serbia	5,932,222	12.8
4	Belarus	4,358,525	9.4
5	Netherlands	1,693,036	3.7
6	Latvia	1,643,202	3.5
7	Moldova	1,448,736	3.1
8	Germany	1,407,939	3.0
9	Austria	1,237,410	2.7
10	Hungary	714,511	1.5
11	Uzbekistan	654,281	1.4
12	Ukraine	606,423	1.3
13	France	469,642	1.0
14	Azerbaijan	227,640	0.5
15	Greece	201,440	0.4
16	Turkey	108,550	0.2
17	Other	212,478	0.5
	TOTAL	46,322,011	100

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Damage caused by the almond witches' broom phytoplasma is expressed in the reduction and complete cessation of fruiting of stone fruits and the subsequent death of trees, as well as in the work on the elimination of fruit plantations during the epiphytotics (Abou-Jawdah *et al.*, 2002).

'*Ca*. P. phoenicium' can enter in the territory of the Russian Federation by import of planting material and stone fruit seedlings. At present the Russian Federation does not import stone fruit seedlings from countries where the spread of the pathogen has been officially confirmed.

'*Ca*. P. phoenicium' was first identified in nectarine trees in 1999, but until 2008, the phytoplasma detection in plants other than almonds was not reported. Almond witches' broom phytoplasma was included in the EPPO Alert List in 2015. In 2017 the phytoplasma was transferred to the EPPO A1 List. In 2018, the specialists of scientific department of the All-Russian Plant Quarantine Center carried out a pest risk analysis (PRA) to determine the phytosanitary status of '*Ca*. P. phoenicium' for the Russian Federation. The main plants that host this pathogen are stone fruit: all varieties of almond (*Prunus dulcis*), peach (*Prunus persica*), nectarine (*Prunus persica* var. Nucipersica), apricot (*Prunus armeniaca*).

The cicadas *Asymmetrasca decedens* that are the main vectors of the pathogen (Abou-Jawdah *et al.*, 2014; Tedeschi *et al.*, 2015) are fairly widespread, including some countries of the EPPO region. In some articles, it was stated that *A. decedens* is distributed in a number of countries, including near-neighbor to Russia. '*Ca*. P. phoenicium' in Lebanon is present in a wide range of altitudes, from 0 to 1,200 m asl, where various climatic conditions are encountered. Climatic conditions have more influence on insect vectors than on phytoplasmas.

In case of introduction into the territory of Russia, acclimatization of '*Ca*. P. phoenicium' is possible in all areas of presence of host plants, which are grown for commercial purposes mainly in the southern part of the Russian Federation, but as ornamental or garden plants, they are also present in the central part of the country (Karimova *et al.*, 2019). It should be noted that the Russian Federation is among the twenty largest apricot producers worldwide. Almond belongs to the light-loving plants that can tolerate strong heat, drought and low temperatures (up to -30°C). To obtain a crop in the territory of the Russian Federation, almonds are grown in the Crimea and in the North Caucasus, the range of cultivation for decorative purposes is much wider. Due to insufficient winter hardiness, peach and nectarine are cultivated in the Russian Federation mainly in the southern regions only.

Based on the PRA carried out, it was proposed to include '*Ca*. P. phoenicium' in the list of the quarantine pests for the Russian Federation. Before including almond witches' broom phytoplasma in this list it is planned to prepare protocols for laboratory diagnostics of the pathogen (Molino Lova *et al.*, 2011; Quaglino *et al.*, 2015). For testing the methods of detection and identification, experts of the Scientific and Experimental Center of All-Russian Plant Quarantine Center take part in the EUPHRESCO 2017-F-234 project (Set up of reliable detection protocols for the specific identification of '*Candidatus* Phytoplasma phoenicium').

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'Candidatus Phytoplasma phoenicium' associated with apricot yellows and peach witches' broom in Iran

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Abstract

During field surveys carried out from 2012 to 2017 in seven provinces of Iran, phytoplasma-like symptoms were observed in apricot (yellows) and peach (witches' broom). The aim of this work was to identify and characterize the agent(s) associated with such diseases by biological assays and molecular analyses. Healthy bitter almond and apricot or peach seedlings, grafted with shoots of symptomatic trees, exhibited phytoplasma symptoms. Nucleotide sequence analyses of the 16S rRNA gene allowed the classification of phytoplasmas associated with apricot yellows and peach witches' broom within the 'Candidatus Phytoplasma phoenicium' species in the subgroup 16SrIX-B and variants. These and previously reported Iranian phytoplasma strains belong to SNP genetic lineages distinct from those found in Lebanon.

Keywords: 16SrIX-B, almond witches' broom, SNP genetic lineages

Introduction

Stone fruit trees can be infected by phytoplasmas belonging to at least eight ribosomal groups. Phytoplasmas in the 16SrIX group are associated with numerous diseases affecting crops and wild plants in different geographic areas worldwide. Almond witches' broom (AlmWB), associated with the presence of 'Candidatus Phytoplasma phoenicium' (subgroup 16SrIX-B and its variants) is an economically important disease in Lebanon and Iran (Abou-Jawdah *et al.*, 2002; Salehi *et al.*, 2006). Previous studies demonstrated the capability of the leafhopper *Asymmetrasca decedens* to transmit '*Ca.* P. phoenicium' in Lebanon (Abou-Jawdah *et al.*, 2014). On the other hand, in Iran, despite the presence of high populations and rearing on almond trees, the leafhopper *A. decedens* was not able to transmit the phytoplasma. Peach, nectarine, GF-677, wild almond, *Anthemis* spp., and *Smilax aspera* are other plant hosts of '*Ca.* P. phoenicium' in these countries (Abou-Jawdah *et al.*, 2009; Salehi *et al.*, 2006, 2015).

In the present study, during field surveys carried out from 2012 to 2017 in seven provinces of Iran, apricot yellows and peach witches' broom were reported and found associated with the presence of '*Ca.* P phoenicium' through biological assays and molecular analyses.

Materials and Methods

During field surveys carried out in orchards in Kerman, Lorestan, Razavi Khorasan, Isfahan, Kurdistan, West Azerbaijan, and Fars provinces of Iran, phytoplasma-like symptoms were observed in apricot trees (yellowing) in the Fars province and in peach trees (witches' broom) in all the seven provinces (Figure 1). Phytoplasma-free seedlings of bitter almond, apricot and peach were side grafted with apricot or peach symptomatic or symptomless shoots. Total nucleic acids, extracted from fresh leaf midrib tissues of field-collected plants and seedlings employed in grafting trials, were used as templates in nested PCR reactions for the amplification of the R16F2n/R2 16S rDNA fragment (Gundersen and Lee, 1996). Obtained PCR products, sequenced by a commercial service (BioNeer, South Korea), were assembled, aligned, and analyzed by the software BioEdit. Attribution to 16Sr group/subgroup was carried out using the *i*PhyClassifier (Zhao *et al.*, 2009). 'Candidatus species' attribution was conducted by comparing the 16S rDNA nucleotide sequences of apricot yellows (AprY) and peach witches' broom (PWiB) phytoplasma strains with '*Ca.* Phytoplasma' species reference strains. The 16S rDNA sequence alignment was used to detect single nucleotide

polymorphisms (SNPs) within group 16SrIX and to conduct Minimum-Evolution phylogenetic analysis.

Figure 1. Symptoms of apricot yellows (left) and peach witches' broom (right).

Results

The agents of AprY and PWiB were graft transmitted respectively from affected apricot and peach trees to seedlings of bitter almond, apricot and peach. The R16F2n/R2 16S rDNA fragment was amplified from all the symptomatic field-collected apricot and peach trees and the seedlings employed in grafting trials. The *i*PhyClassifier analyses showed that the virtual RFLP patterns derived from phytoplasma strains infecting apricot and peach trees were identical (similarity coefficient 1.00) to the pattern of subgroup 16SrIX-B and its variant. AprY and PWiB phytoplasma strains share > 99.5% sequence identity with the '*Ca*. P. phoenicium' reference strain A4, and harbour its species-specific signature sequence. Phylogenetic analysis positioned AprY and PWiB phytoplasma strains with high confidence values in a cluster including 16SrIX phytoplasmas in which the AprY and PWiB strains belong to a distinct subcluster including the '*Ca*. P. phoenicium' strain A4. Thus, AprY and PWIB phytoplasmas belong to '*Ca*. P. phoenicium', which strain members are clearly distinct from phytoplasma strains of other 16SrIX subgroups. This was also confirmed by calculation of average sequence identity of 16SrIX subgroup strains and the reference strain A4. Alignment of 16S rDNA nucleotide sequences of '*Ca*. P. phoenicium' strains (subgroup 16SrIX-B and variants), available in GenBank and identified in the present study, allowed the identification of 21 SNPs in comparison with the sequence of the strain A4. In detail, 13 SNPs were present in the '*Ca*. Phytoplasma' strains identified in Iran, and eight were present in the strains from Lebanon. The combination of such SNPs, mutually exclusive in the phytoplasma strain populations identified in the two countries, allowed the recognition of nine SNP lineages in Lebanon and eight in Iran.

Discussion

Overall results of field surveys, transmission trials and molecular analyses proved that AprY and PWiB diseases in Iran are associated with '*Ca*. P. phoenicium' associated with almond witches' broom. The 16S rDNA sequence analyses highlighted that AprY and PWiB phytoplasmas are distinguished by SNPs found exclusively within Iranian strains and belong to five SNP genetic lineages. The report of specific '*Ca*. P. phoenicium' genetic lineages associated with AprY and PWiB in Iran suggests the possible phytoplasma adaptation to other fruit trees species, as reported for peach and nectarine in Lebanon (Abou-Jawdah *et al.*, 2009). It possible to hypothesize that *Prunus scoparia*, a wild almond species harboring '*Ca*. P. phoenicium' and scattered in the Iranian provinces examined in the present study, play a role in the phytoplasma transmission. In fact, previous study demonstrated that '*Ca*. P. phoenicium' naturally infecting wild almond can be transmitted to peach through grafting (Salehi *et al.*, 2015). Furthermore, based on detection of '*Ca*. P. phoenicium' in insect body and saliva and the presence of consistent populations, the leafhopper *Frutioidea bisignata* can be considered as potential vector of this phytoplasma in Iran. Further investigations are needed in Iran to (i) determine the epidemiological pathways of the phytoplasma associated with AprY and PWiB, (ii) survey the diseases in other provinces, (iii) investigate the susceptibility of apricot and peach varieties.

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Genetic diversity of 'Candidatus Phytoplasma phoenicium' strain populations associated with almond witches' broom in Lebanon and Iran

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Abstract

'Candidatus Phytoplasma phoenicium' (subgroup 16SrIX-B) strains associated with almond witches' broom disease in Lebanon and Iran were typed by PCR-based amplification and sequence analyses of rplV-rpsC and secY genes. Results showed that Iranian and Lebanese AlmWB-associated phytoplasma strain populations constitute at least two distinct genetic lineages, supporting previous evidence obtained through 16S rDNA analysis. Such genetic diversity reflects differences in biological features (i.e. insect vectors and plant hosts) of 'Ca. P. phoenicium' in Lebanon and Iran. Molecular markers identified within rplV-rpsC and secY genes should be employed to improve the knowledge about 'Ca. P. phoenicium' ecology and almond witches' broom epidemiology in the Middle East.

Keywords: 16SrIX-B, ribosomal proteins, secY gene, peach, apricot

Introduction

In the Middle East, 'Candidatus Phytoplasma phoenicium' (subgroup 16SrIX-B) is associated with almond witches' broom (AlmWB), a lethal devastating disease of almond, peach and nectarine trees in Lebanon and Iran (Abou-Jawdah et al., 2002, 2009; Salehi et al., 2006). All almond varieties were affected with this severely damaging disease. Some varieties (i.e. Alwani and Awja in Lebanon and Sangi in Iran) were highly susceptible and developed severe witches' brooms, leading to a rapid death of the trees, while other varieties (i.e. Kachabi) were less affected. Unintentional planting of infected material is supposed to be one of the factors responsible for the AlmWB agent spread. There is a very little information about insect vectors responsible for the local spread of AlmWB phytoplasma; only Asymmetrasca decedens and two Tachycixius species are the identified vectors responsible for spread to almond, peach and nectarines in Lebanon, while there is no insect vector identified in Iran (Abou-Jawdah et al., 2014; Tedeschi et al., 2015). Grafting experiments and further molecular studies revealed that 'Ca. P. phoenicium' does not affect cherry and plum (Abou-Jawdah et al., 2003), while it can infect apricot trees (Salehi et al., 2018) (Table 1). In the present study 'Ca. P. phoenicium' strain populations from Lebanon and Iran were typed by sequence analysis of rplV-rpsC and secY genes.

Table 1. Host range of 'Ca. P. phoenicium' in Lebanon and Iran.

Host	Species	Common name	Country
Plant	Prunus amygdalus	almond	Iran, Lebanon
	Prunus armeniaca	apricot	Iran
	Prunus persica	peach	Iran, Lebanon
	Prunus orientalis	wild almond	Lebanon
	Prunus scoparia	wild almond	Iran
	GF-677	almond x peach	Iran
	Anthemis spp.	chamomilla	Lebanon
	Smilax aspera	rough bindweed	Lebanon
Insect	Asymmetrasca decedens		Lebanon
	Tachycixius spp.		Lebanon

Materials and Methods

'Ca. P. phoenicium' strains, previously identified in AlmWB-diseased trees in Lebanon (Quaglino et al., 2015) and Iran (Kumar et al., 2018) and attributed to subgroup 16SrIX-B,

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were selected as representatives of the strain populations present within the two countries for finer molecular typing. *RplV-rpsC* and *secY* genes were amplified by nested PCRs as described by Lee and colleagues (2012). Obtained PCR products, sequenced by a commercial service (Eurofins Genomics, Germany), were assembled, aligned, and analyzed by the software BioEdit. Nucleotide sequence alignment was used for *in silico* RFLP analysis, detection of single nucleotide polymorphisms (SNPs) within group 16SrIX, and Minimum-Evolution phylogenetic analysis using the software MEGA 7.

Results

Nested PCR-based amplifications of *rplV-rpsC* and *secY* genes, carried out utilizing 16SrIX group-specific primer pairs, confirmed the presence of 16SrIX phytoplasmas in the selected samples. *In silico* RFLP analysis showed that Lebanese and Iranian '*Ca*. P. phoenicium' strains share identical patterns, undistinguishable from those of the previously described subgroups rp(IX)-B1 and secY(IX)-B1 (Lee *et al.*, 2012). Analysis of the sequence alignments identified SNPs at positions that were able to define distinct genetic lineages including strains from Iran and Lebanon. In the case of *rplV-rpsC* genes, based on the presence of 7 SNPs consistently detected, it was possible to identify two rp(IX)-B1 lineages, one ("a") including Lebanese strains and one ("b") Iranian strains. In the case of *secY* gene, based on the presence of 18 consistent SNPs, it was possible to identify two secY(IX)-B1 lineages, one ("a") including Lebanese strains and one ("b") Iranian strains. Clustering within the phylogenetic trees (Figure 1) reinforced this evidence highlighting the presence of genetically distinct lineages among '*Ca*. P. phoenicium' strain populations in Lebanon and Iran.
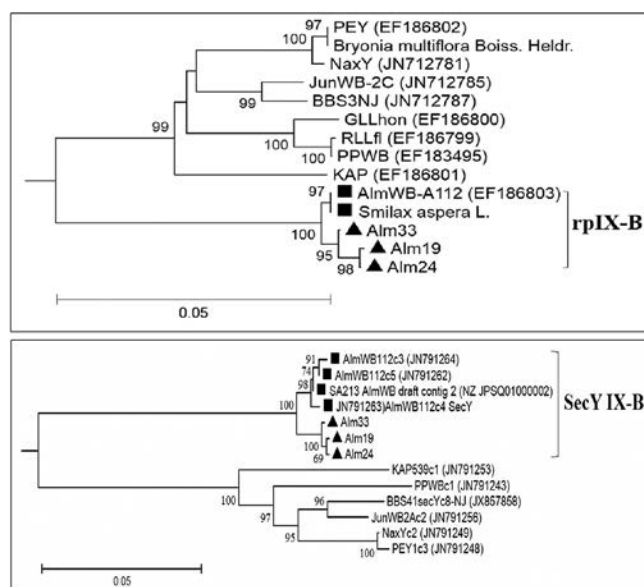
Figure 1. Minimum evolution phylogenetic trees based on nucleotide sequence alignments of *rplV-rpsC* (top) and *secY* (bottom) genes. Phytoplasma strains from Lebanon and Iran are marked by black squares and triangles, respectively.

Discussion

Recent studies reported that, based on 16S rDNA sequence analysis, '*Ca*. P. phoenicium' strains from Lebanon and Iran constitute distinct genetic lineages (Salehi *et al.*, 2018). In order to improve the resolution power of 16S rRNA gene to distinguish closely related phytoplasma strains within the group 16SrIX, less conserved genes have been employed (Lee *et al.*, 2012; Quaglino *et al.*, 2015). Results obtained in the present study, based on sequence analyses of *rplV-rpsC* and *secY* genes, showed that Iranian and Lebanese *Ca*. P. phoenicium' strains constitute at least two distinct genetic lineages. Such genetic diversity can reflect the phytoplasma adaptation to different ecological niches, leading to a distinct host range in the two Countries. Molecular markers identified within *rplV-rpsC* and *secY* genes should be employed to improve the knowledge about '*Ca*. P. phoenicium' ecology and almond witches' broom epidemiology in the Middle East.

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Identification of 16SrIX-B phytoplasmas associated with apricot rosette in Iran

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Abstract

During a survey, samples of apricot (*Prunus armeniaca*) with rosette symptoms were collected from Sistan O Bluchestan province of Iran. DNA was extracted from samples using a CTAB method and nested PCR analysis were performed for amplifying phytoplasma 16S rDNA. The nested PCR products were sequenced and compared with 16S rRNA of available phytoplasma sequences. The 16S rDNA sequences of apricot rosette phytoplasma were deposited in GenBank and their analyses showed that these strains share 99% identity with '*Candidatus* Phytoplasma phoenicium' strains. *In silico* RFLP and phylogenetic analyses of apricot rosette phytoplasma 16S rRNA gene sequence demonstrated that these phytoplasma strains are related to the 16SrIX-B phytoplasma subgroup.

Keywords: apricot rosette phytoplasma, 16SrIX-B, Iran

Introduction

Phytoplasmas are obligate pathogens of agriculturally important plants and live in the plant phloem tissue. They are associated with various symptoms including witches' broom, virescence, yellowing and phyllody in many plant species (Bertaccini and Duduk, 2009). Eight phytoplasmas classified in different 16S rRNA subgroups have been reported in plants of the genus *Prunus* (Marcone *et al.*, 2014; Salehi *et al.*, 2018). During a survey in 2016 and 2017 summers in apricot gardens at Sistan O Bluchestan province (Iran), apricot trees exhibiting symptoms of production of numerous axillary buds (Figure 1), formation of dense leaf rosettes, and shortened internodes were observed. These symptoms are similar to those reported for the peach rosette phytoplasma disease, but were never observed in apricot (*Prunus armeniaca*). The agents associated with the peach rosette are two phytoplasmas assigned to the 16SrIII (X-disease) and 16SrI (aster yellows) phytoplasma groups (Marcone *et al.*, 2014). The aim of this study was to detect and identify the possible phytoplasmas present in these symptomatic apricot trees.

Materials and Methods

Fifteen symptomatic apricot trees showing apricot rosette (AR) symptoms (Figure 1) were sampled in a commercial orchard in the Khash (Sistan O Bluchestan, Iran). The total

DNA was extracted from leaf tissue as described previously (Doyle and Doyle, 1990) and used for nested PCR analysis. The almond witches' broom phytoplasma strain was used as positive control (Ghayeb Zamharir, 2011) and DNA from two asymptomatic apricots were used as negative control. Nested PCR analysis were performed using the phytoplasma universal primer sets P1/Tint (Deng and Hiruki, 1991; Smart *et al.*, 1996) in direct PCR and R16F2n/R16R2, R16mF1/mR1 (Gundersen and Lee, 1996) or 16R758f /16R1232r (=M1/M2) primers (Gibb *et al.* 1995) in nested PCR. The PCR conditions were as follows: for direct PCR, the DNA was amplified by 35 cycles consisting of denaturation at 94°C for 60 seconds (5 minutes for cycle 1), annealing at 53°C for 2 minutes, and primer extension at 72°C for 3 minutes (5 minute for cycle 35). For nested amplification, the thermal conditions were the same except the annealing was at 55°C for 2 minutes. The amplifications were carried out in a programmable thermocycler (Bio-Rad, USA); the PCR products were electrophoresed in 1.2% agarose gels in a TAE buffer and visualized with a UV transilluminator following ethidium bromide staining. The molecular weight of the PCR products was estimated by comparison with a 1 kb DNA ladder (Fermentas, Vilnius, Lithuania). Selected R16mF1/mR1 (1,500 bp) and R16F2n/R2 (1,248 bp) amplified fragments from phytoplasma detected in apricot (APZ1, APZ4, APZ5, APZ7, APZ9) were sequenced directly on both strands by Macrogen Co. (Korea). Sequence alignments were performed using

ClustalW2. The sequences have been analyzed using the close-neighbour-interchange algorithm, and compared with nucleotide sequences in the GenBank database, using BLAST (version BLASTN 2.2.18). A phylogenetic tree was constructed using 16S rDNA sequence from AR samples APZ1, APZ4, APZ5, APZ7, APZ9 and sequences from 29 'Candidatus Phytoplasma' strains with Acholeplasma laidlawii as outgroup. The analysis was replicated 100 times. Bootstrap analysis was performed to estimate stability and support for the inferred clades.

Figure 1. Apricot rosette symptoms (left and center) and bud swelling (right).
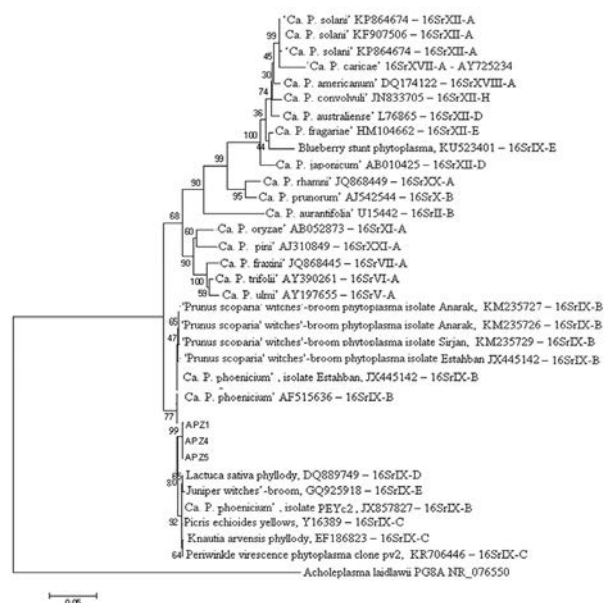
Figure 2. Evolutionary analyses conducted in MEGA6 using the maximum parsimony method on the apricot rosette phytoplasma sequences identified in this work.

Results and Discussion

Amplicons of the expected size were produced only from all symptomatic samples using both R16F2n/R16R2 and M1/M2 primer sets. Sequences from five samples were deposited in GenBank with the accession numbers MG748605, MG748606, MG748607, MG748608 and MG748609. These sequences showed 100% nucleotide identity to each other and shared 99% nucleotide similarity with 'Candidatus Phytoplasma phoenicium' strain PEYc2 and strain NaxYc3 (GenBank accession numbers JX857827 and JN791266, respectively) belonging to 16SrIX-B group (Lee et al., 2012). The obtained aligned 16S rDNA sequences from AR phytoplasmas and 29 other phytoplasma strains show the clustering of the AR with strains in the 16SrIX group as a separate branch (Figure 2). Results of this study indicated that a 'Ca. P. phoenicium' (16SrIX-B) related strain is associated with apricot rosette in Iran. The symptoms identified in Khash were limited to a commercial orchard and were different from those reported recently in apricot yellows in Iran (Salehi et al., 2018). Spread of 16SrIX-B and 16SrIX-C phytoplasmas have been reported in almond (Ghayeb Zamharir, 2011) and peach (Ghayeb Zamharir, 2014), so, it is probable that apricot infection originated from other hosts (peach, grapevine and some vegetables) by possible transmission by insect vectors. The study of potential vectors of phytoplasmas in stone fruits in Iran is critical for preventing the spread of these phytoplasmas to other important stone fruit trees.

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Phytoplasma survey in *Ulmus* species in Belgium

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Abstract

About 600 elm trees were sampled during a dedicated survey in 2017-2018 all over Belgium. The sampled leaves and roots were analysed for phytoplasma presence, using universal primers. In one tree '*Candidatus* Phytoplasma ulmi', EU quarantine regulated pathogen, was detected and in seven trees '*Candidatus* Phytoplasma fragariae' presence was identified in different locations: *Ulmus* is a new host plant for this latter phytoplasma.

Keywords: survey, diagnostics, *Ulmus* sp., elm yellows phytoplasma

Introduction

A survey was conducted in Belgium during the years 2017-2018 to define the status of '*Candidatus* Phytoplasma ulmi'. This quarantine organism is currently being examined at European level to determine whether it should remain or not as Q organism. The wide presence of '*Ca*. P. ulmi' in the EU has been confirmed by various reports in Germany (Mäurer *et al.*, 1993), Italy (Lee *et al.*, 1993), France (Boudon-Padieu *et al.*, 2004), Serbia (Jovíc *et al.*, 2008), the Czech Republic (Navrátil *et al.*, 2009) and the United Kingdom (NPPO UK, 2014). In Italy the presence of this phytoplasma was also detected in some elm bonsai species (Murolo and Romanazzi, 2008). The most important (most susceptible) host plants are *Ulmus americana*, *U. alata*, *U. serotina* and *U. rubra*. Although there was a sharp decline in the number of elms in the last century, as a result of the Dutch elm disease outbreaks (*Ophiostoma novo-ulmi*), elm trees have never completely disappeared from the European landscape. There are still many individual trees, including some notable specimens, present in a large part of the Belgian territory as well.

Materials and Methods

During the survey, the sampling strategy was made of the *Ulmus* species present and their distribution in Belgium, based on available inventory sources. In addition to the geographical spread over the territory, different cultivars were sampled, such as almost 30 *Ulmus* species, *Ulmus* x *hollandica* hybrids and some *Zelkova* species (Table 1). A balanced sampling was also carried out in the different fields of implantation; e.g., in forests, along rivers, in lane and park trees, trees of scenic value, botanical gardens and arboreta. A visual inspection of the sampled trees was carried out on site. All observations were extensively documented in an excel table and all sampled trees were also photographed.

In the laboratory, total genomic DNA was extracted from leaves and roots by a CTAB protocol (Doyle and Doyle, 1990). The 16S rRNA gene was partially amplified using the phytoplasma universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by nested PCR with primer pairs R16F2n/R2 (Lee *et al.*, 1995; Gundersen and Lee, 1996) or a direct PCR with the universal primers Fu5L/R16R2. The phytoplasma identity in the positive samples was confirmed by sequencing.

Table 1. Overview of the sampled *Ulmus* species during the survey (580 samples in total).

Species	Number of samples
Ulmus glabra	71
Ulmus laevis	41
Ulmus minor	50
Ulmus procera	15
Ulmus x hollandica	51
Ulmus sp.	288
other *Ulmus* (*americana, chenmoui*, ...)	33
Celtis aetnensis	1
Zelkova sp.	2
other *Zelkova* (*abelicea, serrata*, ...)	28

Results

The status determination for '*Ca*. P. ulmi' revealed one positive tree, located in Meise, in a botanical garden (De Jonghe *et al*.,

2019). It concerns a young 7-year old *Ulmus minor* subsp. *canescens*. Very intensive monitoring was carried out in the botanical garden (all elms and related species) and in the surrounding streets. No other tree tested positive. An insect vector monitoring was also carried by means of yellow sticky plates. Among the captured Auchenorrhyncha, *Philaenus spumarius* and *Iassus scutellaris* were found on the glue plates, potentially playing a limited role in the spread of the phytoplasma. In addition, other potential vectors belonging to other genera were found. The survey identified the presence in elm of another phytoplasma, '*Ca.* P. fragariae', which was found in 7 locations in elm trees in Belgium. A survey in neighbouring trees also yielded a maple tree (*Acer* sp.) that tested positive for the same '*Ca.* P. fragariae'.

Discussion

The details of the '*Ca.* P. ulmi' observation (Flemish Brabant, in the botanical garden of Meise) were reported to the FASFC, intensively followed up, made known within Europe via the EPPO reporting service (07/2018, art. number 2018/144). The determination was made on an *U. minor* subsp. *canescens*, of which the seed originated from Italy (Messina region, Sicily).

The positive '*Ca.* P. fragariae' (Valiunas *et al.*, 2006) results were obtained on samples from different elms, both on young and old trees from different regions and without symptoms. That is why these are considered as independent reports. Until now, both *Ulmus* sp. and *Acer* sp. were not known to be host plants for this phytoplasma.

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Towards the identification of genes involved in resistence/tolerance to "flavescence dorée"

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Abstract

A list of genes possibly involved in response to "flavescence dorée" (FD) was obtained by the comparison of RNAseq data from grapevine and *Arabidopsis thaliana* infected with FD. From one to four weeks post-inoculation, the relative expression of twenty genes was studied comparing healthy and infected plants. A reverse genetic approach using knockout *A. thaliana* mutants was adopted to validate the gene expression results.

Keywords: phytoplasma, RNAseq, *Vitis vinifera*, *Arabidopsis thaliana*, reverse genetics

Introduction

"Flavescence dorée" (FD) is the main phytopathological problem of southern European viticulture and the associated phytoplasma is included in the European list of quarantine organisms (Jeger *et al.*, 2016). Genes involved in resistance to FD have not been identified yet, and all the cultivars are susceptible to the disease although at different degrees (Eveillard *et al.*, 2016). The management of FD is based exclusively on preventive control strategies, such as mandatory insecticide treatments and replacement of infected plants with healthy plant material. A deeper knowledge on mechanisms of plant defence in response to phytoplasma infection, would help the identification of new sustainable strategies for FD control. Working on a model system, *Arabidopsis thaliana*/FD phytoplasma/*Euscelidius variegatus*, attempt to identify the host metabolic pathways altered by the phytoplasma presence are presented. The integration and comparison of data obtained by RNAseq of both *Vitis vinifera* and *A. thaliana*, naturally and experimentally FD-infected respectively, gave a valuable genetic platform to identify the main mechanisms involved in the plant response to FD presence. The set of genes identified by NGS analysis were biologically validated on FD-infected *A. thaliana* plants and their role further investigated by the use of mutants in a reverse genetics approach.

Materials and Methods

The transcriptome of healthy and FD-infected *V. vinifera* and *A. thaliana* plants was obtained by Illumina sequencing.

Bioinformatics pipelines were developed for the comparison of sequencing data, and the identification of genes possibly involved in the plant response to FD.

Euscelidius variegatus vector was used for experimental inoculation of FD phytoplasma in controlled conditions (Rashidi *et al.*, 2014). Plants of *A. thaliana* Col-0 were singly exposed to four infective insects for a 5 days inoculation period and to four healthy insects as control. For gene expression profile studies, six infected and control plants, were sampled at 1, 2, 3 and four weeks after inoculation.

Expression analyses were performed on quantitative RT-PCR (qRT-PCR) (CFX connect, Biorad) with SYBR Green. The relative expression of each target gene was normalized to the expression of two *V. vinifera* reference genes, actin and ubiquitin, known to be stably expressed during FD phytoplasma infection (Margaria *et al.*, 2014). The gene studies were supported by CFX Manager Software, version 3.0. To assess the statistical significance of the differences between healthy and infected plants, a univariate (two-way ANOVA) method was used. Selected mutants were maintained in a growth chamber at 22°C to 24°C with a photoperiod representing a short day (light, 8 h; dark, 16 h).

Results

The transcriptomes of natural (grapevine) and experimental host (*A. thaliana*) (Figure 1), of FD phytoplasma were obtained by NGS. A bioinformatic pipeline for healthy and infected plant comparison provided a list of differential expressed genes in response to the phytoplasma infection. Twenty genes, mainly belonging to the GO categories of "plant defence",

"hormone metabolism" and "cell development", were selected and their transcription was followed overtime in *A. thaliana* plants. An early activation of plant response to FD infection was found, with modified gene expression detectable one week after the inoculation. The *in vivo* validation, allowed further restriction of the set of genes putatively involved in the plant defence against the phytoplasma. Eight *A. thaliana* mutants were therefore selected to determine the functional role of the identified genes by analyzing mutant phenotypes in a reverse genetic approach (Figure 2).

Figure 1. Symptoms induced by FD phytoplasma in *A. thaliana*, Col-0. Right, healthy plant; left, infected plant.
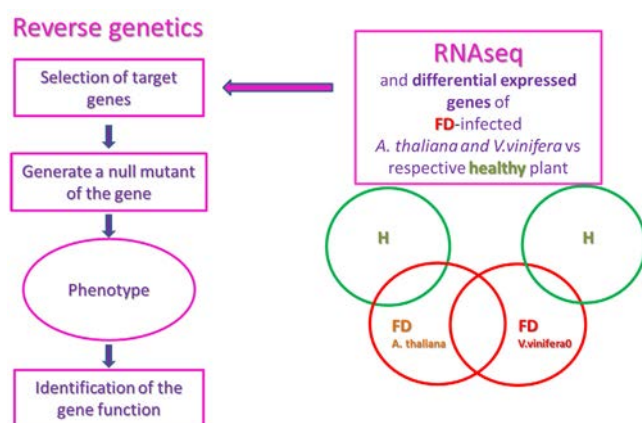
Figure 2. Flow chart of the work described.

Discussion

The use of *A. thaliana* mutants has been fundamental for the functional characterization of phytoplasma effectors (Sugio *et al.*, 2011; Minato *et al.*, 2014). In the same way, *A. thaliana* mutants were analysed to validate the functional characterization of possible resistance/tolerance plant

genes to the phytoplasma infection. The selected mutants, defective for genes putatively involved in the response to FD, need to be further analysed, quantifying both the plant response to the infection and the multiplication rate of the pathogen (Marzachì *et al.*, 2005) in comparison with wild type plants. Identifying genes involved in the susceptibility to infection in a model system represents a preliminary step for the characterization of genes potentially involved in the resistance to FD in grapevine.

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Napier grass stunt disease: effector gene prediction

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Abstract

Napier grass stunt disease (NGSD) is associated with the presence of a 'Candidatus Phytoplasma oryzae'-related strain belonging to the 16SrXI group and can also be found in other grasses such as rice, sugarcane and Bermudagrass. After the infection to plant hosts, phytoplasmas are restricted to the phloem for colonization. However, they secrete effector proteins that change plant morphology via Sec-dependent pathways that requires signal peptide (SP) sequences in the effector genes. In this experiment, twenty contigs of NGSD were analysed for the presence of effector proteins containing SP sequences by the PrediSi program and the result found fourteen candidate proteins. Primers were designed for amplification of these potential effector genes from NGSD and sugarcane white leaf (SCWL) infected samples and the result showed 7 and 2 PCR products from NGSD and SCWL, respectively. These PCR products are now being ligated into plant plasmid vectors and transformed into *Arabidopsis thaliana* plants for symptom observation.

Keywords: phytoplasma, effectors, signal protein, sugarcane white leaf

Introduction

Napier grass (*Pennisetum purpureum* Schumach) is a major grass used for feeding livestock. However, there are some diseases that reduce yield and change the plants morphology, such as head smut caused by a fungus, that makes the infected plants thinner with shorter stems, small leaves and smutted heads, although this disease can be controlled by growing resistant plants. A second serious disease is the napier grass stunt (NGSD), in which the plant is infected with a phytoplasma strain related to the '*Candidatus* Phytoplasma oryzae'. NGSD plants have small yellow leaves, short internodes, large numbers of tillers and the plants become stunted and die prematurely (Asudi *et al.*, 2015; Kawube *et al.*, 2015). Phytoplasmas are pathogenic bacteria that lack cell walls, have small genomes and lack some significant metabolic genes. They have dual life habits because they can live in two hosts; insects and plants (Bertaccini and Duduk, 2009). Phytoplasmas can affect crops worldwide and infected plants show various symptoms such as dwarfism, witches' broom, yellowing, virescence and phyllody (Sugio *et al.*, 2011). Several recent studies have shown that phytoplasmas secrete virulence proteins to change the plant morphology, such as TENGU, SAP11, SAP54 and PHYL (Hoshi *et al.*, 2009, Sugio *et al.*, 2011; Oshima *et al.*, 2013). In this study, NGSD contigs from a whole genome sequencing project have been searched for secreted proteins that could be potential virulence effectors. Furthermore, since the

sugarcane white leaf (SCWL) phytoplasma is in the same 16SrXI group as NGSD it can be predicted that some of the primers designed to amplify the NGSD effectors might be able to amplify the equivalent genes from SCWL.

Materials and Methods

Twenty contigs of the napier grass stunt phytoplasma genome were analysed for Open Reading Frames (ORF) with Sanger program and ORF finder (www.ncbi.nlm.nih.gov) and then each of the ORFs were analysed for signal peptide (SP) sequences with the PrediSi (Prediction of Signal peptides) program. Subsequently, primers were designed for these candidate effector genes using the Primer3 program to amplify candidate potential effector sequences. NGSD and SCWL DNAs were extracted by the CTAB method and checked firstly by P1/P7 and nested PCR with R16mF2/R16mR1 primers (Bertaccini *et al.*, 2019); both genomic DNAs were amplified with effector gene primers and then sequenced and translated to protein and aligned between NGSD and SCWL (https://www.ebi.ac.uk).

Results

From each NGSD contig, coding regions were identified with the Sanger program and ORF finder, and those with predicted signal peptide sequences identified with the PrediSi program. The PrediSi showed either no SP sequences or SP sequences presented the cleavage site where the signal peptidase would

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cut the SP sequences before the protein secretion. Additionally, the PrediSi score is on a scale 0 to 1 and a score larger than 0.5 means that the protein sequences contain an SP as shown in figure 1 and there was a higher peak than at other positions at the cleavage site for the signal peptidase. Subsequently, primers from genes containing SPs were designed and used to amplify sequences from NGSD and SCWL genomic DNAs, and the results showed 7 and 2 PCR products from NGSD and SCWL, respectively. Table 1 shows amino acid sequences from each primer set and Figures 2 and 3 show protein alignments between NGSD and SCWL from Ef1 and Ef2. For Ef1 there are some amino acid differences between the sequences from the two phytoplasmas, whilst for Ef2 the sequences are identical.

Table 1. List of protein similarity from NCBI database.

Primer	Genomic	Protein sequences
Ef1	NGSD	MFKLQNQFKIIRVFLFTFLGFLIINNNQVMAMGNKNSNNNSEISNDKE YALYIQAEIAIKSELTNLQLNNAKKSELLHKQQFITTEIEKYNKRQNNNQ LSTTYGEQPESSRRNISSAINDDDENELSDDELQPMNLIKNNSKCPIDQ
Ef2	NGSD	KYSKIIPYSFKSLILYPISSNSEKVKLHSFIFFIFVLGIFLANTQFPFFSVIL*
Ef3	NGSD	FINRSKISFNCCFSFFFPITSSKVGKSFESLKYLLAAKLLFSFSILALLSIFT SFKVFKASRKETTASLKIFLSCSNSSLLLFNSVISRLYLPISLFKYSFFSISN CLSLLIFRSRKSMNFSLILRNSSFKSLYLVISCWRLLVNFSIPFVKISISFFN*
Ef4	NGSD	KKDNVVINGNISGVIPKIIVNDNKKIFKKSKSDGFLNQLIIKIKGNINK VEIINNLDIELISF*
Ef5	NGSD	KKDNVVINGNISGVIPKIIVNDNKKIFKKSKSDGFLNQLIIKIKGNINK VEIINNLDIELISF*
Ef6	NGSD	LFEIIFFILNLFLNLGFFKHFLYVLFLLTILNLCYNKSVKHSISSDYLEKKLYYA*
Ef7	NGSD	MMQIKNKLYFLPLFLMSFLGLFLLINTNPVIAAPEKNDKGKKIASSEKQE KTTKKDISQYYELYNTLENYSEEDRNKIIQMLSDSQTLKILQEEALKSKK KGSSSKKPDDSKK*
Ef1	SCWL	MMQIKNKLYFLPLFLMSFLGLFLLINTNPVIAAPEKNDKGKKIASSEKQE KTTKKDISQYYELYNTLENYSEEDRNKIIQMLSDSQTLKILQEEALKSKK KGSSSKKPDDSKK*
Ef2	SCWL	KYSKIIPYSFKSLILYPISSNSEKVKLHSFIFFIFVLGIFLANTQFPFFSVIL*
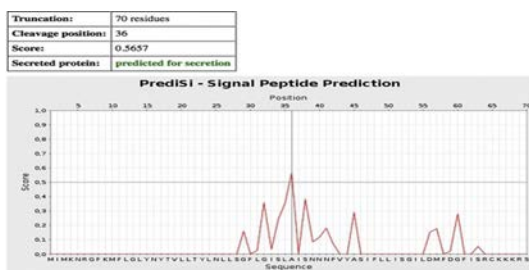
Figure 1. Screenshot of amino acid peak, cleavage site and scores.

Figure 2. Protein alignment of NGSD compared with SCWL strains for the *Ef1* gene.

Figure 3. Protein alignment of NGSD compared with SCWL strain for the *Ef2* gene.

Discussion

Phytoplasma effectors are secreted via Sec-dependent pathways requiring SP sequences for guiding them through cell membranes. SP sequences consist of 3 domains; positive charge, hydrophobic charge and polar charge and the cleavage site is located on the polar charged domain (Hiller *et al.*, 2004). In this study, the SP sequences were predicted by PrediSi whilst Bai *et al.* (2009) analysed AY-WB SP sequences by SignalP combined with TMHMM2.0 and PredictNLS and pSORT to predict nuclear localization signals, which are required for subcellular localization in plant cells, and their result showed 4 SAPs with NLS. In this study, 7 candidate effector proteins were identified in NGSD and 2 candidate effector proteins in SCWL. Additionally, the PCR results revealed that NGSD and SCWL shared some nucleotide sequence and also completely matching protein sequences for Ef2, whilst there were differences in the EF1 between the two phytoplasmas. These candidate effector genes are now being ligated into plant plasmid vectors and transformed into *A. thaliana* for observation of symptoms.

Acknowledgements

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Phylogenetic analyses of phytoplasma replisome proteins demonstrate their distinct and complex evolutionary history

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Abstract

The genus 'Candidatus Phytoplasma' encompasses diverse groups of endocellular bacteria without cell wall that reside within plant phloem and insect cells. They are classified to the class *Mollicutes* together with acholeplasmas, spiroplasmas, mycoplasmas and other related bacteria. One of main characteristics is their small and reduced genomes with the often presence of potential mobile units (PMUs). PMUs are transposon-like genetic elements suggested to contribute to the ability of these pathogens in colonization of wide range of hosts. Replisome genes, such as *dnaB*, *dnaG* and *SSB* are frequently found in PMUs. In this study, their possible role in independent PMU replication is investigated and discussed. Herein, the extensive phylogenetic analysis of DnaB, DnaG and SSB replisome PMU sequences of different members of the class *Mollicutes* was performed. The results indicate a complex evolutionary history of phytoplasma PMU replisome proteins. For DnaB sequences, phylogenetic analysis revealed separation into DnaB1 and DnaB2 clusters with additional clustering of PMU and non-PMU sequences within DnaB1. Separate grouping of the PMU sequences was also shown for DnaG and SSB sequences. These results suggest distinct and independent evolution of non-PMU and PMU replisome sequences and frequent intermixing and recombination of PMU genes among different phytoplasma species enabling their better adaptation to different environment.

Keywords: evolution, phylogeny, phytoplasma, potential mobile unit, replisome

Introduction

The term replisome refers to a complex molecular machine carrying out replication of DNA encompassing a number of proteins including helicase, RFC, PCNA, gyrase/topoisomerase, SSB/RPA, primase, DNA polymerase III, RNAse H, and ligase. Unlike the processes of transcription and translation whose main executable proteins are homologous in all three domains of life (Bacteria, Archaea and Eukarya), most components of the replisome seem to be unrelated between Archaea and Eukarya (Yao and O'Donnell, 2016). While the replication process has been studied in detail for some bacteria such as *Escherichia coli* and *Bacillus subtilis*, data on other bacterial replisomes are less available (Beattie and Reyes-Lamothe, 2015). The genus 'Candidatus Phytoplasma' encompasses diverse groups of endocellular bacteria without cell-wall residing within plant phloem and insect cells. They belong to the class *Mollicutes* consisting of two major clades that diverged early in evolution. Phytoplasmas belong to the AAA clade (orders *Acholeplasmatales* and *Anaeroplasmatales*) and form a monophyletic group in the order *Acholeplasmatales*. The other clade contains the orders *Mycoplasmatales* and

Entomoplasmatales (SEM clade - Spiroplasma, Entomoplasma and Mycoplasma) (Lee *et al.*, 2000). One of the intriguing characteristics of phytoplasma genomes is the presence of potential mobile units (PMUs), putative transposons that are suggested to be associated to phytoplasma genome instability (Bai *et al.*, 2006). These units are thought to increase the phytoplasma fitness in the environment that they encounter cycling between hosts from two kingdoms. An interesting feature of PMUs is that, among other genes, they frequently contain the replisome genes *dnaB*, *dnaG* and *ssb*. In this study, the aim was to investigate the phylogenetic and evolutionary relationships of the replisome elements *dnaB*, *dnaG* and *ssb*, frequently found in phytoplasma PMUs. Their relationship to the same phytoplasma genes that are not found within PMU regions of the genome were also studied.

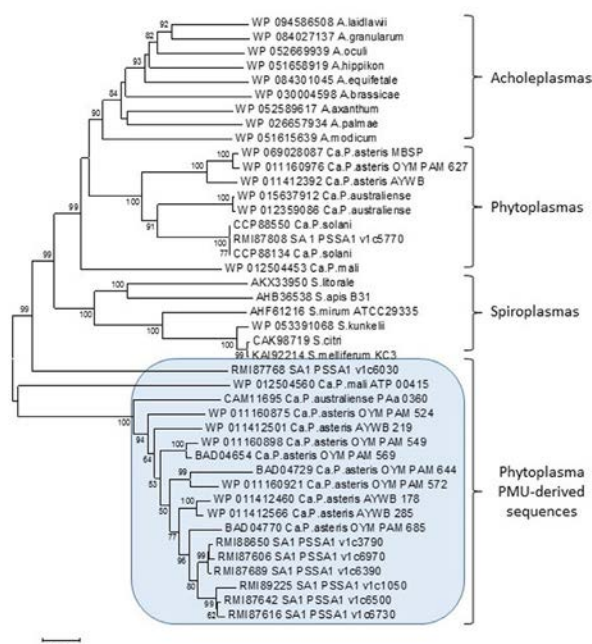## Materials and Methods

DnaG, DnaB and SSB amino-acid sequences from 'Ca. P. solani' strain SA-1 genome were the result of a previous study reporting the genome draft of this strain of 'Candidatus Phytoplasma solani' (Seruga Music *et al.*, 2019). All DnaG protein sequences were subjected to the BLASTp search,

followed by retrieval of other phytoplasma, spiroplasma and acholeplasma sequences from GenBank database. All selected sequences were aligned using ClustalX 2.0 (Thompson *et al.*, 1997). Subsequent phylogenetic analyses were performed by using different methods (neighbour-joining, maximum likelihood or maximum parsimony) and parameters in MEGA 7 software (Kumar *et al.*, 2016).

Results

Phylogenetic analysis of the DnaB protein sequences from different Mollicute members showed clustering into 3 main distinct groups. It has been noted previously that the *dnaB* gene is annotated at least twice in phytoplasmas and *Acholeplasma laidlawii* and that the annotated DnaB2 shows significant differences to DnaB1 in phytoplasmas (Kube *et al.*, 2012). These analyses showed that phytoplasma DnaB sequences segregated into 3 distinct phylogenetic groups clearly making distinction between DnaB1 and DnaB2. DnaB1 sequences roughly segregated into non-PMU and PMU subgroups, while DnaB2 clade encompassed only sequences from non-PMU genomic regions. Phylogenetic analyses of DnaG sequences also demonstrated a separation of PMU and non-PMU sequences, where group of phytoplasma PMU-derived DnaG sequences formed a unique clade among *Mollicutes* (Figure 1). The separation into different clusters encompassing PMU-derived and non-PMU sequences was also shown for SSB amino-acid sequences. Moreover, clustering of SSB sequences originating from non-PMU regions present only in one copy per genome showed conservation among members of the class *Mollicutes*.

Figure 1. Phylogenetic tree constructed by neighbour-joining analysis (number of differences) of the DnaG amino-acid sequences from representative members of the class Mollicutes. Numbers on main branches correspond to bootstrap values as percentages (500 replicates). GenBank accession numbers are indicated left to the taxon name or acronym.

Discussion

The role of frequent existence of genes for replisome proteins DnaB, DnaG and SSB in phytoplasma genomes and particularly in phytoplasma PMUs is still not clear. Toruňo *et al.* (2010) demostrated that PMU1 from AY-WB phytoplasma can excise from the chromosome and replicate independently as an extrachromosomal element. However, the exact mechanism of regulation and excision is still not clear. In this study, phylogenetic analyses of replisome genes among *Mollicutes* showed separation of PMU-derived sequences for all the 3 studied replisome proteins. These results reveal independent and complex evolution of non-PMU and PMU replisome sequences. Moreover, frequent intermixing and recombination of PMU genes among different 'Candidatus Phytoplasma' species enabled their better adaptation to the different plant and insect cell environments. Whether these genes support the replication process of the host cell is not known. Further studies are needed to answer these questions in a more detailed manner.

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Identification of 16SrIII-J phytoplasma effectors using a viral vector

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Abstract

The pathogenicity mechanisms of phytoplasmas has been documented for several ribosomal groups, but there is a lack of information for the 16SrIII group. Phytoplasmas in this ribosomal group were detected in most of the countries of South America, in different crops, from herbaceous to woody species. This works aimed to verify if the *in planta* transient expression of putative phytoplasma effector proteins allow to observe symptoms related to those associated with phytoplasma diseases. Plants infected with *Tobacco mosaic virus* vector carrying 16SrIII-J phytoplasma SAP54 and SAP05 ortologous genes, showed flowering abnormalities in comparison to those not infected or infected with the viral vector alone indicating a putative role of these proteins in 16SrIII-J phytoplasma symptom induction.

Keywords: 16SrIII-J, protein effector, viral vectors, symptomatology

Introduction

Phytoplasmas belonging to 16SrIII-J subgroup were widely reported in South America infecting several crops, from herbaceous to woody host species (Galdeano *et al.*, 2009; González *et al.*, 2011). Even when the association of these phytoplasmas with disease expression is clear, the pathogenicity mechanisms of these bacteria were not elucidated. The advances in next generation sequencing (NGS) together with plant transformation techniques, allowed to identify several phytoplasma possibly secreted peptides, called effectors, that have the ability to trigger symptoms in plants through the interaction with transcription factors (SAP11) or by their link to specific protein motifs that promotes the protein degradation, with a consequent alteration of the plant physiology (SAP54) (Sugio *et al.*, 2011; MacLean *et al.*, 2011). Transgenic *Arabidopsis thaliana* overexpressing a single phytoplasma effector protein showed the typical phytoplasma symptoms. The pathogenicity effector genes, and the number of them vary depending on the phytoplasma and its ability to infect different hosts. The present study is focusing on 16SrIII-J phytoplasmas to clarify their ability to induce different symptoms in a wide spectrum of host plants. The identification of 16SrIII-J phytoplasma effector(s) related with symptom induction in *A. thaliana* as a model of herbaceous host was therefore carried out.

Materials and Methods

To correlate the symptoms observed in plants transiently expressing phytoplasma genes with phytoplasma induced symptoms in *Nicotiana benthamiana* and *A. thaliana* plants, several transmission assays were carried out, collecting specimens of *Paratanus exitiosus*, an efficient vector of 16SrIII-J phytoplasma, in a vineyard infected with the phytoplasma (Longone *et al.*, 2011). Successful infections were confirmed using PCR with R16F2n/R2 primers (Gundersen and Lee, 1996). For the construction of viral vectors carrying phytoplasma genes, the identification of putative effectors sequences that resembled signal peptides related with secretion by Sec translocase system were searched (Siewert *et al.*, 2014), by SIGNALP 4.0 server (http://www.cbs.dtu.dk/services/SignalP) and BLAST tools available in CLC Genomics workbench v8.1. The available draft genome of a 16SrIII-J strain deposited at a local database (Accession Number LLKK01000000) (Zamorano and Fiore, 2016) was used. Two genes were selected (SAP54 and SAP05), based in protein identity with AYWB strain effectors (MacLean *et al.*, 2011; Sugio *et al.*, 2012). After determination of expression levels by quantitative RT-PCR, using *pduL* gene as reference, full length ORF of SAP54 and SAP05, including restriction sites for *Pac*I and *Xho*I to insert a *Tobacco mosaic virus* based vector (pBSG1057) were amplified. This vector carries a GFP gene that can be replaced by the interest gene. Healthy plants of *N. benthamiana* and *A. thaliana* ecotype Shahdara, were infected with RNA of the TMV-based vector, carrying SAP54 and SAP05 genes, obtained by *in vitro* transcription using Ambion mMACHINE T7 kit (AM1344). Efficient infection was determined by detection of TMV and SAP-genes by RT-PCR using primers external to the ligation region (Figure 1).
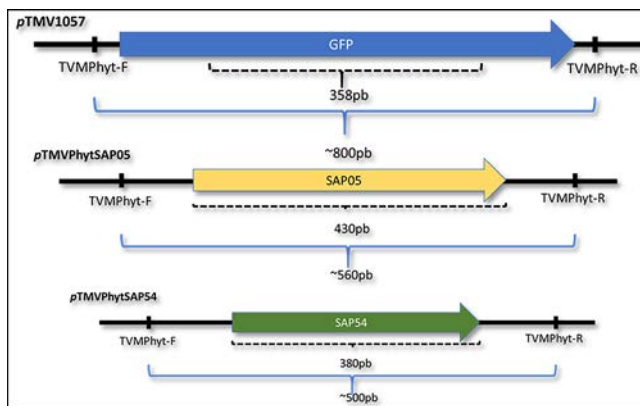
Figure 1. Schematic representation of genes in TMV vector and expected PCR product size using the primers external to the ligation site.

Results

The symptoms observed in *A. thaliana* infected by *P. exitiosus* ranged from dwarfism to the total absence of flowers (Figure 2) and were observed 30 days after the beginning of the transmission assays. None of the plants of *N. benthamiana* resulted positive to phytoplasma after transmission assays. The *N. benthamiana* plant infected with TMV-SAP54 exhibit dwarfism and absence of flowers in comparison with a plant infected with TMV carrying GFP gene 40 days post inoculation (dpi). The *A. thaliana* ecotype Shahdara 35 dpi TMV-SAP54 showed dwarfism and absence of flowers; TMV-SAP05 showed shorter floral stem, meanwhile in the TMV-GFP plant a normal phenotype, with long and abundant floral stems was observed (Figure 3). No flower alterations like phyllody or virescence were observed, in both plant species.

Figure 2. Symptoms observed in *A. thaliana* transmission assays using *Paratanus exitiosus* as 16SrIII-J phytoplasma vector.
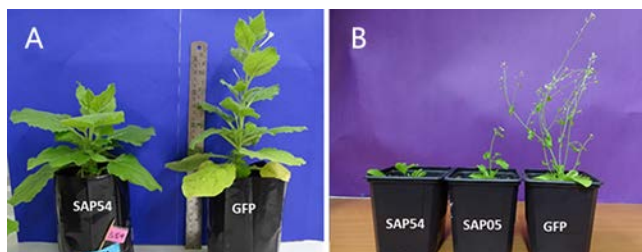

Figure 3. Symptoms observed in: A) *N. benthamiana* infected with SAP54 and TMV-GFP 40 dpi; B) *A. thaliana* infected TMV-SAP54, TMV-SAP05 and TMV-GFP, 35 dpi.

Discussion

The stunting symptoms observed in *A. thaliana* infected by 16SrIII-J phytoplasma as expected, are similar to those observed in other *Brassicaceae* species in Brazil (Eckstein *et al.*, 2013). This symptom was accompanied by poor flowering to absence of flowers, the same symptom observed in TMV-SAP54 infected in both model plants. Intriguingly, in AYWB phytoplasmas these symptoms were reported as the result of a combination of two effectors, SAP11 and SAP54, but they were observed only with transient expression of SAP54. The results of this experiment are in agreement with those reported by Hoshi *et al.* (2009) who associated a single virulence factor (Tengu) to flower abnormalities and dwarfism. In the other hand, TMV-SAP05 infected plants, showed early flowering and aerial rosettes. Transcriptomic analyses are in progress to achieve deeper knowledge of the genes affected by the presence of these effectors.

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PM19_00185 of '*Candidatus* Phytoplasma mali' is a protein that functions as a E3 ubiquitin ligase

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Abstract

Phytoplasmas are the agents associated with numerous diseases in several plant species all over the world, including important food crops. The mode of phytoplasma infection is poorly understood and often based on genomic data. A yeast two-hybrid screening was used to find new protein-protein interactions between '*Candidatus* Phytoplasma mali', the phytoplasma associated with apple proliferation and its host plant. '*Ca.* P. mali' strain PM19 genome encodes a protein PM19_00185 that interacts with at least five different ubiquitin conjugating enzymes (UBC, E2) of *Arabidopsis thaliana*. The *in vitro* ubiquitination assay shows that PM19_00185 is enzymatically active as E3 ligase with *A. thaliana* E2 UBC09.

Keywords: '*Candidatus* Phytoplasma mali', host-pathogen interaction, ubiquitination, E3 ligase

Introduction

'*Candidatus* Phytoplasma mali' is the agent associated with the apple proliferation (AP) disease that leads to considerable yield losses and economic damage in apple production all over Europe. The availability of an increasing amount of phytoplasma draft genome sequences in GenBank (NCBI) give valuable insights into the genetic makeup of these pathogens and provide important background information in phytoplasma research.

Gene prediction algorithms have become more sensitive, but it is still difficult to predict gene and protein function accurately from genomic data. A proteomic approach can be helpful to evaluate genomic data. To better understand how phytoplasmas spread throughout the plant after infection and induce specific symptoms, a search for protein-protein interactions between '*Ca.* P. mali' and plant proteins using library-scale yeast-two hybrid (Y2H) screens was performed. The selection of 23 candidate proteins of '*Ca.* P. mali' that were so far undescribed and annotated as hypothetical proteins with at least one potential transmembrane region by Phobius prediction using a combined transmembrane topology and signal peptide algorithm (Käll *et al.*, 2004) was done, and search for new protein interactions using Y2H screening with a *Arabidopsis thaliana* library was carried out. One of the candidate proteins, named PM19_00185 (GenBank: accession number MK552110) according to its corresponding protein ATP_RS00185 of the sequenced strain AT (Kube *et al.*, 2008), showed promising results in the Y2H screen. Therefore, this protein was further analysed in detail for biochemical properties and effects on plant defence mechanisms.

Materials and Methods

Transient protein expression *in planta* and protoplast preparation was performed by agroinfiltration as described (Schöb *et al.*, 1997). Y2H screening was performed according to manufacturer's protocol (Invitrogen). Expression and purification of His-AtUBC9-His and His-MdUBC10-His and His-MBP-PM19-00185-DTM-His were as recommend by manufacture´s protocol (Novagen). *In vitro* ubiquitination assays were performed using Ubiquitylation Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's protocol.

Results

In Y2H screening, about one thousand clones were found to activate all reporter genes. The 10% of the positive clones were randomly selected and the plasmids were isolated and sequenced. The sequences data were analysed *via* BLAST in NCBI database. The results show that all identified genes belong to a superfamily encoding *A. thaliana* ubiquitin conjugating enzymes (AtUBC, E2) and can be categorized

into three groups: AtUBC group no. III (3% of screened clones) with AtUBC2, AtUBC group no. V (14% of screened clones) with AtUBC07 and AtUBC14 and AtUBC group no. VI (83% of screened clones) with AtUBC08, AtUBC09 and AtUBC10 (Figure 1, left panel). To further study the possible function of PM19_00185 interaction with *Arabidopsis* E2 proteins, it was recombinantly expressed as a fusion maltose binding protein (MBP) and one of the E2 proteins identified in the Y2H screen, AtUBC09 was used for an *in vitro* assay. In an ubiquitin assay PM19_00185 was found to function as a E3 ligase. The result showed that a high-molecular mass smear was detected only in the reaction including MBP-PM19_00185-ΔTM and AtUBC09. (Figure 1C, right panel). Since the smear is detectable by using α-MBP, it indicates the formation of poly ubiquitinated forms of MBP-PM19_00185-ΔTM. E3 ligases are crucial for the transfer of the activated ubiquitin to a substrate.
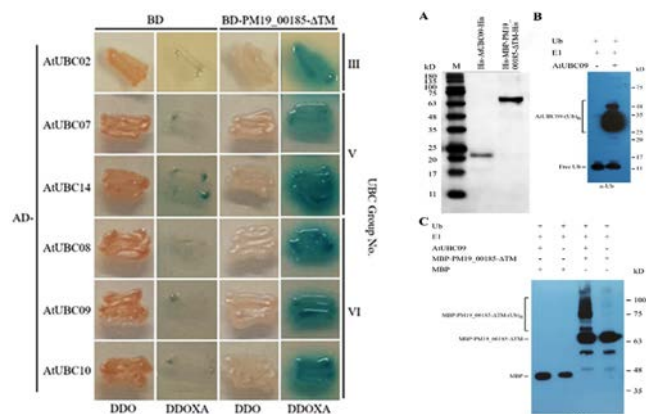
The activated ubiquitin is either directly transferred to the E3-bound substrate, or a E3-ubiquitin intermediate is formed before the ubiquitin is transferred (Glickman *et al.*, 2002). Although no other additional E3 protein was added into the reactions, the ubiquitin chains were transferred from E2 protein AtUBC09 to His-MBP-PM19_00185-ΔTM-His. This result suggests that PM19_00185 has a E3 ligase activity and could transfer the ubiquitin chain to a specific target via a E3-ubiquitin intermediate complex.

Discussion

In contrast to the phytoplasma effector SAP54 which promotes degradation of MADS-box transcription factors by binding RAD23, a plant protein, and shuttling the substrate to 26S proteasome (MacLean *et al.*, 2014), the PM19_00185 has E3 ligase activity and probably binds to target proteins prior to loading to the ubiquitin-proteasome system (UPS). Nevertheless, these findings suggest that phytoplasmas may affect the ubiquitin pathway, exploiting weak points in this system, thus resulting in increased pathogen fitness. This is the first experiment showing that a phytoplasma effector protein has an activity like E3 ligase proteins. To fully understand how PM19_00185 affects the plant host ubiquitin system and to elucidate the suppressing activity of the protein on the plant defence, further investigations are required.

Figure 1. Left panel. PM19_00185 binds six UBC proteins of *A. thaliana* (AtUBC) belonging to three groups. A yeast two-hybrid screen was performed using the binding-domain (BD) fused PM19_00185-ΔTM resulting in expression plasmid pGBKT7-PM19_00185-ΔTM and an expression plasmid carrying the activation domain (AD) fused to one of the six AtUBC previously identified in the Y2H. As negative control empty pGBKT7 expressing only BD was used. Co-transformed yeast cells were patched on double drop-out medium (DDO) to select for the presence of both expressing plasmids and DDO media containing aureobasidin A and X-α-Gal (DDOXA) to select for protein interaction.

Right panel. A) Recombinant expressed PM19_00185-ΔTM acts as E3 ubiquitin ligase in presence of AtUBC09. A SDS-PAGE analysis of recombinant expressed and purified his-MBP-PM19_00185-ΔTM-his and his-AtUBC09-his. His-tagged proteins were expressed in *Escherichia coli* and purified using Ni-NTA-based purification. After separating using SDS-PAGE, the gel was stained with Coomassie brilliant blue. M: protein marker. B) *In vitro* ubiquitination assay confirms the E2 activity of the purified his-AtUBC09-his. Poly ubiquitinated forms of his-AtUBC09-his [AtUBC09-(Ub)n] can be detected as high-molecular mass smear by Western blotting using á-Ub. Reaction without his-AtUBC09-his is used as a negative control. C) *In vitro* ubiquitination assay suggests E3 ligase activity of PM19_00185. In presence of E1 and purified his-AtUBC09-his as E2, his-MBP-PM19_00185-ΔTM-his is poly ubiquitinated. Poly ubiquitinated forms of his-MBP-PM19_00185-ΔTM-his [MBP-PM19_00185-ΔTM-(Ub)n] were detected in Western blotting using α-MBP. Reactions without his-AtUBC09-his or only MBP were used as negative controls. All *in vitro* ubiquitination assays have been repeated at least twice with similar result.

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Association of AAA+ ATPases and FtsH proteases with phloem bleeding of proliferation-diseased apple trees

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Abstract

To examine the mechanisms involved in the virulence of the apple proliferation pathogen 'Candidatus Phytoplasma mali' a considerable number of genes encoding ATPase AP460 and FtsH protease AP382 were sequenced. Analysis showed that these and other AAA+ proteins are closely related to the virulence of 'Ca. P. mali'. From the deduced protein sequences of AP382 and AP460 markers were developed that are specific for virulence and avirulence. However, the interpretation of the results was sometimes difficult by either shifts in the phytoplasma population or by a too narrow specificity of the markers. For this it is planned to develop additional markers based on ATPase protein AP406.

Keywords: apple proliferation, 'Candidatus Phytoplasma mali', phloem bleeding, AAA+ proteins, virulence

Introduction

Apple proliferation (AP) is associated with the presence of 'Candidatus Phytoplasma mali'. Previous analysis of the full-length sequence of a strain of this pathogen revealed that phytoplasmas possess an unusually high number of ATPases associated with various cellular activities (AAA+ proteins). In 'Ca. P. mali' 12 of these proteins are present, consisting of 6 AAA+ ATPases and 6 FtsH proteases. Due to this finding and the presence of reports that AAA+ proteins are essential in several bacterial diseases of human and animals, the relationship of these proteins in the AP disease development was previously examined by sequence analysis and PCR amplification. This research revealed that the foliar symptoms and vigor of the trees are closely related to the presence of these proteins. The work reported is focusing on the phenomenon of phloem bleeding and is dealing with the relationship of the AAA+ proteins in this phenomenon.

Materials and Methods

Phloem sap was collected from seven-year-old apple cultivar Golden Delicious trees by making incisions in the conducting phloem of the trunk or major scaffold limbs from October to December 2014 (Figure 1). Four nonsymptomatic trees and 20 trees inoculated with 7 different phytoplasma accessions were used. To compare the virulence of the accessions with the selected virulence markers ATPase AP460 and FtsH protease AP382, they were examined by PCR amplification or sequencing. DNA extraction, cloning,

sequencing and PCR amplification were performed as described (Seemüller *et al.*, 2013, 2018).

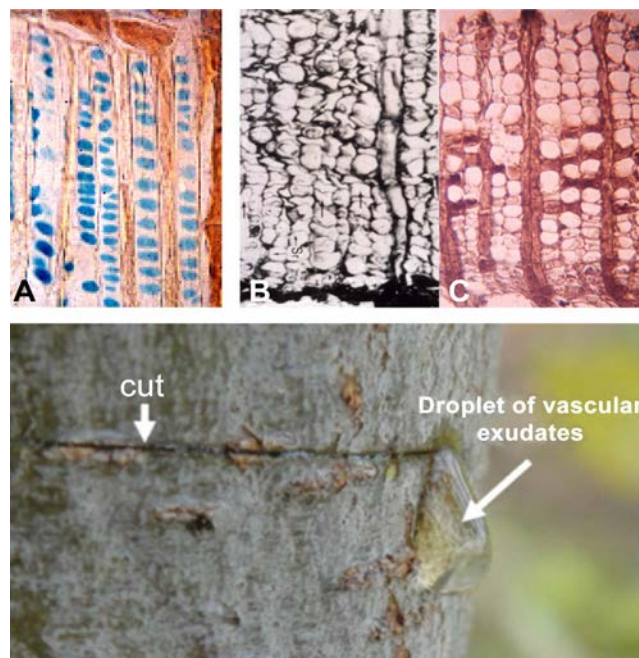**Figure 1.** Phloem of infected apple tree showing severe histopathological modifications such as callose deposition on sieve plates A), sieve tube necrosis of annual phloem B) and healthy annual phloem C). Below: wounding-induced vascular exudates that can be collected from severely affected trees, but not from infected non-symptomatic or healthy trees.

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Results

All inoculated trees examined showed at sampling moderate or severe symptoms. For this reason it was expected that the presence of markers for virulence dominate. However, this was only true for the majority of the samples. In some cases, in trees with similar symptoms, markers of avirulence occurred more often than the markers for virulence. This was observed even after the sampling was repeated over several years. However, considerable differences between the two proteins were also observed (Table 1).

Table 1. Distribution of markers for virulence (vir) and avirulence (avir) in multiply infected accessions of '*Ca*. P. mali' as determined by protein sequence analysis.

Inoculated accessions	FtsH protease382		ATPase 460	
	Vir	Avir	Vir	Avir
4/93	24	6	25	21
14/93	28	8	26	17
16/93	9	82	9	123
3/4	1	28	40	19
3/5	32	0	53	0
3/6	30	0	6	27
3/8	20	21	21	17

Discussion

Previous work showed indication that most or all the AAA+ proteins are involved in the development of the foliar symptoms of the apple proliferation disease. This also seems to be true for the phloem bleeding that is associated with several other phytoplasma diseases and was first described for apple proliferation by Kollar *et al.* (1989). This finding is likely to be useful for studying several aspects of AP disease or other aspects of phytoplasma diseases, such as the impairment of the sieve tube membrane or the phloem transport.

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Investigating the interaction between the apple proliferation effector SAP11$_{CaPM}$ and its targets in susceptible and resistant *Malus* accessions

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Abstract

SAP11$_{CaPM}$ is the only bacterial effector known to date to be identified as secreted by the apple proliferation phytoplasma. In *Malus* spp. this effector binds and deactivates two transcription factors of the TCP family, subgroup CIN-class 2. To elucidate the role of SAP11$_{CaPM}$ and its targets in the context of resistance against apple proliferation phytoplasma (AP), the sequences of the target genes from susceptible and resistant *Malus* have been compared. Interestingly, some mutations exclusively present in AP resistant *Malus x domestica* TCP 25 (MdTCP25) could be observed. The MdTCP25 sequences of many other AP-resistant plants were sequenced and a good degree of correlation was found between resistance and presence of the previously detected resistant exclusive mutations. Using a yeast two hybrid-based reporter gene assay, the interaction strength between SAP11$_{CaPM}$ and MdTCP25 from resistant plants was evaluated. However, no visible differences in interaction strengths between SAP11$_{CaPM}$ and MdTCP25 from susceptible or resistant *Malus* could be observed.

Keywords: apple proliferation, TCP transcription factors, SAP11

Introduction

Apple proliferation (AP) is a severe disease widespread in apple-growing areas in Europe. Typical symptoms comprise foliar reddening, shoot proliferation, small leaves with altered shape and undersized, tasteless and colourless fruits. The agent associated with the disease is '*Candidatus* Phytoplasma mali'. Phytoplasmas release proteins, called effectors, in the host cells altering the physiological processes and providing a fitness advantage to the bacteria (Bai *et al.*, 2008). The genome analysis of different strains of '*Ca.* P. mali' revealed the presence of the effector SAP11$_{CaPM}$ (Siewert *et al.*, 2014), which has been observed to target at least two apple class II TCP (TEOSINTE BRANCHED/CYCLOIDEA/ PROLIFERATING CELL FACTOR) transcription factors, namely MdTCP24 and MdTCP25 (homologs to AtTCP13 and AtTCP4, respectively) (Janik *et al.*, 2017), in accordance with what was described for other plant species (Sugio *et al.*, 2011a). These two TFs are likely to be involved in developmental processes and defence responses, such as leaf and root growth, phosphate tolerance and jasmonic acid biosynthesis pathway (Martín-Trillo and Cubas, 2010; Xua *et al.*, 2014). The SAP11-CIN-TCP binding leads to the degradation of the TFs (Sugio *et al.*, 2011b), although a direct link between TF deactivation and symptom development is lacking. In the last years, resistance against AP was observed in some experimental rootstock selections: in these plants the phytoplasmas were not detected or difficult to detect and they either never developed symptoms or recovered within a few years (Seemüller *et al.*, 2010), but the physiological and molecular basis of the resistance are mostly unknown.

Materials and Methods

The full length MdTCP24 and MdTCP25 genes from the DNA of two AP susceptible-Golden Delicious and M9- and three resistant-*Malus sieboldii*, 4608, D2218- (Schmidt, 1964) plants were amplified and sequenced. Sequencing data were compared with the sequences of the 117 *Malus* accessions (Duan *et al.*, 2017) leading to the identification of the most interesting mutations in the two genes. By comparing the results with preliminary screening tests for AP resistance, 46 interesting genotypes were selected and sampled for further analyses. The regions of interest were amplified and sequenced with Illumina NGS. The full-length MdTCP25 genes from resistant and susceptible plants were cloned into the yeast expression vector pGAD-HA and co-transformed with the SAP11$_{CaPM}$ expressing bait vector in *Saccharomyces cerevisiae* NMY51, a Y2H reporter strain. The interaction strength was evaluated by transferring positive clones on

selective medium containing increasing concentrations of the interaction inhibitor 3-amino-1,2,4-triazole (3-AT).

Results

Two non-synonymous substitutions were found in MdTCP24 genes of AP-resistant plants, but the comparison with other *Malus* accessions proved them to be common allelic varieties that were thus not included in the analysis. On the contrary, some of the amino acid substitutions between resistant and susceptible MdTCP25 genes were quite rare and located in highly conserved sites (Aggarwal *et al.*, 2010) and were thus further analysed. A total of 4 different TCP25 alleles was identified, whose details are summarized in Table 1. Results of the Illumina sequencing are presented in Figure 1, cross-correlated with the resistance screening tests. Only two plants that exhibited resistance or tolerance against AP carry neither the "yellow" nor the "blue" allele. Positive colonies expressing TCP25 and SAP11$_{CaPM}$ grew on selective media for each allele tested proving the interaction between those two. No differences were detected in growth on 3-AT-containing selective medium, with all the clones growing on concentrations lower than 100 mM. No growth was observed with 3-AT concentrations higher than 150 mM.

Table 1. MdTCP25 alleles found in susceptible and resistant apple plants. In the second and third columns amino acid substitution and its position are compared to the wild type (WT).

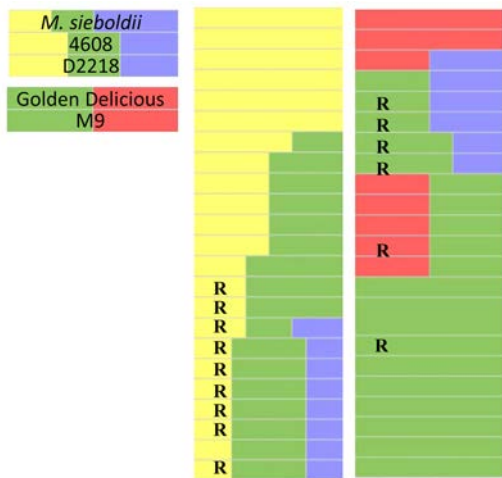| Allele	Substitution	Pos(AA)	Variety
WT	...	...	Golden Delicious, M9
Yellow	Lys ▶ Ala	66	*M. sieboldii*, 4608, D2218
	Ala ▶ Ser	122	
Red	Glu ▶ Lys	99	Golden Delicious, M9
	Asn ▶ Asp	136	
Blue	Ser ▶ Arg	94	*M. sieboldii*, 4608, D2218

Figure 1. Results of the MdTCP25 Illumina sequencing performed on plants screened for resistance and promising ones. Colours indicate presence of mutation as described in Table 1. "R" = plants resulted resistant in the preliminary test; no letter means either data not available or test in progress.

Discussion

The Y2H-based interaction analysis resulted in no detectable difference between the interaction strength of SAP11$_{CaPM}$ and MdTCP25 derived from resistant or susceptible *Malus* accessions. Although these results are yet to be confirmed by further analysis, they suggest that the interaction strength itself is not involved in the molecular mechanisms conferring resistance. On the other side, there is a correlation between the presence of some rare amino acid substitutions allele and resistance. Beside the functional elucidation, the potential role of MdTCP25 as a marker of resistance is promising. Hence this should be further investigated and confirmed by extending the pool of plants analysed and screened for resistance.

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In search of the molecular targets and the function of apple proliferation phytoplasma effector proteins

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Abstract

The plant pathogen '*Candidatus* Phytoplasma mali' infects apple trees and is associated with apple proliferation, an important disease in European apple growing regions. '*Ca*. P. mali' manipulates its plant host by secreting so called effector proteins. The characterization of these effector proteins and the determination of their *in planta* targets is important to unravel the molecular mechanisms underlying disease and symptom development. Preliminary results show that a '*Ca*. P. mali' effector targets the ubiquitination system of the plant, transcription factors of different classes and other host proteins that are involved in immune response regulation. Furthermore a regional genetic variant of another potential '*Ca*. P. mali' effector that functionally differs from the variant of the '*Ca*. P. mali' strain AT was found.

Keywords: '*Candidatus* Phytoplasma mali', effector protein, apple proliferation, ubiquitin, transcription factors

Introduction

The plant pathogen '*Candidatus* Phytoplasma mali' is the bacterial agent associated with apple proliferation, a disease of economic importance in apple-growing areas in Europe. Even though this pathogen is of economic relevance, little is known about the molecular mechanisms of the phytoplasma-host interactions. These interactions can be mediated by secreted phytoplasma proteins, so called effector proteins that alter host-cell structure and function (Hogenhout *et al.*, 2009). The characterization of effector protein function and the identification of their *in planta* targets is crucial for our understanding of how '*Ca*. P. mali' manipulates its plant host. Preliminary results indicate that a '*Ca*. P. mali' effector targets the plants ubiquitination system, transcription factors of different classes and other host proteins that are involved in immune response regulation. While the effector protein SAP11 from aster yellows witches' broom phytoplasma is present in homologue forms in different phytoplasmas (Sugio *et al.*, 2011, 2014; Janik *et al.*, 2017) a regional genetic variant of a potential '*Ca*. P. mali' effector was found that functionally differs from the variant of the '*Ca*. P. mali' AT strain used as reference (NCBI:txid 37692).

Materials and Methods

The potential '*Ca*. P. mali' effector proteins PME_002-HV, PME_002-Kube and PME_010 were subcloned into the yeast-two-hybrid (Y2H) vector pLexA-N and a Y2H screen and identification of the *Malus domestica* interaction partners was performed as described elsewhere (Janik *et al.*, 2017; Janik und Schlink, 2017). To confirm the interaction *in planta*, the potential effector and its proteinaceous *Malus* interaction partner identified in the Y2H were subjected to bimolecular fluorescence complementation (BiFC) analysis as described (Janik *et al.*, 2019).

Results and Discussion

The Y2H screening with PME_010 as bait against the *Malus domestica* cDNA library revealed interactors that are involved in the ubiquitination system and different transcription factors that regulate manifold physiological processes in the plant. Furthermore, this effector is binding proteins that are involved in the *Malus domestica* hypersensitive-induced response. These interactions are currently under further characterization applying BiFC and other molecular techniques. A Y2H screen with PME_002-Kube based on the sequence published by Kube *et al.* (2008) revealed eight different *Malus domestica* interaction factors from different functional protein groups. The confirmation of these interaction factors is currently in progress. Interestingly in '*Ca*. P. mali' strains that are predominantly found in South Tyrol (Alto Adige, North-Eastern Italy) a variant of this potential phytoplasma effector protein

was detected (PME_002-HV). A Y2H screen and a co-transformation of the proteins interacting with PME_002-Kube confirmed the presence of only one interactor, *i.e.* a *Malus* protein of unknown function.

The results of this study – even though they are partially at a preliminary status – clearly indicate that the described effector(s) of '*Ca*. P. mali' target the plants ubiquitination system and might manipulate the plant hosts physiology and its immune response. Considering the other identified targets of PME_010, it is further underlined that the plants immune system is in the focus of this potential '*Ca*. P. mali' effector. The results of the interaction studies with PME_002 indicate that regional differences might exist regarding the effector repertoire and function of '*Ca*. P. mali' and thus underline the necessity to gain more whole genome information of phytoplasma strains from different regions. This will broaden the understanding of how '*Ca*. P. mali' interacts with its host plant apple and which genes are indispensable for the bacterial life cycle.

Acknowledgements

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The timetree of phytoplasmas reveals new insights into the relationships with their insect and plant hosts

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Abstract

Phytoplasmas are a diverse monophyletic group of phytopathogenic bacteria. No attempts have yet been made to the estimate the divergence times of phytoplasma lineages. Reltime molecular divergence time analyses using 16S rRNA gene sequence data of Acholeplasmataceae was performed. The timetree of phytoplasmas, provided here for the first time, and based on prior divergence time estimates for two nodes within the *Mollicutes*, estimates the split between phytoplasma and *Acholeplasma* at about 663 million years ago (Ma), and initial diversification of the crown phytoplasma clade at about 330 Ma. Overall, these results suggest that phytoplasmas have been associated with insect and plant hosts at least since the carboniferous period.

Keywords: bacteria, divergence time, diversification, evolution

Introduction

Phytoplasmas are a diversified monophyletic group of phytopathogenic bacteria (phylum *Tenericute*s, class *Mollicutes*, family Acheloplasmataceae) (Gundersen *et al.*, 1994), strictly associated with phloem sieve elements of the plants and internal organs of the sap-sucking hemipteran insects (mainly Auchenorrhyncha and Psyllidae) that are their only known vectors (Weintraub and Beanland, 2006). Due to difficulties in *in-vitro* cultivation, phytoplasmas are either classified as taxa based on phylogenetic analysis of molecular data (Lee *et al.*, 2000) or as provisional '*Candidatus*' species according to bacterial nomenclature rules (Murray and Stackebrandt, 1995; IRPCM, 2004). Thirty-five ribosomal groups and 169 subgroups have been recognized based on 16Sr genetic data (Trivellone, 2019). To facilitate finer-scale characterization of some subgroups, other markers, *e.g. secY, tuf,* and *secA,* have been used (Hodgetts and Dickinson, 2010). Despite the availability of molecular phylogenies of phytoplasmas and a general timetree for bacteria (Marin *et al.*, 2016), no attempts have yet been made to estimate divergence times of the detected phytoplasma lineages.

Materials and Methods

The 16S rRNA gene sequence data were employed to reconstruct the phylogenetic relationships of Acholeplasmataceae, including all designated subgroups/ '*Candidatus* species' of phytoplasmas and acholeplasmas, as well as 25 other *Mollicutes* and 2 *Firmicutes* species as outgroups. The maximum likelihood analysis was performed on RAxML v8.2.11 using GTRGAMMAI model with 1,000 bootstrap replicates. To estimate phytoplasma divergence times, RelTime molecular divergence time analyses in MEGA X (Mello, 2018) was performed and applied to two calibrated nodes: the split of *Acholeplasmatales* from *Mycoplasmatales* + *Entomoplasmatales* (1,704.5-2,070 million years ago, Ma) (Sheridan *et al.*, 2003; Marin *et al.*, 2016), and the split of *Mycoplasmatales* from *Entomoplasmatales* (1,581-1,679.4 Ma) (Sjöstrand *et al.*, 2014; Marin *et al.*, 2016).

Results and Discussion

A timetree focused on phytoplasmas is provided here for the first time. The 16S rDNA Maximum Likehold tree supported phytoplasmas as a monophyletic group derived from *Acholeplasma*, saprophytic or parasitic bacteria associated with various plant and animal hosts (Hanajima *et al.*, 2015). Phytoplasmas are sister to a clade containing the plant pathogen *Acholeplasma palmae* and the horse parasite *A. parvum* (Tully *et al.*, 1994; Atobe *et al.*, 1983). The split between phytoplasmas and *Acholeplasma* was estimated at about 663 Ma, much earlier than the emergence of land plants (481-584 Ma) (Kumar *et al.*, 2017), suggesting that the phytoplasma ancestor was originally associated only with animals. Plant parasitism apparently evolved independently in several lineages of Acholeplasmataceae, including phytoplasmas after their divergence from *Acholeplasma*.

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The crown clade of phytoplasmas began to diversify about 330 Ma, which corresponds to the rise of *Spermatophyta* (289-337 Ma) (Kumar *et al.*, 2017) and early radiations of phytophagous Hemiptera (about 300-386 Ma) (Johnson *et al.*, 2018), suggesting that the rise of seed plants and insect vectors played a crucial role in the phytoplasma diversification. Two major phytoplasma clades of phytoplasmas were recovered: one comprised the ribosomal groups 16SrI, XII, XIII, XVI-XVIII, XXIII, XXVIII and XXXI, which began diversifying about 130 Ma ago; the other contained the remaining groups and began to diversify about 257 Ma ago. The timetree suggests that a rapid radiation of phytoplasmas began after the emergence of angiosperms (168-194 Ma) (Kumar *et al.*, 2017) and that many of the numbered 16S ribosomal groups appeared between 20 and 50 Ma ago. Overall, these results suggest that the carboniferous was an important period for the initial spread and diversification of phytoplasmas and, therefore, that the diversity of phytoplasmas may have been driven by co-evolution, and co-radiations, with plants and insect vectors. Besides providing crucial information for understanding the evolutionary history of lineages, this first timetree of phytoplasmas offers new insights into the understanding of the biological relationships with their insect, plant and ancestor hosts. To overcome the lack of a fossil record in bacteria, which could provide calibration points closer to the tips of the phylogeny, more detailed analyses of the 16SrV group using faster-evolving genes are underway. These will incorporate a date estimates for more recent divergence events that can be used to refine the overall divergence time estimates for phytoplasmas.

Acknowledgements

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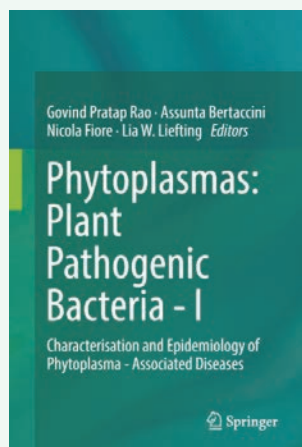
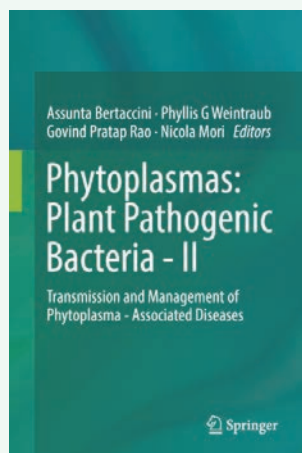Editors: G.P. Rao, A. Bertaccini, N. Fiore, L.W. Liefting

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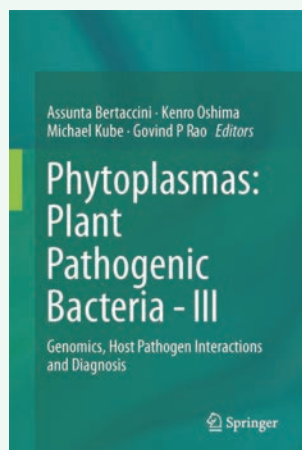Editors: A. Bertaccini, P.G. Weintraub, G.P. Rao, N. Mori

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