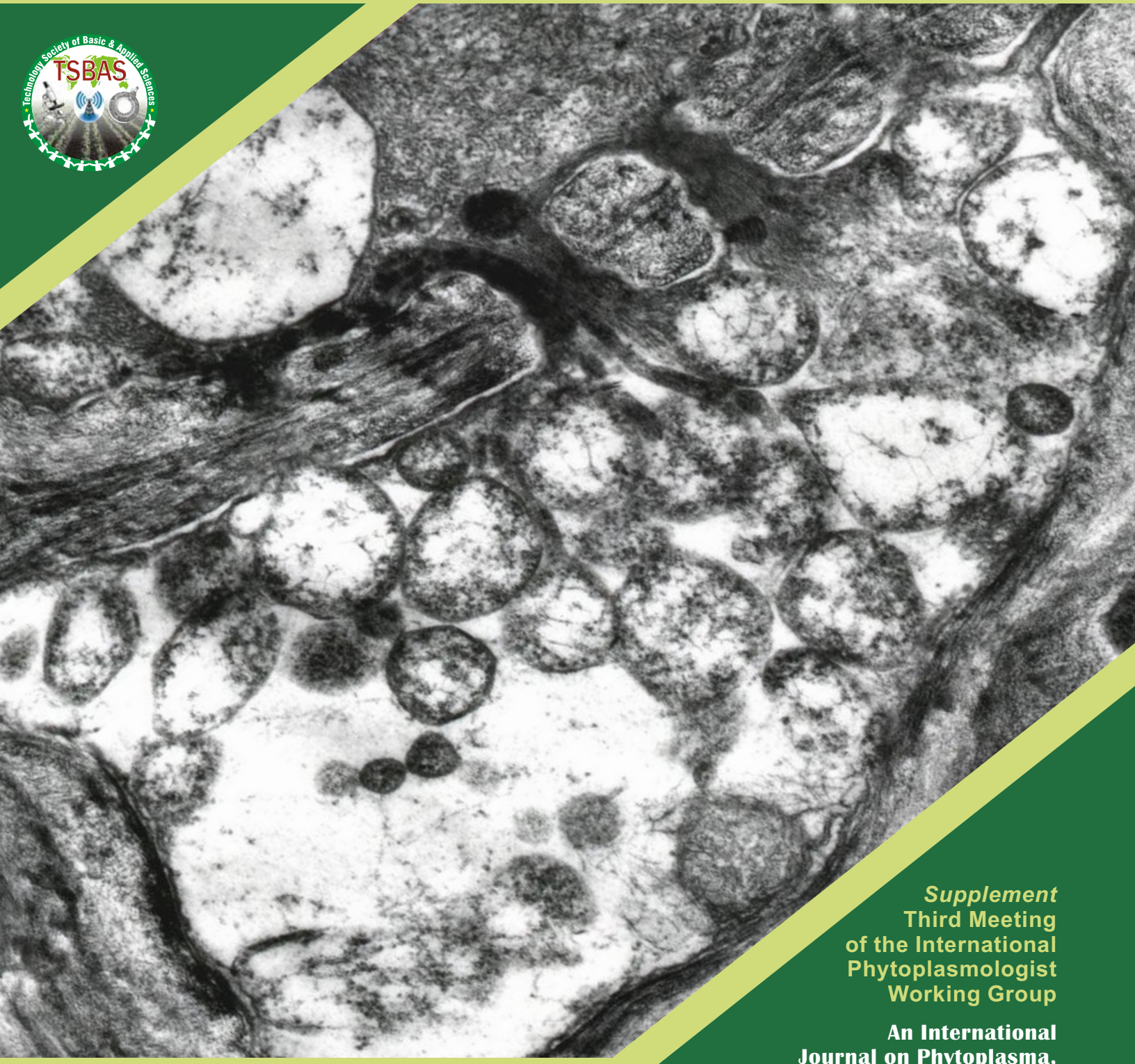
Vol. 5 (1-Supplement), January 2015 • Print-ISSN : 2249-4669 • Online-ISSN : 2249-4677

Phytopathogenic Mollicutes



Supplement
Third Meeting of the International Phytoplasmologist Working Group

An International Journal on Phytoplasma, Spiroplasma and other 'Pholem-limited Plant Pathogens'

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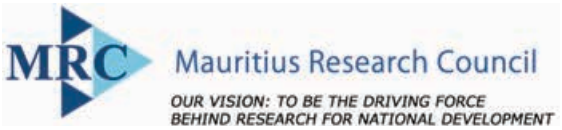

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Third International Phytoplasmologist Working Group Meeting

Mauritius
January 14-17, 2015

Edited by Assunta Bertaccini and Govind P. Rao

This meeting is dedicated to the 100th birthday of
Prof. Karl Maramorosch

Preface

"Phytoplasma research has progressed greatly during the forty years since the fastidious pathogens have been identified by plant pathologists and entomologists in Japan. The intricate interactions between insect vectors and phytoplasmas continue to attract plant pathologists, entomologists, and molecular biologists, who contribute new findings from laboratories around the world. I still remember the time when phytoplasma diseases were believed to be caused by viruses. I also recall the time when tubercle bacteria could not yet be cultured in artificial media and,together with leprosy and syphilis pathogens, were considered fastidious. Until 1960 mycoplasmas were called fastidious PPLO, but Channok and Hayflick succeeded in growing them in a culture medium and the PPLO name was changed to mycoplasmas. Phytoplasmas multiply in plants and specific invertebrate animal vectors, but not yet in artificial culture media. I hope that collaboration between phytoplasma researchers and microbiologists will eventually result in the cultivation of the fastidious phytoplasmas. [...] Science recognizes no political, religious, ethnic, or geographic borders and scientists speak only one language – the language of science. Scientists can collaborate with each other, irrespective of background and political believes [...] (from the letter to 1st IPWG 2007)

Best wishes to Assunta Bertaccini and all who came to the 3rd IPWG meeting in Mauritius today. Although I am unable to be with you in person on my 100th birthday, I am happy to be with you in my thoughts." (November 2014)

Karl Maramorosch

Welcome by the editors

We are delighted to edit the extended abstract book of the "Third International Phytoplasmologist Working Group Meeting" that will be held in Mauritius next January 2015.
The group born in Bologna in 2007 is actively working in entomology, molecular biology, and plant pathology to increase and expand knowledge about phytoplasma diseases worldwide. The meeting is the forum for sharing information and strength and/or built new and more intense interactions among participants.

Exciting finds since the last meeting in 2011 in Germany in the phytoplasmology field continue to be published: more pathogenicity factors unveiled, insect vector-phytoplasma interaction mechanisms further clarified, some metabolic pathways discovered by mining the full sequences of a number of phytoplasma genomes and last and hopefully not least first steps toward their cultivation in axenic culture and more that will be presented during this meeting.
The European founded project COST FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems, that ended in December 2013, was helping the realization of harmonized and sustainable management of phytoplasmas diseases avoiding the use of antibiotics and further disseminate the knowledge and the skills to work in this research field. The only way to control the insect vector populations and diseases is an integrated approach based on solid scientific knowledge of biological and epidemiologic feature of phytoplasma associated diseases.
This meeting in Mauritius is a great opportunity for all of us, as both organizers and participants, to share and further disseminate mutual experience in such important field to agriculture in important areas of the world where phytoplasma diseases are among the most dangerous to agriculture and landscaping.

It is a pleasure of the editorial board of the Phytopathogenic Mollicutes to accept extended abstract by experts in Phytoplasmology, the new bacteriology branch very strictly related to plant pathology, entomology, genetics and molecular biology.

Over the days of the conference, there will be 55 presentations distributed over 7 sessions; to the traditional topics it was added one session about mixed infection of phytoplasmas and other pathogens to open the field to the concept of pathological communities as agent of plant diseases. Two round tables on "burning" topics such as phytoplasma classification and yellowing diseases of palm are also planned. The invited presentation finally will try to show guidelines for future research that to lead the field to fruitful results to agriculture improvement and valorisation.
All the papers published in this issue have been reviewed and accepted by the IPWG Scientific Committee. 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.
As editors we would like to extend our gratitude to all of the Scientific Committee members. Many thanks also to Fabio Montanari for its great assistance during the preparation of the extended abstract book of the third IPWG meeting published as a Supplement issue of the Phytopathogenic Mollicutes 2015 vol. 1.

The scientific committee members and the editors discharged their refereeing responsibilities. Any errors could be indicated, till May 31, 2015, by authors sending e-mail to the Phytopathogenic Mollicutes secretary. Unintentional substantial mistakes will be corrected adding an errata corrige, as a last page of the 'electronic reprint', on the online version of the third IPWG meeting extended abstract book.
Online information concerning the IPWG proceedings is available at the following web addresses:
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An International Journal on Phytoplasma, Spiroplasma and other Phloem-limited Plant Pathogens

Published by :
Technology Society of Basic and Applied Sciences

mollicutes.indianjournals.com

Print ISSN : 2249-4669
Online ISSN : 2249-4677

Volume 5 (1-Supplement), January 2015

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S1-S4

doi: 10.5958/2249-4677.2015.00001.8

Phytoplasma research between past and future: what directions?

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Abstract

The great majority of research in phytoplasma field was devoted in the last decades to classify and differentiate these prokaryotes on the basis of molecular and bioinformatic tools. Inspite to the development of a robust and quite exhaustive classification system that allowed to further develop phytoplasma multilocus typing on diverse genes, biology represents still the very unknown part in phytoplasma research. However a deep and sound knowledge of phytoplasma biology is needed to allow the definition of feasible solutions to reduce phytoplasma disease impact on worldwide agriculture.

Keywords: phytoplasmas, taxonomy, biology, insect vector, plant disease, epidemiology

Introduction

In the past years the great majority of research in phytoplasma field was devoted to classify and differentiate these prokaryotes on the basis of molecular and bioinformatic tools. This part of research is well defined by the following sentence: "...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" (Mayer, 1942). The development of a robust and quite exhaustive classification system allowed to further develop phytoplasma multilocus typing on several diverse genes, mainly according to the different '*Candidatus* Phytoplasma' species' (IRPCM, 2004).

However this research could end in a sterile play, producing shopping lists of genotypes, if the knowledge of phytoplasma biology is not accompanying their taxonomy. A lot of information were achieved by full genome sequencing, however these are mainly related to putative biochemical pathways, all showing that phytoplasmas are very special microorganisms because they lack a lot for relevant features such as cell wall, mobility, key enzymes, pathways. However the small phytoplasma chromosome is very efficiently working and code tricky metabolic pathways that allow phytoplasmas to a trans kingdom life of interaction with both plant and insect hosts.

Phytoplasma presence, beside inducing severe and very rapidly spreading diseases, can increase metabolic activity of their hosts, modify insect fitness, increase plant shoot production and change flower shape and color (Figure 1). In evolutionary sense phytoplasmas seem to evolve toward becoming permanent cell hosts. However they are still far from loosing independence and freedom as they still act as very dangerous pathogens for many relevant agricultural crops.

Biology represents the very unknown part in phytoplasma research. Recently small steps toward these needed biologic studies were achieved, and should allow confirmation of the numerous molecular data reported about potential pathogenetic and metabolic aspects. The knowledge of phytoplasma biology will help in improved experimental definition of feasible solutions to reduce their impact on worldwide agriculture suggesting the best management. It is a well know strategy that in order to be able to control the enemy it must be known very well.

Figure 1. On the left symptoms of phyllody and virescence in a chrysanthemum inflorescence infected by 16SrI-B phytoplasmas (from Bertaccini *et al.*, 1990). On the right tomato plant showing symptoms due to "stolbur" phytoplasma only on the top part of the plant; regular tomato are produced in the bottom asymptomatic part of the plant.

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What we know

Phytoplasmas are introduced by insect vectors during feeding activity into plant sieve tube elements from which they move systemically through the plants (Figure 2). They are mainly spread among plants by insects in the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers), and Psyllidae (psyllids), which feed on the phloem sap of infected plants, therefore phytoplasma host range is strongly dependent upon feeding preferences of insect vectors. Phytoplasmas may overwinter in insect vectors or in perennial plant hosts, and interact in various ways with insects: examples of both reduced and enhanced fitness while they are in the vectors (Sugio *et al*., 2011) and transovarial transmission in certain phytoplasma-vector combinations were demonstrated (Alma *et al*., 1997; Kawakita *et al*., 2000; Hanboonsong *et al*., 2002; Tedeschi *et al*., 2006). The possibility of seed transmission was also shown on commercial seedlings of alfalfa, tomato, corn and winter oil seed rape (Khan *et al*., 2002; Botti and Bertaccini, 2006; Calari *et al*., 2011; Chung and Jeong, 2014). Phytoplasmas can also be efficiently spread via vegetative propagation such as the grafting of infected plant tissues as shoots onto healthy plants, the vegetative propagation through cuttings, and the micropropagation practices and other methods used to multiply plant materials.

Phytoplasmas possess the smallest genome among bacteria, however gene duplication and redundancy are well represented: it was estimated that in the genome of the onion yellows (OY) phytoplasma the 18% of gene complement is represented by multiple redundant copies of only five genes, that are generally present in single copy (Oshima *et al*., 2004). However extrachromosomal DNA or plasmids of various sizes have also been found in all members of the aster yellows phytoplasma group (16SrI) and "stolbur" (16SrXII), as well as in some members of the X-disease (16SrIII) and the clover proliferation (16SrVI) groups (see in Bertaccini *et al*., 2014).

Since many phytoplasma genes encode transporter systems, some of which are present as multiple copies it was suggested that they aggressively import many metabolites from their host cells, inducing metabolic imbalance and disease symptoms in plants. It was also demonstrated the lack of APT synthase genes that is suggesting that ATP synthesis may be strongly dependent on glycolysis. Interestingly, two sets of 5 glycolytic enzymes were encoded in a duplicated genomic region of '*Candidatus* Phytoplasma asteris' strain OY-W, a strongly pathogenic strain, while mild strains and other aster yellows strains do not possess this duplication, suggesting this feature as unique to this strain as possible pathogenicity mechanism (Oshima *et al*., 2007). Glycolysis genes are completely absent in '*Ca*. P. mali', that possesses a gene encoding 2-dehydro-3-deoxyphosphogluconate aldolase leading to the hypothesis that in this phytoplasma pyruvate is formed independently from glycolysis (Kube *et al*., 2012). It is possible that pathogenic mechanisms may be different according with the strain and/or the diverse environmental conditions such as different host species.

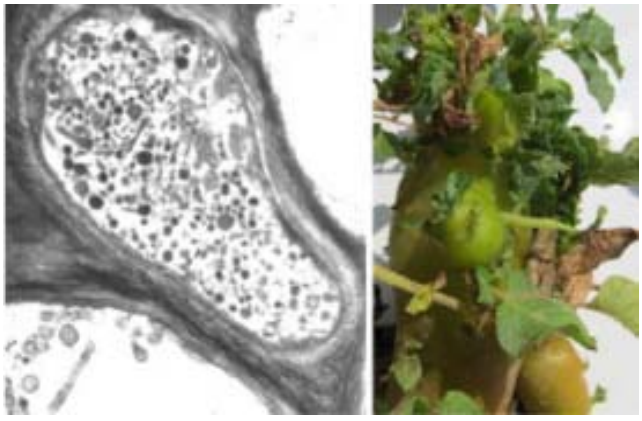

Figure 2. On the left electron microscopy picture of cross section of sieve tubes with phytoplasmas (X 6,000) and on the right symptoms associated with phytoplasma presence in potato tubers.

The occurrence of major surface epitopes, that are unique to each phytoplasma species, suggests that these proteins are key participants in specific interactions with host cells. Genes encoding these proteins have been identified in several phytoplasma groups and are classified into: immunodominant membrane protein (Imp), immunodominant membrane protein A and antigenic membrane protein (Amp). The Amp protein of the OY phytoplasma forms a complex with insect microfilament composed by actin and myosin that was correlated with the phytoplasma-transmitting capacity of leafhoppers (Suzuki *et al*., 2006; Galetto *et al*., 2011). Genes encoding Imp were not correlated with polymorphism of the *16S rRNA* gene suggesting that the variability of immunodominant membrane proteins reflects factors other than evolutionary time. The sequence identity of Imp was found to be low among the different phytoplasma groups, however the gene organizations flanking Imp were well conserved in most phytoplasmas (Kakizawa *et al*., 2009).

Since phytoplasmas are intracellular parasites of both plants and insects, their ability to adapt to two diverse environments is of considerable interest. The genes encoded in the PMUs of '*Ca*. P. asteris' strain AY-WB are more highly expressed in insects than in plants, most likely due to increased production of the extrachromosomal circular type of PMU during insect infection (Toruno *et al*., 2010). Microarray analysis of '*Ca*. P. asteris' strain OY-M revealed that expression of approximately 33% of the genes changes during host switching between plant and insect (Oshima *et al*., 2011) and the phytoplasma may use transporters, secreted proteins, and metabolic enzymes in a host-specific manner. Differential gene expression between plant and insect hosts has been also reported in the same strain, in which TENGU, a small secreted protein identified in the phytoplasma genome, was shown to induce in transgenic *Arabidopsis thaliana* symptoms similar to those induced by phytoplasma presence; moreover the protein is more highly expressed in plant than in insect hosts (Hoshi *et al*., 2009).

Phytoplasmas were also shown to possess two known secretion systems, the YidC system for the integration of membrane proteins, and the Sec system for the integration and secretion of proteins into the host cell cytoplasm; a functional Sec system seems to be common to most or all phytoplasmas. The identification of effector proteins such as SAP11, SAP54 and P38 suggest that phytoplasmas could also aggressively induce symptoms by secretion of effector proteins and modification of plant-gene activity (Sugio *et al*., 2011; MacLean *et al*., 2011; Neriya *et al*., 2014).

What is new

Recently a preliminary evidence that phytoplasmas can be grown in or on cell free laboratory media has been reported (Bertaccini *et al*., 2010; Contaldo *et al*., 2012; 2013). This is an important breakthrough in the study of their biology since, despite a reduced genome size in comparison to their ancestors, phytoplasmas retain an independent metabolism that allows them to survive as parasites in environments as diverse as plant phloem and insect haemolymph. This versatility is a unique property among microbes, shared only with some animal- or plant-infecting viruses and with a few other microorganisms such as the causal agent of malaria. Employing specific commercially available media, it was possible to achieve axenic growth of phytoplasmas belonging to diverse ribosomal groups from micropropagated periwinkle shoots, infected with phytoplasmas, as an initial source of inoculum. In spite of the relatively long time required for incubation in liquid medium, colony growth of phytoplasmas on agar usually occurs within two to five days, as for the majority of cultured bacteria. Although further research is needed and is in progress in order to optimize the culture system, the prospect of routine cultivation is now an option for researchers. When observed with a binocular microscope, the phytoplasma colonies on the surface of agar medium are comparable in size and appearance to those of other cultured *Mollicutes* (Figure 3).

PCR assays using phytoplasma specific primers confirmed that phytoplasma DNA was present in the growing cells used as a source of DNA template. Identification using RFLP analysis and direct sequencing of selected amplicons also confirmed the phytoplasma identity. The more recent success of phytoplasma isolation was achieved from naturally infected plants such as grapevine infected with "flavescence dorée" and "bois noir" associated respectively with the presence of 16SrV and 16SrXII phytoplasmas. This confirms the feasibility of further studies on the biology of these organisms using strains that are in epidemic phases, therefore should help in better understanding and confirmation of their pathogenicity characteristics. Typical phytoplasma colonies were obtained in 24 to 72 hours from tubes inoculated with symptomatic material after appropriated incubation time (Figure 4). Specific PCR/RFLP assays on 16S rDNA and direct sequencing confirmed the identity of phytoplasmas in colonies, to the ones detected in the original grapevine samples (Contaldo *et al*., 2014).

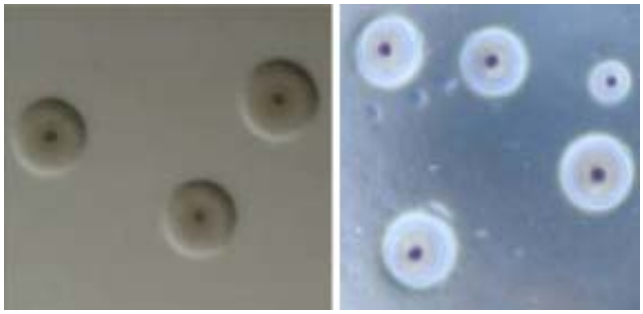

Figure 3. Mollicutes colonies growing on agar: on the left *Acholeplasma* colonies and on the right aster yellows phytoplasma colonies grown on PivS medium (courtesy David and Helena Windsor on the left and of Nicoletta Contaldo on the right).

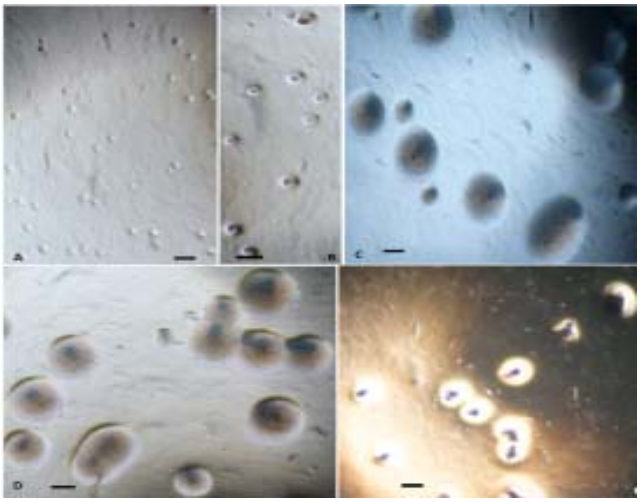

Figure 4. Phytoplasma colonies from infected grapevine, on PivS medium at 25 + 1°C photographed at 40 × magnification. A) B) and C), colonies derived from phytoplasma infected grapevine incubated for 24, 48 and 72 hours respectively; D), and E) phytoplasma colonies photographed after 4 days incubation: D) "flavescence dorée" phytoplasmas and E), "bois noir" phytoplasmas (Bars = 250 µm) (courtesy Nicoletta Contaldo).

One of the most immediate benefits to axenic culture of phytoplasmas is a genome sequencing to compare and contrast gene complement and metabolic pathways of strains representative of each phytoplasma 16Sr group. Currently genome sequencing is very inefficient, largely because of the intimate association of phytoplasmas with their respective host plants and insect vectors, therefore the availability of phytoplasma colonies should help. It is likely that whole genome comparisons will emerge as the premier tool for phytoplasma identification in the near future. Alternatively, or in concert with genomics, biochemical and physiological tests should now be feasible and used to implement phytoplasma taxonomy.

As a consequence, strategies targeting treatment or prevention of phytoplasma diseases could be better defined and more effective. In addition, more knowledge about basic mechanisms that regulate the survival of phytoplasmas, which are among the smallest known self replicating

organisms, will be obtained. The recognition of durable resistance to phytoplasma diseases is the only effective long term mean of controlling these diseases. Selection and screening of plants resistant to phytoplasma infection as well as the study of the modes of colonization by phytoplasmas of plant and insect vectors are other possible applications. As a consequence strategies aiming to treat and/or prevent phytoplasma related plant diseases could be better defined and more effective.

Acknowledgements

The author like to thanks all colleagues in the phytoplasma field worldwide with who she has, have and will have collaborations, and in particular the Bologna Phytobacteriology laboratory team.

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Phytoplasma genomic

Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S7-S8

doi: 10.5958/2249-4677.2015.00002.X

High-throughput transcriptome analysis of the leafy flower transition of *Catharanthus roseus* induced by peanut witches' broom phytoplasma infection

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Abstract

Peanut witches' broom (PnWB) phytoplasmas are obligate bacteria that cause leafy flower symptoms in *Catharanthus roseus*. The PnWB-mediated leafy flower transitions were studied to understand the mechanisms underlying the pathogen-host interaction. The whole transcriptome profiles from healthy flowers and stage 4 (S4) PnWB-infected leafy flowers of *C. roseus* were investigated using next-generation sequencing (NGS). More than 60,000 contigs were generated using a *de novo* assembly approach, and 34.2% of the contigs (20,711 genes) were annotated as putative genes through name-calling, open reading frame determination, and gene ontology analyses. Furthermore, a customized microarray based on this sequence information was designed and used to further analyze samples at various stages. In the NGS profile, 87.8% of the genes showed expression levels that were consistent with those in the microarray profiles, suggesting that accurate gene expression levels can be detected using NGS. The data revealed that defense-related and flowering gene expression levels were altered in S4 PnWB-infected leafy flowers, indicating that the immunity and reproductive stages of *C. roseus* were compromised. The network analysis suggested that the expression levels of more than 1,000 candidate genes were highly associated with *CrSVP1/2* and *CrFT* gene expression, which might be crucial in the leafy flower transition. This study provides a new perspective for understanding the mechanisms underlying the leafy flowering transition caused by host-pathogen interactions through analyzing bioinformatics data obtained using a powerful, rapid high-throughput technique.

Keywords: next-generation sequencing, microarray, *Catharanthus roseus*, peanut witches' broom phytoplasma, transcriptome, leafy flower

Introduction

Incomplete genomic information is a limiting factor for investigating the mechanisms of unique phenomena in non-model organisms. After the publication of a next-generation sequencing (NGS) study by Margulies *et al.* (2005), many high-throughput approaches based on this concepts have been developed in various areas that include *de novo* transcriptome analysis. *De novo* transcriptome analysis with NGS has revolutionized the study of gene expression profiles in non-model organisms under various conditions or treatments.

Catharanthus roseus was used as a host for study phytoplasma infection (Liu *et al.*, 2014). Various phytoplasmas, such as peanut witches' broom (PnWB) and aster yellows witches' broom, are associated with the presence of leafy flower symptoms in *C. roseus* and *Arabidopsis* (Liu *et al.*, 2014; MacLean *et al.*, 2014). In this study the whole transcriptome profiles of *C. roseus* were analyzed using next-generation sequencing (NGS), and resulted in the identification of 20,711 genes. These sequences were used to design a customized microarray for verifying the gene expression levels during PnWB infection. The gene expression accuracy was evaluated by comparing the NGS data with the microarray profiles. In addition, an entire leafy flower transition network was generated by connecting ABC model genes, and genes involved in the PnWB-mediated leafy flower transition were discovered. The mechanism of the leafy flower transition in *C. roseus* during PnWB infection was investigated.

Materials and Methods

Total RNA was extracted from 10 healthy flowers (HF) stage 4 (S4) leafy flowers. The whole transcriptome profiles of the HF and S4 flowers were analyzed using an Illumina Hiseq 2000. The paired-end reads were then assembled using the standard protocol of *de novo* assembly in CLC genomics Workbench. The transcriptome expression profiles and the database were analyzed and constructed using the ContigViews server (http://contigviews. csbb.ntu.edu.tw). The gene expression profiles generated from the customized

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microarrays were subjected to network analysis to investigate PnWB-mediated leafy flower development.

Results

PnWB-infected *C. roseus* flowers exhibit various floral abnormalities, including discoloration, virescence, and phyllody, depending on the number of days after infection. Figure 1 shows 5 categories of floral abnormalities in PnWB-infected *C. roseus* plants. Stage 1 (S1) flowers show abnormal petals with partial virescence compared to healthy flowers; stage 2 (S2) flowers show smaller and virescent petals. Phyllody malformations develop in stage 3 (S3) flowers, which are fully virescent and have partially pink petals. A leaf vein pattern was observed in the stage 4 (S4) flowers, which also had phyllody in petals. Finally, full phyllody in petals characterize stage 5 (S5) flowers. The corolla tube was shorter in infected floral organs than in healthy organs, and it shortened as the infection developed (S1 to S5). A swollen ovary was observed in S3 and S4, and a cracked swollen ovary was occasionally observed.

Moreover, the flowers developed whorl-like structures and small leaves in S5. Through the mixed reads *de novo* assembly approach, the longest 60,580 *C. roseus* contigs were identified among 253 million transcriptome reads. Analyzing the efficiency of these contigs and identifying the crucial genes involved in the PnWB-mediated leafy flower transition are major areas of interest.

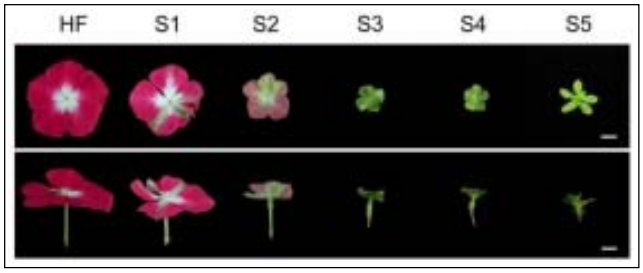

Figure 1. Five categories of PnWB-infected leafy flower symptoms compared with healthy flowers of *C. roseus*. S1 (Stage 1): abnormal petals and partial virescence; S2 (Stage 2): small and virescent petals; S3 (Stage 3): fully phyllody and partially pink petals; S4 (Stage 4): phyllody in petals with leaf vein pattern; S5 (Stage 5): fully phyllody in petals; HF: healthy flower. Bar = 1 cm. (from Liu *et al.*, 2014).

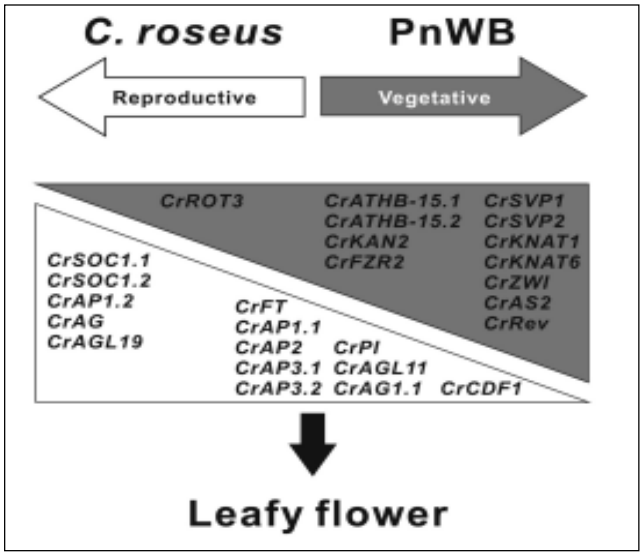

Figure 2. Model for leafy flower formation during PnWB phytoplasma infection. The arrows represent the direction of host plant development. The triangles represent gene expression levels. The gray color represents the vegetative development. The white color represents the reproductive development. The black arrow represents the leafy flower transition (from Liu *et al.*, 2014).

Discussion

In this study, a pipeline for whole-transcriptome analysis in non-model organism based on NGS and customized microarray profiles was designed. The *C. roseus* and PnWB interaction increases the interest in discovering the mechanism of the PnWB-mediated leafy flower transition. A model for the PnWB-mediated leafy flower transition shown in Figure 2 is proposed. In the *C. roseus* floral meristem, gene expression is predicted to promote a switch from the vegetative stage to the reproductive stage. However, in PnWB-infected *C. roseus*, the pathogen represses certain flowering genes, but turns on leaf development genes. Moreover, the flowering repressors *CrSVP1* and *CrSVP2* are highly expressed and are triggered by PnWB to suppress the reproductive stage. These alterations switch the development of the host to the vegetative stage. However, the original developmental direction in the floral meristem of *C. roseus* is from vegetative to flowering, and certain unrepressed flowering genes, were still highly expressed in the PnWB-infected floral meristem. Therefore, 2 groups of genes (reproductive and vegetative) were abnormally co-expressed in the floral meristem, resulting in leafy flower formation.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S9-S10

doi: 10.5958/2249-4677.2015.00003.1

High-throughput analysis and characterization of *Ziziphus jujube* transcriptome jujube witches' broom phytoplasma infected

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Abstract

Jujube witches' broom disease caused devastating damages in some Chinese orchards. To study this disease, transcriptome sequencing was used for obtaining information on gene expression that can greatly facilitate studies of this plant species for which a sequenced genome is not available. In the present study 45,132 unigenes were identified from contigs dataset, and functional annotation was carried out in different systems. The results show the presence of some pathogenesis-related pathways in jujube during phytoplasma infection.

Keywords: high-throughput analysis, jujube witches' broom phytoplasma, transcriptome sequence

Introduction

Chinese jujube (*Ziziphus jujuba* Mill.) is a native fruit and medicinal plant in China. However, jujube witches' broom (JWB) disease causes serious problems for its industry. Large quantities of infected jujube plants have been cut down, and consequently the jujube cultivation in China is declining. For a long time, the work was carried out for the characterization of the disease pathogen, while information on its genetic background is scarce and there is little transcriptome research on the disease. Transcriptome sequencing would provide a basis for detailed studies of gene expression and genetic relationships to plant secondary metabolism (Marquez *et al*., 2012). Transcriptome data contain a large amount of genetic information that can be used for many purposes including: investigations of alternative splicing; generation of gene sequences from non-model organisms that lack a reference genome, discovery of novel genes, analysis of gene expression, identification of SSRs and single-nucleotide polymorphism. While RNA-seq technology has been successfully used in species such as potato, grapevine and peanut (Garg *et al*., 2011; Yuan *et al*., 2012), to date, little is known about gene expression related to JWB, by analysing jujube gene expression while is infected by phytoplasmas. Identification of candidate genes involved in the biosynthetic pathway present in infected plants will significantly contribute to the understanding the physiology and the methabolism of the infected plant (Guozhong *et al*., 1994). Transcriptomes and metabolic pathways were therefore analyzed to evaluate the compounds in diseased plant and to explore this approach usefulness to provide possible new tools for disease control.

Materials and Methods

Samples of jujube (cultivar Langzao) (10 year-old, 6 m) represented by flowers and leaves were collected from symptomatic (PF, PL) and healthy (HF, HL) plant in Pomology Institute, Shanxi Academy of Agricultural Sciences (Taigu, China). Total RNA was extracted from pooled samples of leaves using a total RNA kit (RNA simply; Tiangen, Beijing, China) according to the manufacturer's instructions. The total RNA extracted was employed for sequencing using Illumina HISeq2000. cDNA library was produced and after sequence assembly, the unigenes determined were used to build up a gene expression profile. The putative unigene function annotation was carried out by NT, NR, Swiss-Prot, and KEGG, Interpro and gene ontology, GO classification, to determine the metabolic pathways of differentially expressed genes.

Results and Discussion

A total of 45,132 unigenes (DEGS) were identified from contigs dataset (Figure 1), and about 41,456 ORF were identified. A further functional classification of all unigenes was performed using a set of plant-specific GO slims (Figure 2). A total of 77,553 unigenes (98.37%) were assigned to 47 functional groups using GO assignments, including biochemistry, cell apoptosis, growth, development, and metabolism. Within each of the three main GO categories, biological process (31,434 unigenes), cellular component (17,650 unigenes) and molecular function (28,469 unigenes) were identified. To further evaluate the completeness of the transcriptome library and the effectiveness of annotation

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process, the annotated unigene sequences were used to search for the genes in the COG classifications.

Among the 24 COG categories (Figure 3) that were assigned to unigenes, following the expression study by cluster 3.0 analysis all genes and unigenes were determined (Figure 4). A total of 686 unigene sequences were assigned to 260 KEGG pathways. The most relevant pathways were "metabolic pathways" and "genetic information processing". The metabolism pathways were involved in the amino acid and secondary metabolism. The results show that RNA1 polyprotein OS (*Cowpea mosaic virus*, *Broad bean wilt virus 1*) has an high expression; 96 unigenes take part in the regulation of amino acid metabolism, including amino acid related enzymes, Ala. Asp. Glu. Gly. Ser. Thr. Arg. Pro. Lyr. Vsl. Ile. Phe. Vsl. Trp. Except in Phe, about 6 genes were down regulated and only 2 genes upregulated. Metabolism of terpenoids, polyketides and peroxidase activity show an increase in diseased samples.

For phytoplasma infected *Z. jujuba* the transcriptome sequencing provided large transcript information, which can be a useful tool for studying the molecular and genetic mechanisms of plant infection. The increase of the peroxidase activity can promote the formation of jujube witches' broom symptoms. If terpenoids and polyketides compounds have a role in plant resistance needs further studies. During secondary metabolites biosynthesis, a cytochrome P450 upregulation was identified that also need to be further studied.

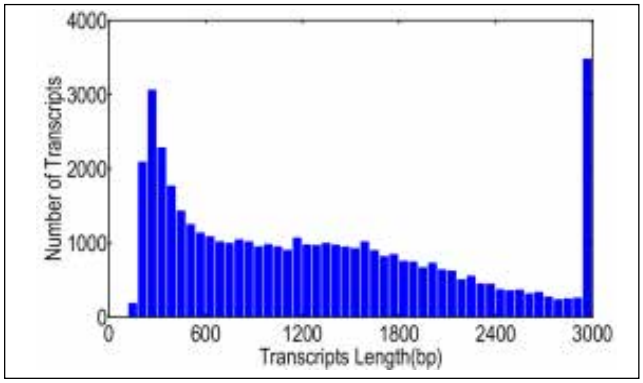

Figure 1. Number of DEGS identified from contigs dataset after high-throughput analysis.

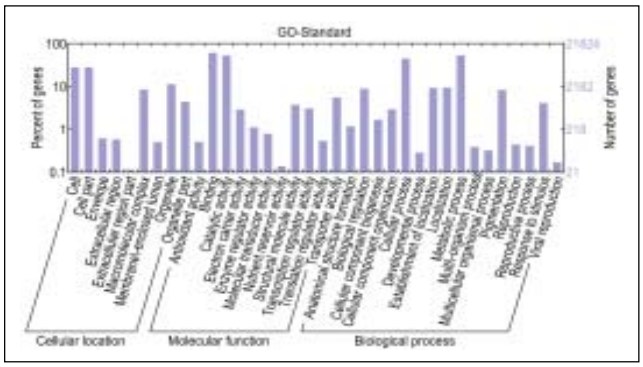

Figure 2. GO classification of the DEGs in transcriptomes.

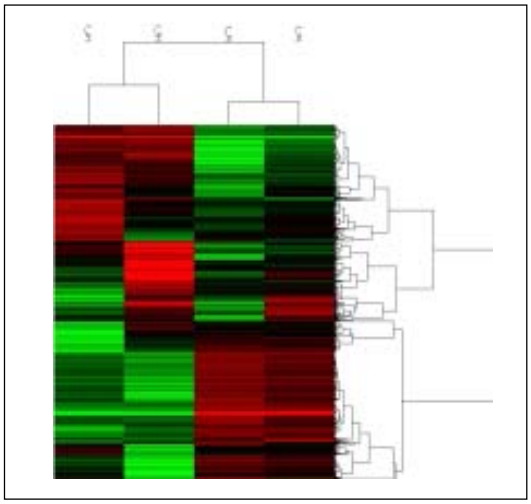

Figure 3. COG classification assigned to the unigenes. Histogram presentation of clusters of orthologous groups (COG) classification.

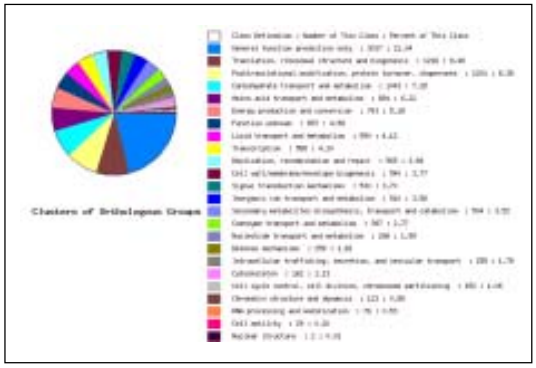

Figure 4. Cluster of the DEGs.

Acknowledgements

We would like to thank Shanxi Scholarship Council of China and Department of Human Resources and Social Security of Shanxi Province for funding.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S11-S12

doi: 10.5958/2249-4677.2015.00004.3

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Utilization of *dnaB* gene for characterisation of phytoplasmas associated with toria, brinjal and *Phlox* in India

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Abstract

In the present study a new set of primers was designed, which amplified 961 bp fragment of *dnaB* gene. The *dnaB* gene-based primers were successfully utilized for identification of phytoplasmas belonging to 16SrVI and 16SrXI groups associated with phlox little leaf and yellowing, toria phyllody and brinjal little leaf. This *dnaB* gene primer set could be used as an alternative PCR-based diagnostic tool for rapid and specific assay for both specific detecting and confirming the presence of phytoplasmas that affiliate with 16SrVI and 16SrXI ribosomal groups.

Keywords: *dnaB* gene, phytoplasma, 16SrIX, 16SrVI, India

Introduction

Phytoplasma classification and '*Candidatus* Phytoplasma' taxon are mainly based on 16S rDNA sequence. This procedure does not always provide molecular distinction of closely related phytoplasma strains. More variable single copy genes, such as ribosomal protein (*rpl22* and *rpS3*), *secA, secY, tuf,* and *groEL* were employed for finer differentiation (Marcone *et al*., 2000; Lee *et al*., 2004; 2006; Martini *et al*., 2007; Mitrovic *et al*., 2011). Very limited attempt has been made with *DnaA, dnaB* and *dnaK* genes for characterization of phytoplasmas (Music *et al*., 2007; Balakishiyeva *et al*., 2011). The DnaB helicase (product of the *dnaB* gene) in bacteria opens the double helix during DNA replication (Fass *et al*., 2009). In the present study, a PCR system was developed for *dnaB* gene and it was tested and validated with phytoplasma infected samples of *Toria* phyllody (HM559246) 16SrIX (Azadavar *et al*., 2010), *Phlox* little leaf (KC178678) 16SrIX (Madhupriya *et al*., 2013), and brinjal little leaf (JQ409544) 16SrVI (Azadavar and Baranwal, 2012).

Materials and Methods

The nucleic acid from leaf midribs of symptomatic toria, brinjal and *Phlox*, was extracted following the procedure described by Ahrens and Seemüller (1992). The phytoplasma presence was confirmed using nested polymerase chain reaction with phytoplasma specific primers for amplification of *16SrRNA* gene. Negative control were represented by DNA extracted from healthy samples from the same species. For amplification of *dnaB* gene dnaBF/dnaBR (5'-AAACCCATTTTCCCCAAGAT-3'/5'-TCAGGGGA TTT TCCTTGGTA-3') group specific primers were designed. Reactions were carried out in a thermal cycler (Eppendorf, Germany) and the cycling protocol used for the PCR assays was 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 62°C (P1/P6), 56°C (R16F2n/R16R2) and 57°C (dnaBF/dnaBR) for 1 minute, and extension at 72°C for 2 minutes, with extension in the final cycle for 10 minutes, the resulting PCR products were detected in a gel documentation unit (XR documentation system, Bio-Rad, USA). PCR purified products were directly sequenced. The *dnaB* gene sequences were assembled using BioEdit software. A database search of homologous sequences was performed by BLAST analysis at NCBI (http://ncbi.nlm. nih.gov/BLAST). The *dnaB* gene sequences were aligned with phytoplasma group/subgroup representatives available in GenBank using ClustalW. Phylogenetic relationship of the three tested phytoplasmas with other group representative phytoplasma sequences were assessed based on sequences of *16SrRNA* and *dnaB* genes. Sequence alignments were performed using CLUSTAL W. Phylogenetic tree and molecular evolutionary analyses were performed with MEGA 5.0 software (Tamura *et al*., 2011) using the neighbour-joining method with default values for bootstrap analysis. *Acholeplasma laidawaii was* used as the outgroup to root the trees.

Results and Discussion

Approximately 961 bp amplicon was obtained from all samples from the three symptomatic plants using dnaBF/dnaBR primer pair. No amplification was obtained from healthy samples (data not shown). The consensus sequence of *dnaB* gene of phytoplasmas from *Phlox* (KC290925, 754 bp); brinjal (KC290924, 759 bp) and toria, (KC290923, 803 bp) were submitted to GenBank. BLAST analysis of the 16S rDNA partial sequence of toria and *Phlox* phytoplasmas revealed maximum identity of 98-99% with pigeon pea witches' broom (16SrIX) while brinjal little leaf phytoplasma showed 99% similarity with clover proliferation (16SrVI) phytoplasma group.

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Phylogenetic analysis of *dnaB* gene sequences (Figure 1) showed that phytoplasmas from toria, phlox and brinjal grouped with strains belonging to 16SrIX and 16SrVI groups respectively, and this result was further supported by phylogenetic analyses of *23S rRNA* gene (Figure 2). Both toria and phlox phytoplasma strains clustered in one group along with strain related to '*Candidatus* Phytoplasma phoenicium'. Comparison of 754 and 803 bp sequences of *Phlox* and toria strains showed that the phytoplasma associated with toria phyllody had maximum identity of 99% to the *dnaB* gene sequence of *Phlox* which belongs to 16SrIX group, while brinjal little leaf phytoplasma strain (759 bp) cluster with members of 16SrVI group. The *23S rRNA* and *dnaB* gene phylogenies confirmed that *Phlox* and toria phytoplasmas are clustering with pigeon pea witches' broom phytoplasmas (16SrIX) group, while brinjal little leaf cluster with phytoplasmas in the clover proliferation (16SrVI) group. Music *et al*., (2007) used *dnaB* pseudogene single-strand conformation polymorphism analysis for discriminating phytoplasma strains from grapevines and reported some polymorphism unnoticed by RFLP analysis. Balakishiyeva *et al.,* (2011) also characterized '*Ca.* P. brasiliense' strains (group 16SrXV-A) on *dnaK-dnaJ* gene. In the present study, usefulness of *dnaB* gene in identification of phytoplasmas in 16SrVI and 16SrIX groups from different symptomatic plants sources was shown.

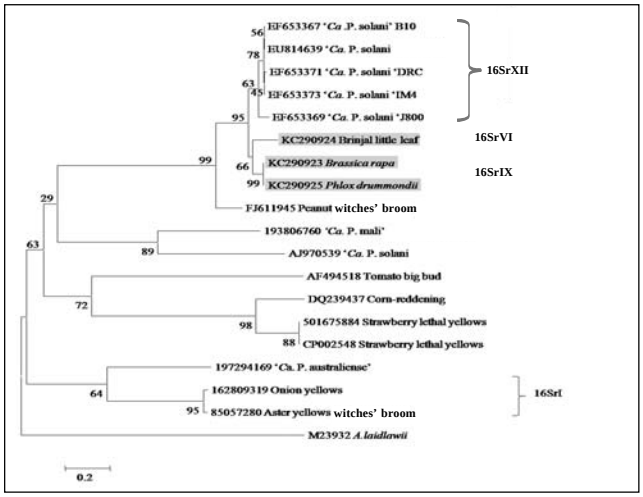

Figure 1. Phylogenetic tree showing the relationships among *dnaB* gene of *Phlox,* toria and brinjal phytoplasmas, and reference phytoplasma strains.

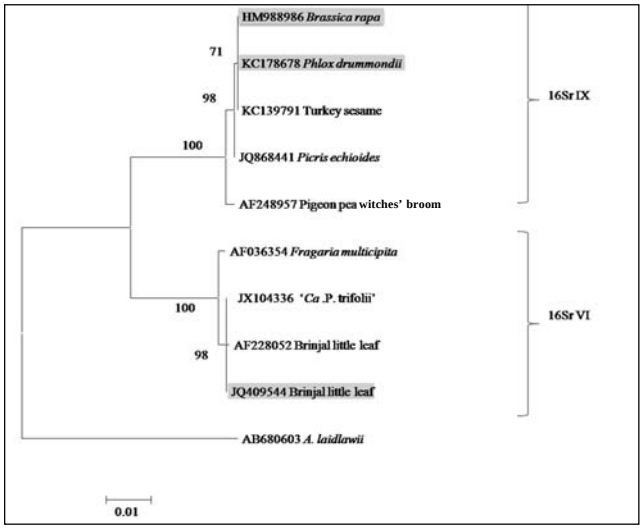

Figure 2. Phylogenetic tree showing the relationships among the *23S rRNA* gene of *Phlox,* toria and brinjal phytoplasmas.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S13-S14

doi: 10.5958/2249-4677.2015.00005.5

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Post-translational cleavage and self-interaction of the phytoplasma effector SAP11

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Abstract

Phytoplasmas are insect-transmitted intracellular plant bacterial pathogens that secreted effectors into host cells to interfere with host developmental or metabolic processes. Recently, the secreted aster yellows phytoplasma strain witches' broom protein 11 (SAP11) has been shown to play as virulence factor that alters plant development, hormone biosynthesis, phosphate homeostasis and defense responses. Here it is shown that SAP11 undergoes proteolytic processing *in planta* and interacts with itself *in vitro*. These biochemical studies provide basic information for understanding the functional mechanisms of SAP11, but the biological relevance of post-translational cleavage and self-interaction of SAP11 need further investigation.

Keywords: phytoplasma, SAP11, post-translational modification

Introduction

The identification of phytoplasma-secreted proteins has revealed that phytoplasma effectors are virulence factors responsible for inducing morphological changes in infected plants (Oshima *et al*., 2013). For example, SAP11, secreted by the aster yellows phytoplasma strain witches' broom (AY-WB) and TENGU, secreted by the onion yellows phytoplasma strain M (OY-M), induce the proliferation of branches similar to the witches' broom symptom (Hoshi *et al*., 2009; Sugio *et al*., 2011). SAP54 of AY-WB was reported to induce green leaf-like flowers symptomatic of phyllody and virescence (MacLean *et al*., 2011). In addition to altering the plant morphology, SAP11 enhanced insect reproduction through the suppression of jasmonate (JA) synthesis and modulated phosphate (Pi) homeostasis by triggering Pi starvation responses (Lu *et al*., 2014). While these findings provide novel insights into understanding the pathogenesis of phytoplasmas, the molecular mechanisms underlying the role of phytoplasma effectors remain largely unknown.

A recent study showed that TENGU, a 38-amino acid protein, is modified through post-translational cleavage by an unidentified host protease (Sugawara *et al*., 2013). The protein was found to be processed into small functional peptides, including an 11-amino acid sequence at the N-terminus that is necessary to induce branch proliferation. Previously, SAP11 was found to bind to and destabilize CIN-TCPs, which inhibits LOX2 expression and reduces JA biosynthesis (Sugio *et al*., 2011; 2014). In the present study, we showed that SAP11 undergoes proteolytic processing *in planta* and self-interaction *in vitro*. These biochemical characterizations of SAP11 are essential for providing a better understanding of the molecular basis of phytoplasma pathogenesis.

Materials and Methods

The *35S::SAP11* transgenic plants used in this study have been described previously (Lu *et al*., 2014). To construct *35S::SAP11ΔC*, a codon-optimized version of SAP11 was used as template for PCR amplification and subcloned into pBA002 vector. The plasmid was introduced into *Agrobacterium tumefaciens* and transformed into *Arabidopsis thaliana* for generation of transgenic plants. An anti-SAP11 antibody was used in the study (Lu *et al*., 2014). To detect SAP11 derivatives, 3-week-old seedlings were ground, and total cell extracts were prepared by directly adding SDS buffer into grinded samples. Proteins were then separated by Tricine SDS-PAGE and blotted onto a PVDF membrane. The chemiluminescence signals were captured with Amersham ECL western-blotting reagents, using an ImageQuant LAS 4000 Mini (GE Healthcare).

Results

Using a specific antibody against SAP11 of AY-WB two SAP11-related bands with a molecular weight (MW) between 11 kDa and 17 kDa among the total cell extracts of *35S::SAP11* transgenic plants (Figure 1A) were found. To further examine the sizes of these SAP11-related bands, the SAP11 recombinant protein was constructed. A PCR product encoding SAP11 was subcloned into the SUMO- pET vector. The N-terminal His-SUMO fusion SAP11 (23.7 kDa) was induced in *Escherichia coli* BL21 (DE3) cells and purified by Ni^{2+}-NTA affinity chromatography. The His-SUMO tag (12.6 kDa) was then cleaved by an ubiquitin-like specific protease 1, and the reaction mixture was further loaded onto a size-exclusion chromatography column (HiPrep 16/60 Sephacryl

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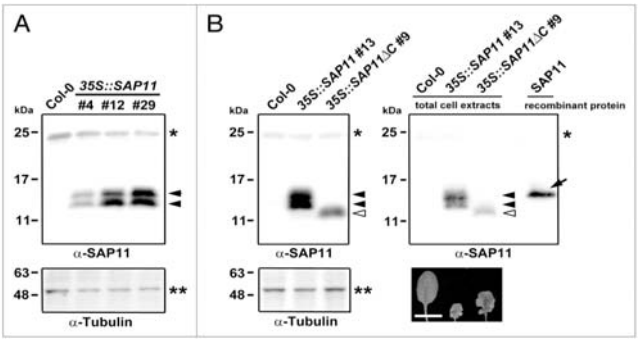

Figure 1. SAP11 undergoes proteolytic processing in *planta*.

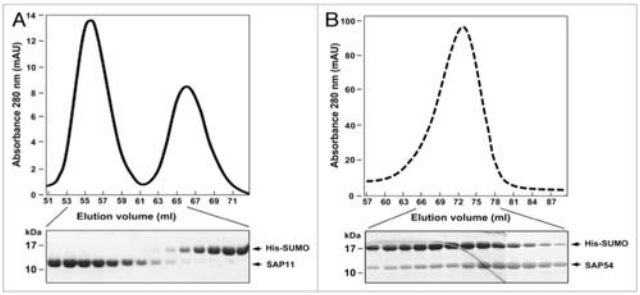

Figure 2. SAP11 interacts with itself *in vitro*.

S-200 HR column); thus the SAP11 protein (11.1 kDa) was obtained. Using the recombinant SAP11 as molecular weight standard, the upper band of SAP11-related proteins that appeared in the immunoblotting experiments had a size similar to the recombinant protein whereas the lower band did not (Figure 1B). This result indicates that the lower band of SAP11-related proteins in *35S::SAP11* transgenic plants is a protein processed through post-translational cleavage.

SAP11 contains a bipartite nuclear localization signal in the N-terminal region and a coiled-coil domain in the C-terminal region, which are important for nuclear targeting and CIN-TCP destabilization. In this study, SAP11ΔC-overexpressing plants in which the conserved C-terminal motif (-GSSSKQPDDSKK-) of SAP11 was truncated, were generated. Although SAP11ΔC-overexpressing plants continue to exhibit a crinkling-leaf phenotype, only one protein band was detected by the anti-SAP11 antibody in the total cell extracts (Figure 1B). This result suggests that the C-terminal motif of SAP11 is required for proteolytic processing *in planta*. During the purification stage the lower MW fractions of SAP11 was eluted earlier than the higher MW fractions of His-SUMO tag in the size-exclusion chromatography experiment (Figure 2A). This result suggests that SAP11 tends to preserve its self-associated state *in vitro*. As a control, SAP54 was subcloned into the SUMO-pET vector to obtain the N-terminal His-SUMO fusion SAP54. After cleavage by an ubiquitin-like specific protease 1, the reaction mixture was loaded onto a size-exclusion chromatography column. As expected, the higher MW fractions of His-SUMO tag was eluted earlier than the lower MW fractions of SAP54 (10.8 kDa) (Figure 2B). However, differently from SAP11, SAP54 was not able to be separated from the His-SUMO tag (Figure 2B).

Discussion

SAP11 is known to cause crinkling-leaf and branching shoots phenotypes, reduce JA biosynthesis, and increase Pi content in plants (Lu *et al.*, 2014). Although the biological relevance of the proteolytic processing and self-interaction of SAP11 to these phenotypes requires further investigation, these findings provide fundamental molecular mechanisms that functionally characterize SAP11 and advance the understanding of morphological and physiological changes in plants that are altered by SAP11.

Acknowledgements

This work was supported by the National Science Council (NSC100-2321-B-005-007-MY3, NSC103-2911-I-005-301) and Ministry of Education (ATU plan), Taiwan.

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Phytoplasma detection and characterisation

Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S17-S18

doi: 10.5958/2249-4677.2015.00006.7

Identification and molecular characterization of the blueberry stunt phytoplasma in Canada

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Abstract

Blueberry farms in Quebec, Canada, were recently surveyed for phytoplasma-associated disease presence. Farmers previously reported a number of high bush blueberries showing stunt symptoms in three blueberry farms. Bushes were tested for phytoplasma based on the *16S rRNA* gene. Nested PCR with universal phytoplasma primers resulted in amplification of phytoplasma DNA from blueberry bushes in samples from two of the farms surveyed. The 16S rDNA of the blueberry stunt phytoplasma from Quebec showed a 99% sequence identity with that of the blueberry stunt phytoplasma from Michigan (AY265220) from group 16SrI '*Candidatus* Phytoplasma asteris' subgroup 16SrI-E. Phylogeny results confirmed the clustering of the blueberry stunt phytoplasma from Quebec within the same phylogenetic branch of phytoplasmas of group 16SrI, closely related to subgroup 16SrI-E. Preliminary virtual RFLP confirmed the sequence and phylogeny classification results. Further actual and virtual analyses are in progress to verify the presence of other possible phytoplasma strains from the Quebec blueberry farms.

Keywords: 16SrI phytoplasma, blueberry, RFLP, '*Candidatus* Phytoplasma'

Introduction

Blueberries have been steadily growing in popularity in Canada and worldwide due to increasing awareness of the health benefits associated with their consumption. Canada is the world's second largest producer of blueberries, second only to the USA (AAFC, 2010). Canadian ministers will invest to the grower representative body to encourage blueberry exports focusing on international export markets including China (www.freshfruitportal.com).

Blueberry plants are susceptible to a range of diseases including blueberry stunt (BBS), which can cause substantial economic damages to their production and quality (Bagadia *et al*., 2013). BBS disease was first observed in New Jersey in 1928, and became widespread in Arkansas, Maine, Massachusetts, New Hampshire, New York, Michigan, North Carolina, Pennsylvania, Maryland, Virginia and Canada (Ramsdell and Stretch, 1987). BBS symptoms include witches' broom growths, stunting, small and deformed leaves, cupping of the leaves, unseasonal discoloration, and shortened internodes. PCR, sequencing, RFLP and phylogeny analyses were used in the present study to characterize the BBS phytoplasma in Canada from which very little information is available. A possible origin of the disease is suggested and information will be made available to farmers in Quebec to support further studies on the BBS phytoplasma for the development of effective control strategies.

Materials and Methods

Total DNA was extracted from midribs of randomly collected leaf samples from three farms of highbush blueberry (*Vaccinium corymbosum* L.), located within a 100 kilometers radius from Montreal, which exhibited BBS-like symptoms (Figures 1A and B). Total DNA was used as a template for nested PCR assays with universal primers that target the phytoplasma *16SrRNA* gene R16mF2/R1 (Gundersen and Lee, 1996) for the first PCR reaction, and either R16F2n/R2 or fU5/rU3 (Lorenz *et al*., 1995) for the nested reaction.

Representatives of R16F2n/R2 PCR amplicons were purified (Omega Bio-Tek, USA), cloned (pGEM-T Easy Vector, Promega), and sequenced bi-directionally. Consensus sequences were compared with GenBank reference sequences and aligned using Clustal W. Preliminary RFLP analysis was conducted using iPhyClassifier (Zhao *et al*., 2013).

A phylogenetic consensus tree was constructed using the neighbour-joining method with MEGA4.0 with default values and 1,000 replicates for bootstrap analysis. Actual and *in silico* restriction analysis pDRAW32 (www.acaclone.com), and PCR-sequencing of non-ribosomal genes are in progress to verify the presence of specific phytoplasma strains in the Quebec blueberry fields.

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Figure 1. BBS symptoms that include small and deformed leaves with cupping and seasonal discoloration (A) and stunting and short internodes (B) observed on highbush blueberries from southern Quebec blueberry farms.

Results

Phytoplasma DNA was amplified from highbush blueberries of two Quebec farms which were bearing typical BBS symptoms. No PCR amplicons were obtained from symptomless blueberry bushes. Comparisons with other BBS phytoplasma strains from North America and Europe, and preliminary virtual RFLP patterns of the 16S rDNA sequence of the BBS phytoplasma from Quebec placed it as a member of group 16SrI. BLAST results showed the highest score and 99% of the 16S rDNA sequence identity with a Michigan BBS phytoplasma (AY265220). Phylogeny analysis indicated that the Quebec BBS phytoplasma clustered within the phylogenetic branch of the 16SrI phytoplasmas of subgroups 16SrI-E, 16SrI-F and 16SrI-P, closely related to subgroup 16SrI-E.

Discussion

Phytoplasmas associated with BBS have been mainly identified as members of the group 16SrI, subgroup E in the USA (Lee *et al.*, 2004; Bagadia *et al.*, 2013). Phytoplasma diseases of blueberry have been also reported in Europe including the Netherlands and Sweden (Valiunas *et al.*, 2004). The group 16SrIII '*Candidatus* Phytoplasma pruni' was reported from wild European blueberry (*Vaccinium myrtillus* L.) exhibiting symptoms of shoot proliferation in Lithuania, and later a '*Ca.* P. trifolii'-related (16SrVI) strain was described in Austria (Borroto Fernández *et al.*, 2007). Recently, Bagadia *et al.*, (2013) identified phytoplasmas of the group 16SrIX '*Ca.* P. phoenicium', subgroup 16SrIX-E in blueberry bushes from New Jersey (USA).

Although the BBS incidence in Michigan is low, and Quebec blueberry growers obtain most of the planting material from New Jersey, the fact that the Quebec BBS phytoplasma was identified as an isolate of the Michigan 16SrI BBS phytoplasma raises suspicions of a possible BBS spread from the United States. The fact that closest strains have been found in different geographical locations in North America suggests that these phytoplasmas may have a complex epidemiology.

Acknowledgements

We acknowledge the support for field surveys from Ornella Piccolli, Istituto Comprensivo di Fagagna Scuole dell'Infanzia, Elementari e Media, Fagagna, Udine, Italy.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S19-S20

doi: 10.5958/2249-4677.2015.00007.9

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Molecular characterisation of phytoplasmas infecting *Dimorphandra* spp. in Brazil

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Abstract

Dimorphandra sp., known as "fava d'anta" and "faveiro", is an endemic species in several South American countries. Symptomatic trees, with witches' broom growths and other symptoms characteristic of diseases associated with phytoplasma presence, were found harbouring phytoplasmas showing differences from those reported so far in Brazil. Ribosomal gene sequencing of one of these strains indicated that it shared 99% of its identity with phytoplasmas enclosed in 16SrXIII group, detected in Mexico, Argentina and Bolivia. RFLP analyses on the ribosomal gene, and further characterisation of the *rp* gene, also showed the similarity of these with phytoplasmas detected in *Turnera* sp. in Brazil.

Keywords: *Dimorphandra gardneriana*, *Dimorphandra mollis*, witches' broom, 16S ribosomal group, classification

Introduction

"Fava d'anta" trees, *Dimorphandra gardneriana* and *D. mollis*, exhibiting witches' broom growths, reduced leaf and yellowing, have been observed in several locations in Brazil, including the states of Ceará and Maranhão. Although in the trees the presence of phytoplasmas was demonstrated (Montano *et al*., 2007), identification was not achieved. Therefore, the identification of phytoplasmas in the *16SrRNA* gene, and their further RFLP characterisation on *rp* and *tuf* genes, were carried out in the present work.

Materials and Methods

Samples from *D. gardneriana*, exhibiting shoot proliferation, reduced leaf size and yellowing, were subjected to nucleic acid extraction and preliminarily amplified to verify phytoplasma presence as reported (Montano *et al*., 2007). Universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and R16F2n/R2 (Gundersen and Lee, 1996) were used to prime the amplification of phytoplasma 16S rDNA in nested PCR assays under reported conditions (Montano *et al*., 2007). PCR amplification, with primers for the *tuf* gene, in a cocktail reaction (Makarova *et al*., 2012), followed by RFLP analyses were also conducted. Other phytoplasma-infected samples collected in different geographic areas of Brazil (Figure 1) were also employed for RFLP characterisation, together with referenced phytoplasma strains from the collection maintained at DipSA (Bertaccini, 2010). Further nested PCR amplification under reported conditions was carried out on one of the phytoplasma positive samples, named DG1, with primers F1/B6 (Duduk *et al*., 2004). Direct sequencing in both directions was also performed, using primers F1 and M1 (=16R758f), (Gibbs *et al*., 1995) as forward primers, and P7 as a reverse primer, after amplicon purification with a QIAquick PCR Purification Kit (Qiagen). The obtained sequence was assembled using Sequencher 4.1 software and compared with selected nucleotide sequences in the GenBank database using BLAST (version BLASTN 2.2.18; NCBI). The 1.2 kb of this sequence was also submitted to the iPhyClassifier online tool for virtual RFLP analyses (Zhao *et al*., 2009). Additional amplification was carried out on the DG1 phytoplasma with primers rpF1C/rp(I)R1A (Martini *et al*., 2007), amplifying the *rpl22* and *rps3* genes. Thirty-eight PCR cycles were conducted as follows: 1 minute (2 minutes for the first cycle) at 94°C for the denaturation step, 2 minutes at 55°C for annealing, and 3 minutes (10 minutes for the last cycle) at 72°C for primer extension. RFLP analyses of R16F2n/R2 and rpF1C/rp(I)R1A amplicons with *Tru*I, *Taq*I, and *Alu*I were then performed. The products of digestion were analysed by using electrophoresis through a 6.7% polyacrylamide gel followed by staining with ethidium bromide and visualising the DNA bands with a UV transilluminator. The RFLP patterns were compared with previously published results, in respect of real or virtually-generated gels (Lee *et al*., 1998; Martini *et al*., 2007; Contaldo *et al*., 2011; Zhao *et al*., 2009).

Results and Discussion

The nested amplification procedure allowed the amplification of fragments of the expected lengths from all five symptomatic samples of *D. gardneriana* tested by using primers R16F2n/R2. The RFLP analyses with selected restriction enzymes (Figures 1A and B, and data not shown) indicated that the profiles of the samples were identical to

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each other, but were distinguishable from all other phytoplasma strains employed as references. The 16Sr DNA sequence assembly allowed a 1,491 bp fragment to be obtained that showed 99% homology with the following phytoplasma sequences deposited in GenBank: two chinaberry yellows strains from Argentina (accession numbers: DQ444264; DQ444265) and one from Bolivia (accession number: AF495882) and a Mexican periwinkle virescence strain (accession number AF248960).

The virtual RFLP analyses showed a profile referable to the one observed in the *Turnera* sp. strain which enables it to be assigned to the phytoplasma group 16SrXIII (Figure 1). The RFLP profiles in phytoplasmas from *D. gardneriana* showed, with all the enzymes, the presence of more bands than those present in the virtual profiles (Figure 1 and data not shown).

It was not possible to identify a profile referable to another phytoplasma. Therefore, it is also possible that interoperon heterogeneity is present. The RFLP analyses carried out on *tuf* amplicons confirmed that the *D. gardneriana* phytoplasma profiles were identical to each other and to phytoplasmas detected in *Turnera* sp. On the other hand, the amplification of the *rp* gene allowed amplicons of the expected lengths (about 1,200 bp) to be obtained, and their RFLP analyses showed identical profiles to each other, but they were different from all those available from the literature. The fragments obtained from virtual RFLP analyses of the *rp* gene of the Mexican periwinkle virescence are comparable to those obtained from real RFLP analyses of *D. gardneriana* phytoplasmas, confirming the clustering of these phytoplasmas with those assigned to the 16SrXIII group.

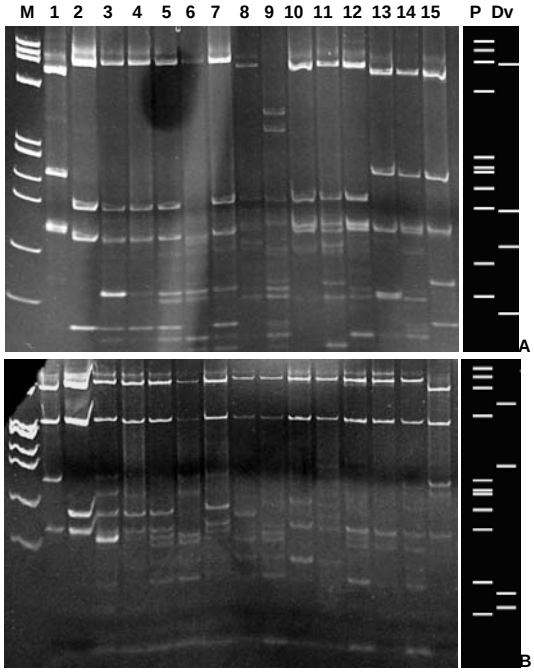

Figure 1. RFLP results obtained after digestion with *Alu*I (A) and *Taq*I (B) on amplicons from symptomatic *D. gardneriana* (samples 3-7), other phytoplasma-infected species from Brazil, and reference strains in periwinkle amplified with R16F2n/R2 primers. M and P, marker Phi X174 RF *Hae*III digest, in real and virtual RFLP gels, respectively. Samples from Brazil: 1, *Erigeron* sp.; 2, *Turnera* sp.; 8, *Hibiscus* sp.. Reference strains: 9, GLAWC (aster yellows in gladiolus, 16SrI-B); 10, WBDL (witches' broom disease of lime, 16SrII-B); 11, LN1 (plum leptonecrosis, 16SrIII-B); 12, BF (X disease, 16SrIII-A); 13, EY1 (elm yellows, 16SrV-A); 14, CPS (cleome phyllody; 16SrVI); 15, ASHY3 (ash yellows, 16SrVII-A); Dv, virtual digestion of the sequence obtained from *D. gardneriana*.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S21-S22

doi: 10.5958/2249-4677.2015.00008.0

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Multigene characterization of aster yellows phytoplasmas infecting grapevine in South Africa

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Abstract

The genetic diversity of aster yellows phytoplasmas in grapevine collected in two locations in South Africa (Vredendal and Robertson) was investigated by the use of multilocus analysis of gene sequences amplified by polymerase chain reaction. Based on results from restriction fragment length polymorphism profiling and nucleotide sequence alignments of *16S rRNA, rp, amp, groEL* and *secY* genes in comparison with aster yellows strains from Italy and reference aster yellows strains, four lineages have been detected in samples from South Africa.

Keywords: aster yellow phytoplasmas, multiple gene analysis, PCR, RFLP, amplicon sequencing

Introduction

The main agents associated with grapevine yellows (GY) in South Africa are phytoplasmas belonging to the 16SrI-B subgroup (aster yellows) (Engelbrecht *et al*., 2010; Carstens *et al*., 2011). In order to clarify the epidemiology of these diseases, variability of non-ribosomal RNA and house-keeping genes such as *amp, groEL, ribosomal protein* and *secY* was investigated among selected aster yellows phytoplasmas infecting South African grapevines.

Materials and Methods

Leaf samples were collected from 28 symptomatic grapevine plants in three vineyards in Vredendal and one in Roberston, South Africa. Total DNA was extracted from leaves using both a commercial kit and a chloroform/phenol based extraction protocol (Prince *et al*., 1993). Nested-PCR assays were carried out for amplifying the genes *16S rRNA* using primers R16F2n/R2 (Gundersen and Lee, 1996), *rp* using primers Rp(I)F1/Rp(I)R1A (Martini *et al*., 2007) and *groEL* using primers AY groelF1/AY groelR1 (Mitrovic *et al*., 2011) on direct amplicons obtained as described in literature. Further PCR analyses were carried out on *amp* [primers Amp N1/C1 (Kakizawa *et al*., 2006)] and *secY* [primers AYsecYF1/AYsecYR1 (Lee *et al*., 2006)] genes. PCR products were digested by the following restriction enzymes: *Hha*I, *Tru*1I (16S rDNA); *Tru*1I and *Alu*I (*rp, amp, groEL, secY*). RFLP profiles were then compared to those obtained from two aster yellows strains from Italy (FD-T and REPT) and from reference strains maintained in a periwinkle collection: NJAY and GDI (16SrI-A), MBS and AY-J (16SrI-B), AVUT (16SrI-B/M), KVF and KVG (16SrI-C), ACLR-AY (16SrI-F).

Selected amplicons obtained from different genes were sequenced in both directions, and sequences were assembled using CLC genomics workbench 7 software and then aligned using Clustal X (Thompson *et al*., 1997).

Results and Discussion

Aster yellows- related strains (16SrI-B) were identified in all the symptomatic grapevines collected in the different vineyards. After phytoplasma identification and characterization of the 16S rRNA gene, 15 samples were

Figure 1. Grapevine plant in Vredendal showing symptoms of yellowing and downward curling on the leaves in Chardonnay associated with the presence of aster yellows phytoplasmas.

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Table 1. Differential restriction profiles obtained from samples infected with grapevine yellows from South Africa and aster yellows strains from Italy.

Samples	16S rDNA subgroup	Rp		GroEL		Amp		SecY		Lineages
		*Tru*1I	*Alu*I	*Tru*1I	*Alu*I	*Tru*1I	*Alu*I	*Tru*1I	*Alu*I	
P	I-B	A	E	F	F	-	-	-	-	nd
1Y	I-B	A	E	F	F	-	-	B	B	nd
2Y	I-B	A	E	F	F	C	A	B	B	AYG-I
S1	I-B	A	E	F	F	-	-	-	-	nd
S3	I-B	A	E	F	F	-	-	-	-	nd
S4	I-B	A	E	F	F	C	A	B	B	AYG-I
S7	I-B	A	E	F	F	C	B	B	-	nd
S8	I-B	A	E	F	F	C	B	B	-	nd
S13	I-B	A	E	F	F	C	A	E	B	AYG-II
S14	I-B	A	E	F	F	C	A	E	B	AYG-II
S15	I-B	B	E	F	F	F	A	E	D	AYG-IV
S17	I-B	A	E	F	F	C	A	E	B	AYG-II
S18	I-B	A	E	F	F	C	A	-	-	nd
Robertson 1	I-B	A	E	F	F	C	A	F	E	AYG-III
Robertson 2	I-B	A	E	F	F	C	A	F	E	AYG-III
APW (control)	I-B	A	E	F	F	C	C	B	B	AYG-V
Aster yellows strains from Italy										
FD-T	I-B	B	B	D	D	-	-	B	B	nd
Reptalus	I-B	B	B	-	-	-	-	-	-	nd

selected for further multigene analyses. Amplicons of the *rp* gene were successfully generated from all samples. Restriction analysis with *Tru*I and *Alu*I enzymes showed that all the samples but one (S15) had an identical profile, and all were different from those available in literature (Table 1). In the case of *groEL* gene typing, *Tru*I and *Alu*I restriction analysis yielded fragments that were identical among all samples, and also identical to that of reference strain AY-J (16SrI-B) but different from the Italian strain FD-T. The RFLP analyses carried out on *amp* amplicons showed some variability among the South African grapevine samples, including the positive control APW (from periwinkle), however four samples were not amplified. Finally, the RFLP analysis carried out on the gene *secY*, showed the presence of five distinct groups. Restriction patterns of fragments from samples 1Y, 2Y, S4 and APW were identical; another profile was detected in samples S13, S14, S17, while samples Robertson 1 and Robertson 2 showed a third type of profile. A unique profile was also observed in sample S15 (E-D; table 1). The *secY* gene showed great RFLP variability and the four groups delineated do not match any reported profile. In order to confirm the results obtained by the RFLP technique, selected amplicons were sequenced. The analyses of obtained sequences confirmed differences in some of the analyzed genes while considerable variability was present in the *secY* gene. This gene could be therefore a more informative marker for finer differentiation of South African aster yellows grapevine strains. Through this analysis it was possible to differentiate for the first time these aster yellows strains in order to carry out better-focused epidemiological studies on the spread of the disease in the region.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S23-S24

doi: 10.5958/2249-4677.2015.00009.2

Saving livestock fodder in East Africa: development of a rapid penside diagnostic assay for detection of napier grass stunt phytoplasma

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Abstract

Napier grass stunt is a disease associated with phytoplasmas belonging to the 16SrXI subgroup. The disease affects *Pennisetum purpureum*, a perennial grass species with significant importance in dairy livestock nutrition and ecological management of pests and parasitic weeds. The present study explores the development, testing and validation of field-friendly diagnostic tools for napier grass stunt phytoplasma detection in East Africa, based on an isothermal assay targeting the gene encoding the immunodominant protein of napier grass stunt phytoplasma.

Keywords: napier grass stunt, phytoplasma, diagnostic assay, *imp* gene

Introduction

Napier grass or elephant grass (*Pennisetum purpureum*) is a perennial grass indigenous to Africa. In East Africa, napier grass is most valued and relied on as a fodder crop by more than 70% of dairy livestock farmers (Khan *et al*., 2001). Additionally, napier grass attracts cereal stem borers, while remaining unaffected by the pest and has therefore been incorporated into an ecological push-pull pest management strategy for cereal stem borers (Khan *et al*., 2001). The utilization of napier grass in this strategy has enabled vulnerable smallholder farmers in the region, to reduce stem borer infestation on cereals by 50% thereby increasing crop yield, while also sustaining high milk production from their dairy animals through the perennial supply of fodder from the grass (Khan *et al*., 2008).

However, the widespread occurrence of napier grass stunt disease (NGS) threatens to curtail the benefits derived from this grass. NGS is a disease associated with the presence of phytoplasmas belonging to the 16SrXI group that are vectored by the leafhopper *Maiestas banda*. Although the disease agent was recently characterized in East Africa, the spreading of the phytoplasma through vegetative propagation of infected planting stock has seen the rampant spread of diseases in the region (Jones *et al*., 2004). NGS is characterized by leaf yellowing, and stunted growth in affected plants that are evident only in advanced disease stages. Stunting and loss of biomass is progressive with every subsequent cutting, eventually culminating in drying and death of affected plants. The standard molecular diagnostic test for NGS is dependent on amplification and sequencing of 16S rDNA. However, this assay requires two rounds of amplification (nested PCR), which prolongates the duration of detection to over 4 hrs and renders the assay prone to false positive due to contamination. Interpretation of sequence data output may also pose challenges in diagnostic specificity, where there are minimal differences in 16S rDNA sequences in two or more phytoplasmas. The 16S rDNA nested PCR assay also requires a molecular laboratory and trained personnel. Therefore, this assay has had limited use in low-resource laboratory settings that are common in rural East Africa. There remains a direct need for field-friendly diagnostic tools to aid early detection and monitoring spread of NGS in the region. This research therefore aims to develop a penside diagnostic assay for NGS detection and field surveillance.

Materials and Methods

The utility of the NGS immunodominant protein (*imp*) gene as a specific diagnostic target was examined. Based on the knowledge that an ideal diagnostic target should be non-variable in a population, the genetic diversity of the NGS imp in infected field samples using PCR and SSCP methods was evaluated. The Imp locus was amplified in a one-round PCR from infected napier grass DNA samples, the amplicons were then sequenced and analyzed. A penside isothermal test is under development and will be validated for specificity and sensitivity against the gold standard nested 16S rDNA PCR assay. The utility of the assay will be further tested and validated under field settings.

Results

Amplification of the Imp locus of samples tested in direct PCR assays gave the same results as the nested 16S rDNA PCR assay carried out for comparison. Sequence analyses of the Imp PCR amplicons reveal minimal genetic diversity in

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the infected field samples tested. The initial efforts for the development of an isothermal penside assay indicate amplification of the imp locus at a constant temperature range of 36 – 40°C within 30 minutes in a water bath.

Discussion

Results indicate that the NGS imp is a promising diagnostic target with the potential to accelerate specific identification of NGS phytoplasma in East Africa. The adaptation of Imp PCR into an isothermal penside assay will contribute towards an effective surveillance scheme of NGS phytoplasma presence in farmers' fields and low-resource laboratories with modest demands on skilled personnel and laboratory facilities. Further, the development, testing and validation of a penside assay for NGS phytoplasma envisaged in this study should encourage the judicious distribution of disease-free napier grass planting material, thereby curtailing the spread of NGS within East Africa and beyond its borders. Ultimately, the outputs of this study will contribute towards widespread adoption of the push-pull pest management strategy, resulting in improved livelihoods of rural smallholder farmers in East Africa through enhanced yield and productivity of crops and livestock.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S25-S26

doi: 10.5958/2249-4677.2015.00010.9

Phytoplasmas associated with naturally infected *Catharanthus roseus* in La Paz, Baja California Sur, Mexico

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Abstract

Phytoplasma infection was detected in periwinkle (*Catharanthus roseus*) with multiple yellows-type disease symptoms including virescence, phyllody, virescence of petals and proliferation of shoots in La Paz, Baja California Sur, Mexico. The phytoplasma presence was first studied by scanning electron microscopy. Using nested PCR, cloning, sequencing and virtual RFLP analysis phytoplasmas belonging to two ribosomal subgroups have been identified and classified.

Keywords: phytoplasma, periwinkle, SEM, nested PCR, virtual RFLP

Introduction

Catharanthus roseus G. Don (Apocinaceae), or periwinkle, is a tropical perennial species native and endemic to Madagascar, and was introduced to various regions of the world as an ornamental plant with very high medicinal value, known from ancient Indian and Chinese traditional medicine. Periwinkle has been identified as a natural host of spiroplasmas and phytoplasmas and is used as an experimental host for these mollicutes to which these pathogens are transmitted mainly grafting and by dodder, to phytoplasma strain collections and to study pathogen-plant interaction (Marcone *et al.,* 1997; Chang, 1998; Favali *et al*., 2008).

The natural infection of periwinkle plants by phytoplasmas belonging to distinct groups has been reported (Chen *et al*., 2011; Barbosa *et al*., 2012; Nejat *et al*., 2013) and one phytoplasma strain from Mexico, Mexican periwinkle virescence, phytoplasma was submitted to GenBank (accession number AF248960) as a reference strain of a new taxon 16SrXIII. In La Paz, Baja California Sur (BCS), periwinkle is a part of the natural ecosystem, grown in gardens, streets and backyards. Symptoms of different floral and foliar malformations in this plant species were observed during surveys to verify disease presence and the occurrence of phytoplasmas was first determined by scanning electron microscopy (SEM) (Poghosyan and Lebsky, 2010).

Molecular techniques were applied after SEM analysis to identify and classify phytoplasmas associated with periwinkle symptomatic plants in La Paz.

Materials and Methods

Symptoms of reduction of size in apical and internodal leaves, intervenal and vein chlorosis in mature leaves and different floral abnormalities: petals and sepals deformed and reduced in size, virescence, phyllody, proliferation of shoots were observed in some periwinkle plants growing in La Paz (Figure 1). Samples were taken from symptomatic plants growing in two different locations, three samples at each place, as well as from one asymptomatic periwinkle plant. Collected samples of midribs, leaf stalks and floral parts from symptomatic and asymptomatic plants were processed for SEM (S-3000N Hitachi) analysis as reported Poghosyan and Lebsky (2010) and for molecular assays. Total DNA was extracted following the method described by Zhang *et al.* (1998) with minor modification. Nested PCR assay was performed using universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by R16F2n/R16R2 (Gundersen and Lee, 1996). Amplicons of approximately 1,250 kb were purified using a Purification Kit (GE Healthcare Life Sciences), cloned into pGEM-T-easy vector (Promega) and sequenced (Genewiz, USA). Sequence analysis was performed using BlastN program in GenBank database. Nucleotide sequences of six clones were subjected to virtual RFLP analysis (Wei *et al*., 2008).

Figure 1. Symptoms of floral size reduction and virescence.

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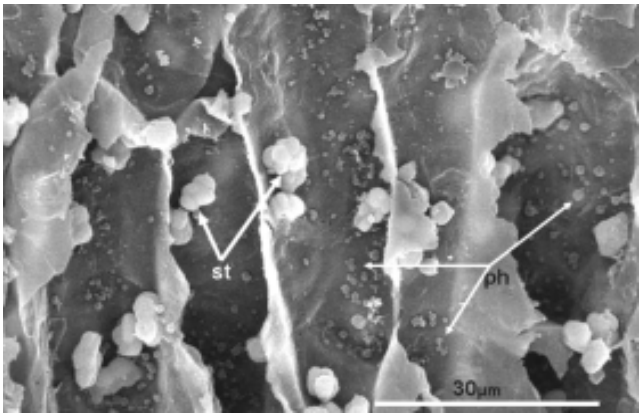

Figure 2. Phytoplasmas (ph) and starch granules (st) in phloem tissue of flower calyx.

Results

In phloem tissue of samples from symptomatic plants phytoplasmas ranging from 500 nm to 1,500 nm were detected in leaf midribs, leafstalks, pedicels, petals and stamens. A high concentration of starch grains was observed in all phloem cells (Figure 2), confirming previous reports (Poghosyan and Lebsky, 2010; Lebsky *et al*., 2012). Nested PCR assay allow to obtain amplicons of approximately 1,250 kb in all symptomatic analyzed samples, supporting the presence of phytoplasmas in symptomatic samples from both plots. Amplified products from samples collected at the two locations were cloned and sequenced. Analysis of sequences of four amplicons, obtained from samples in the first plot, shared the highest identity (more than 99%) with phytoplasmas from 16SrIII phytoplasmas (X-disease group), were deposited in GenBank database under accession numbers HQ87661, HQ8762, HQ8763 and HQ8764). Sequences of two clones from samples collected in the second plot displayed the highest sequence similarity with 16SrI group, '*Candidatus* Phytoplasma asteris'-related phytoplasmas. Virtual RFLP analysis of sequences from the first plot revealed the higher similarity with reference profiles of the 16SrIII-J subgroup phytoplasmas, while two strains from the second location were classified as members of 16SrI-B ribosomal subgroup.

Discussion

The presence of phytoplasmas belonging to two different subgroups, 16SrI-B, and 16SrIII-J in the Mexican state BCS was supported by SEM and molecular analysis. Phytoplasma strains, *'Ca*. P. asteris'-related, subgroup 16SrI-B, were recently discovered in La Paz in kumquat (*Citrus japonica*) with "huanglongbing" symptoms (A. Poghosyan *et al*., unpublished). Phytoplasmas belonging to 16SrIII, X-disease group were earlier found in BCS in pepper, co-infected with two begomoviruses (Lebsky *et al*., 2011). Natural infection of periwinkle with begomviruses was already reported from Pakistan (Ilyas *et al*., 2013). An additional investigation is needed to define the role of periwinkle as a possible reservoir of those pathogens in BCS.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S27-S28

doi: 10.5958/2249-4677.2015.00011.0

Rubus stunt in raspberries: a multiplex TaqMan qPCR assay for sensitive detection of phytoplasmas in *Rubus* species

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Abstract

Rubus stunt is an economically important disease in the production of raspberries, blackberries, and loganberries. A fast, sensitive, and reliable detection of phytoplasmas, the agents associated with the disease, is of prime importance to stop its spread by vegetative propagation and by insect vectors. Therefore, two multiplex qPCR assays using TaqMan probes in combination with up to four different kinds of fluorophores in the same reaction were developed, allowing the detection of phytoplasmas. Moreover the specific detection of different phytoplasma groups that have previously been identified in *Rubus* species was achieved. This assay is applied to monitor presence and distribution of phytoplasmas in *Rubus* plants of different geographic origins, cultivars and cultivation systems as well as in putative insect vectors.

Keywords: phytoplasma, rubus stunt, insect vectors, real-time PCR, taqMan, detection

Introduction

Worldwide, wild and cultivated *Rubus* species like raspberry (*Rubus ideaeus* L.), blackberry (*Rubus fruticosus* L.), and loganberry (*Rubus x loganobaccus*) are frequently infected with phytoplasmas which are associated with a disease referred to as rubus stunt. Symptoms include stunted growth, shoot proliferation, small leaves, short internodes, enlarged sepals, phyllody, and flower proliferation as well as fruit malformations (Mäurer and Seemüller, 1994). Thus far, phytoplasmas belonging to the 16Sr groups of elm yellows (16SrV), X disease (16SrIII), aster yellows (16SrI), and "stolbur" (16SrXII) have been identified in *Rubus* species (Cieslinska, 2011). As the time between the infection of a plant and the development of disease symptoms can take up to 1 year and *Rubus* plants are produced by vegetative propagation, an early detection of phytoplasmas using highly sensitive and rapid molecular methods is of great importance to minimize their spread. Therefore, two multiplex qPCR assays using TaqMan probes in combination with up to 4 different kinds of fluorophores in the same reaction were developed. The assay combines TaqMan probes previously published in literature with newly designed probes, allowing a rapid and simultaneous detection of phytoplasmas in general, as well as a more specific detection of the above mentioned groups of phytoplasmas infecting *Rubus* species.

Materials and Methods

Elm yellows group specific primers and TaqMan probes were designed for the *secY* and *degV* genes of '*Candidatus* Phytoplasma rubi'. The *secY* gene of '*Ca.* P. pruni' was used to generate X disease specific primers and probes and the *tuf* gene of '*Ca.* P. asteris' was used for the aster yellows group. All primers and probes were designed using PrimerQuest by Integrated DNA Technologies. For the universal detection of phytoplasmas a primer and probe pair from Christensen *et al.* (2004) was used. In addition, a primer and probe set developed by Oberhänsli *et al.* (2011) for 18S rDNA detection of plant DNA was used as an internal control to allow the detection of plant host DNA in the same reaction, enabling the confirmation of a successful DNA extraction and to exclude false negatives due to potential inhibition of the PCR reaction. TaqMan probes were labelled with FAM, HEX, ROX, and Cy5, allowing simultaneous detection of up to 4 targets in a single reaction.

DNA was extracted from 1 g of leaf or root tissue of healthy and symptomatic *Rubus* species using an initial phytoplasma enrichment step with phosphate grinding buffer prior to the extraction according to Prince *et al.* (1993).

Primers and probes for the qPCR were run in 20 µl reactions employing the iQ Multiplex Powermix (Biorad) on an iQ5 real-time thermal cycler (Biorad) with an initial denaturation step of 2 minutes at 95°C followed by 45 cycles with 10 seconds denaturation at 95°C and 60 seconds annealing and elongation at 55°C. Nested PCR was run with primer pairs P1/P7 (Schneider *et al.*, 1995) followed by R16F2n/R16R2 (Gundersen and Lee, 1996).

Results

A multiplex qPCR for detection of phytoplasmas infecting *Rubus* species was successfully developed. The internal control allowed detection of false negative results caused by substances in the DNA extract inhibiting PCR or failed DNA extractions. It was possible to detect phytoplasmas in DNA

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extract dilutions from undiluted to 1: 1000 diluted with Ct (Cycle threshold) values ranging between 23 and 38 for phytoplasmas (Figure 1). Out of 34 tested samples from raspberry plants, 8 were positive by nested PCR and 9 were positive by qPCR.

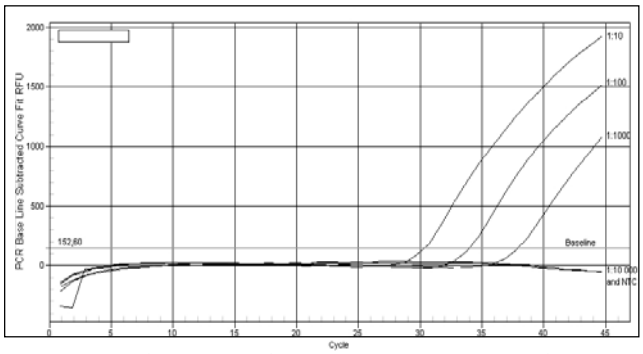

Figure 1. Amplification chart for the elm yellows group specific TaqMan probe generated from the *secY* gene of '*Ca*. P. rubi'. The DNA extract of raspberry roots was assayed at 4 different dilutions, namely 1:10, 1:100, 1:1,000, and 1:10,000 with an additional no template control (NTC). Ct values reflect the cycle number at which the fluorescence generated within a reaction crosses the baseline threshold. In this case, at Ct values of 30.38 for the 1:10, at 33.99 for the 1:100 and at 37.97 for the 1:1,000 dilution of the samples. There was no amplification for the 1:10,000 dilution and the NTC.

Discussion

TaqMan assays for detection of phytoplasmas were shown to be at least as sensitive as nested PCR (Smart *et al*., 1996; Angelini *et al*., 2007), but less susceptible to inhibitors. This leads to higher detection sensitivity of TaqMan assays due to the fact that DNA extracts could be used less diluted (Oberhänsli *et al*., 2011). Compared to nested PCR, the multiplex TaqMan assay developed in this study is time saving, has a reduced risk of contaminations and is therefore more reliable. These facts, in addition to the simultaneous detection of different groups of phytoplasmas and the presence of an internal control, make the assay developed in this study a solid tool for the screening of mother plants in nurseries or during plant propagation. In addition the assay is now used to monitor presence and distribution of phytoplasmas in *Rubus* plants of different geographic origins, cultivars and cultivation systems as well as in putative insect vectors like leafhoppers.

Acknowledgements

The project is supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S29-S30

doi: 10.5958/2249-4677.2015.00012.2

Local distribution of '*Candidatus* Phytoplasma mali' genetic variants in South Tyrol (Italy) based on a MLST study

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Abstract

Apple proliferation is one of the economically most important apple diseases in Europe and infection rates are increasing over the last years in many apple growing areas. '*Candidatus* Phytoplasma mali', the agent associated with the disease, is transmitted by two psyllid species, *Cacopsylla melanoneura* and *C. picta*. A multilocus sequence typing analysis was performed to determine genetic variability of '*Ca.* P. mali' in infected trees and insect vectors. Two major genetic clusters were identified and occurrence of sequence variants is different in the geographic areas of South Tyrol (North Italy).

Keywords: apple proliferation, '*Candidatus* Phytoplasma mali', *Cacopsylla*, multilocus sequence typing

Introduction

Apple proliferation (AP) associated with the presence of '*Candidatus* Phytoplasma mali' is characterized by symptoms like witches' brooms, enlarged stipules, early leaf reddening or chlorosis, formation of leaf rosettes and undersized fruit of poor quality. In South Tyrol (northern Italy), the largest contiguous apple growing area in Europe where apples are cultivated on more than 18,000 hectares, AP infection rates increased dramatically over the last years and led to heavy economic losses. Interestingly, some areas show alarmingly high infection rates, whereas other regions are less affected. '*Ca.* P. mali' is transmitted by the psyllid *Cacopsylla picta* and, with less efficiency, by *C. melanoneura*. However, both vectors are present in the orchards.

In this study the genetic variability of '*Ca.* P. mali' and its local distribution within South Tyrol was studied by a multilocus sequence typing (MLST). The analysis was based on five fragments of genes that code potentially functional and hypothetical phytoplasma proteins.

Materials and Methods

Root samples of AP-infected trees were collected from 2012 to 2013 in commercial orchards, whereas *Cacopsylla* specimens were collected in abandoned orchards as well.

Total DNA from individual samples was extracted according to a modified CTAB method (Marzachì *et al.,* 1998) or by Plant DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). Presence of '*Ca.* P. mali' in the insect vector was determined by real-time PCR as described (Baric and Dalla-Via, 2004; Galetto *et al.*, 2005). Phytoplasma gene fragments were amplified by specific primers (K. Janik *et al.*, unpublished). DNA fragments were cloned according to standard procedures and sequenced, or sequencing was carried out directly on amplicons (GATC Biotech AG, Konstanz, Germany). Alignment and sequence analysis were performed with Sequencher v.4.8 (Ann Arbor, USA) and Geneious v.7.0.06 software (Biomatters Ltd., Auckland, New Zealand). Genotype networks based on the sequence variants of the five genes were constructed by eBURST (Feil *et al.*, 2004).

Results

Sequence analysis of five phytoplasma genes from trees and vectors revealed 3 to 17 different genotypes per gene fragment. The analysis of multiple locus variants performed with eBURST identified two major genetic '*Ca.* P. mali' clusters specified as MLST group-1 and -2. Phytoplasmas belonging to group-1 could be detected in more than 90% of the root samples. The sequence type 3-2-3-00-00 was defined as cluster founder of group-1 and is the most frequent variant found in South Tyrol (Figure 1).

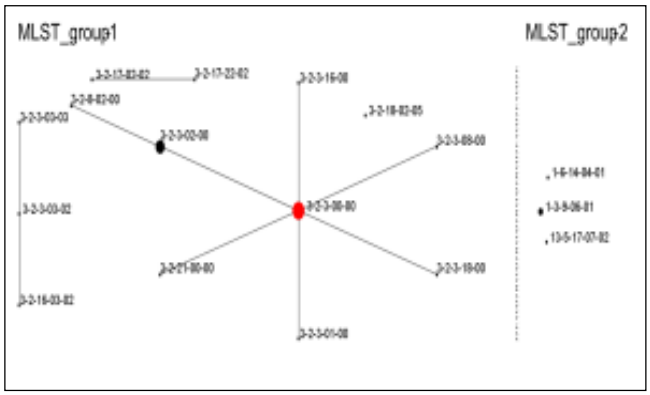

Figure 1. MLST analysis performed with eBURST revealed two different genetic clusters for '*Ca.* P. mali' in root samples. MLST_group-1 is predominant in South Tyrol, MLST_group-2 was detected in less than 10% of the infected trees.

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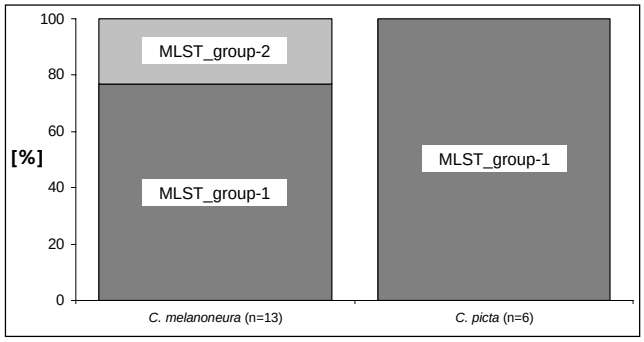

Figure 2. Occurrence of '*Ca.* P. mali' sequence variants of the different MLST groups in the insect vectors *Cacopsylla melanoneura* and *C. picta*.

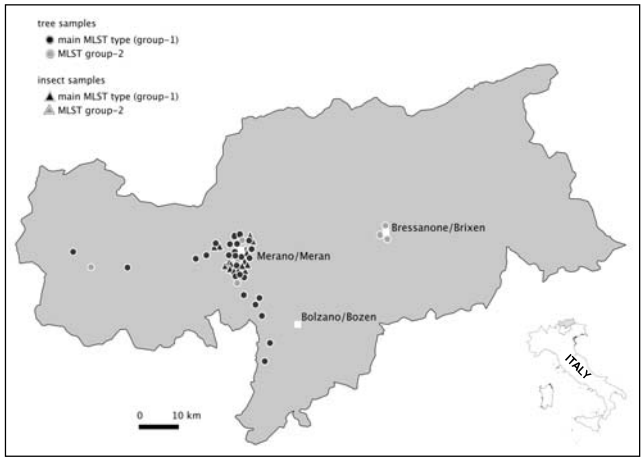

Figure 3. Multilocus variants of '*Ca.* P. mali' identified in South Tyrol. Two major genetic clusters could be identified. MLST_group-1 covers the sequence type 3-2-3-00-00 determined as founder and its locus variants. The MLST_group-2 is formed by the phytoplasma sequence variants of the less frequent cluster.

Discussion

Multilocus sequence variants for '*Ca.* P. mali' from roots and insect vectors collected in South Tyrol were determined. Sequence analysis of phytoplasma gene fragments showed that none of the multilocus sequence types corresponded in all five loci to the genotype described by Kube *et al.* (2008). MLST analysis identified two major genetic clusters of the AP and the sequence variants show a local distribution within South Tyrol (Figure 3). The less frequent variant MLST_group-2, which comprises 11% of the analyzed samples, is the predominant type in an area that is less affected by AP disease. This cluster appears only sporadically in the areas with high infection rates.

This study shows that '*Ca.* P. mali' is subjected to high genetic dynamics even on a small geographic scale. Moreover, linkage of sequences variants to areas with varying infection rates may indicate the correlation of '*Ca.* P. mali' genetic variants with AP infection rates.

Acknowledgements

We thank the South Tyrolean Extension Service for root sampling, E. Mitterrutzner and Y. Reyes Dominguez for providing insect samples, C. Kerschbamer and B. Panassiti for technical assistance. The work was performed as part of the Strategic Project APPL2.0 on apple proliferation and was partially funded by the Autonomous Province of Bozen/Bolzano, Italy (Department 31) and the South Tyrolean Apple Consortium.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S31-S32

doi: 10.5958/2249-4677.2015.00013.4

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Molecular diversity of phytoplasmas infecting cherry trees in Poland

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Abstract

Sour cherry with shoot proliferation and dieback symptoms and sweet cherry showing leaf roll and yellowing of the leaves were observed in orchards located in west and central parts of Poland. Results of phylogenetic and RFLP analysis of the partial sequence of 16S rDNA and 16S-23S rDNA spacer region virtually digested with 16 restriction enzymes showed the presence of '*Candidatus* Phytoplasma prunorum' in sweet cherry and '*Ca.* P. asteris' in sour cherry trees.

Keywords: cherry, phytoplasma, detection, PCR-RFLP, sequencing, phylogeny

Introduction

The wide range of diseases of stone fruit trees collectively named European stone fruit yellows (ESFY) disease are associated with infection by '*Candidatus* Phytoplasma prunorum'. This phytoplasma is associated with apricot chlorotic leaf roll, plum leptonecrosis, and decline diseases in plum, peach and almond. *Prunus* species plants can be also infected by '*Ca.* P. mali', '*Ca.* P. pyri', '*Ca.* P. asteris', '*Ca.* P. aurantifolia' '*Ca.* P. ziziphi', '*Ca.* P. phoenicium', '*Ca.* P. pruni' and '*Ca.* P. solani'. On the basis of 16S rDNA analysis, these '*Candidatus* Phytoplasma' species were classified into eight distinct ribosomal groups: 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVII, 16SrIX, 16SrX, and 16SrXII. The aim of this study was to determine the possible association of phytoplasmas with symptoms of shoot proliferation in cherry trees.

Materials and Methods

Total DNA was extracted from phloem tissue of symptomatic and asymptomatic cherry trees using the DNeasy Plant Mini Kit (Qiagen, Germany). Two-steps PCR protocols were conducted with P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by F1/B6 (Duduk *et al.*, 2004) universal primer pairs for amplification of the partial sequence of 16S rDNA and 16S-23S rDNA spacer region of phytoplasmas. Primers R16(I)F1/R and R16(X)F1/R1 (Lee *et al.*, 1994) detecting phytoplasmas from 16SrI and 16SrX group, respectively, were also employed in nested PCR. The amplicons obtained with F1/B6 primers were sequenced, analyzed using the software package Lasergene 5 (DNASTAR, Inc., USA) and compared with the sequences available on NCBI GenBank database. The phylogenetic relationships among phytoplasmas from infected trees were analyzed using MEGA4 (Tamura *et al.*, 2007). Virtual RFLP analysis was conducted using pDRAW32 program (http://www.acaclone.com).

Results

PCR products (~1.7 kb) amplified with F1/B6 primers were obtained from two samples of sweet cherry (varieties Kordia II.14 and Trzebnica) showing leaf roll and yellowing of the leaves and one sample from sour cherry (cherry XVI.12) with shoot proliferation and dieback symptoms. Products of the nested PCR with R16(X)F1/R1 primers specific for apple proliferation (16SrX group) were obtained for 16S rDNA of phytoplasmas from two sweet cherry trees. Phytoplasma rDNA fragments of phytoplasmas from cherry XVI.12 was amplified by nested PCR with primer pair R16(I)F1/R1 specific for aster yellows group, but not with apple proliferation-specific primers.

Sequence and phylogenetic analyses of the 16S rDNA fragments confirmed the presence of two different phytoplasmas in infected cherry trees. Multiple alignments revealed that the nucleotide sequence of the phytoplasma from two sweet cherry trees was identical and shared 99.6-100% similarity with those of '*Ca.* P. prunorum' strains G1, G2, 215, ESFY and 2102 (GenBank ID: AY542544, AJ542545, AJ575105, AM933142, AY029540). Phylogenetic analysis showed that the phytoplasma infecting cherry XVI.12 was genetically related to the '*Ca.* P. asteris' strains (16SrI group) and formed a monophyletic cluster with the reference strains PaWB, AVUT, HyPH1, AY1, OAY (GenBank ID: AY265206, AY265209, AY265207, AF322644, M30790), belonging to subgroup 16SrI-B and strain ChLL (GenBank ID: AY034089) belonging to subgroup 16SrI-Q (Figure 1). The resulting virtual RFLP patterns of the 16S rDNA sequences singly digested with 16 restriction enzymes also revealed differences among phytoplasmas infecting cherry trees (data not shown).

Discussion

'*Ca.* P. prunorum', the phytoplasma associated with ESFY disease has caused severe outbreaks in the Mediterranean

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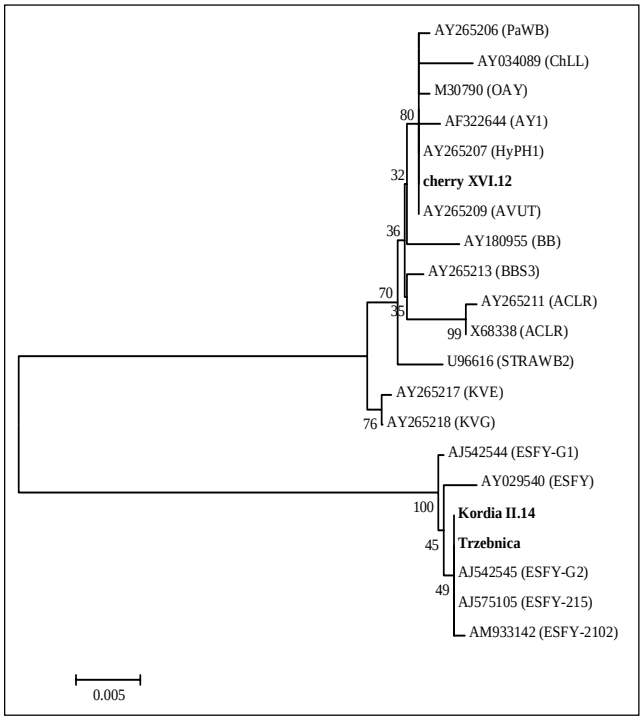

Figure 1. Phylogenetic tree constructed using the neighbour-joining analysis of rDNA of phytoplasmas infecting cherry trees and representative phytoplasma strains of '*Ca*. P. asteris' (16SrI) and '*Ca*. P. prunorum' (16SrX). Bootstrap values are shown on branches.

basin. It is known that infection with '*Ca*. P. prunorum' does not lead to devastation of sweet and sour cherry orchards, as these trees are asymptomatically infected or show only mild symptoms (Giunchedi *et al*., 1982). Although '*Ca*. P. prunorum' rarely infects cherry trees it was detected in the Czech Republic and Poland (Ludvikova *et al*., 2011; Cieslińska and Morgas, 2011). Navràtil *et al*. (2001) reported that in the Czech Republic sweet cherry showed stunting, leaf rolling, and yellowing. Sour cherry trees with small leaves, reduced vigor, and die-back were mainly infected by '*Ca*. P. asteris'. This agent was also associated with cherry little leaf disease of sour cherry in Lithuania showing shoot proliferation, small leaves, and decline symptoms and was classified to a new subgroup 16SrI-Q (Valiunas *et al*., 2009). Similar symptoms were observed on 'cherry XVI.12' during this study and results showed that it was infected by a '*Ca*. P. asteris' strain closely related to reference strains from the 16SrI-B subgroup. Phytoplasmas of subgroup 16SrI-B have reported to infect plum and sour cherry in Lithuania (Valiunas *et al*., 2007) as well as *Prunus* Mahaleb in Hungary (Varga *et al*., 2001). Phytoplasmas from 16SrI group are mainly associated with diseases of herbaceous plants, however their host range is broader and included also fruit trees.

Acknowledgements

This research was conducted in frame of COST Action FA1104 and financially supported by Ministry of Science and Higher Education (project UMO-2013/08/M/NZ9/00138).

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S33-S34

doi: 10.5958/2249-4677.2015.00014.6

Phytoplasma strain collections: the Q-Bank database and the Q-collect project

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Abstract

The setting of the Q-Bank database freely available on line for quarantine phytoplasmas and also for general phytoplasma identification was linked with a new project Q-collect in order to made widely available the identification of relevant plant pests by nucleic acid sequence comparison of their barcodes and also by providing infected samples that are verified by quality standard for correct phytoplasma identification.

Keywords: Collection, phytoplasmas, reference strains, network, sequencing, molecular identification

Introduction

International trade is increasing tremendously in recent years and within this context there is also the increasing risk of importing unwanted organisms via this trade. Also climate change may increase the ability of plant pests to survive regions other than those of their origin. To regulate and control plant pathogens there is an increasing need for efficient and reliable identification and detection tools. For their development and validation, good and well maintained collections containing relevant species are indispensable. An European project (Q-BOL) and its following network (Q-Bank) allowed to produce and deposit barcode sequences of a number of phytoplasmas collected worldwide, in a dedicated portal (Bertaccini *et al*., 2011; Contaldo *et al*., 2014). The majority of these strains are currently maintained in alive shoots in a micropropagated collection (Bertaccini, 2014) (Figure 1). In this frame the new "Coordination and Collaboration between reference collections of plant pests and diseases for EU Plant Health Policy" (Q-collect) project allowed to develop and finalize the inventory of the existing strains in this and other phytoplasma collections. Setting up of quality standards for these materials in order to make them available to worldwide researchers and routine laboratories are among the aims of this project.

Materials and Methods

Q-collect includes an inventory on characteristics of phytosanitary important collections within Europe, guidelines on how to improve quality standards and access and to design and build sustainable networks of reference collections. DNA based sequencing, fingerprinting and barcoding technologies are supportive and/or determinative for developing an improved taxonomy based on traditional methods and keys in the selection and authentication of appropriate reference specimens. Databases of DNA barcodes generated through the recent Q-BOL project and included into the Q-Bank database offer rapid and efficient means to verify phytoplasma identity and will have an increasingly important role in quality control. Phytoplasma infected plant material from periwinkle or from natural plant hosts was used for both micropropagation (Bertaccini *et al*., 1992) and nucleic acid extraction (Prince *et al*., 1993).

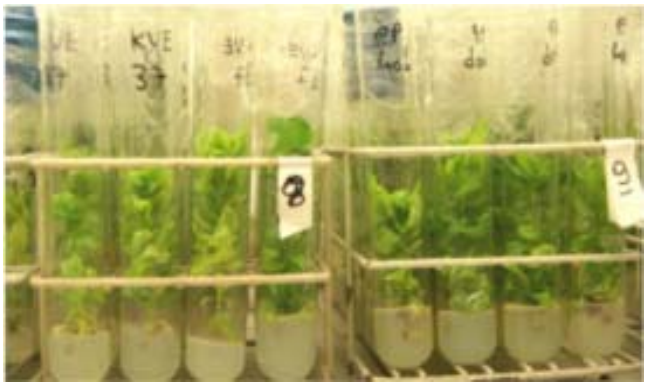

Figure 1. Collection of phytoplasma strains in shoots of micropropagated periwinkle.

Figure 2. Q-collect logo.

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Phytoplasma collections of strains in micropropagation and of their DNAs and/or barcode sequences contain relevant quarantine organisms, their close relatives and other diagnostically similar organisms (look-a-likes). Attached to these collections, taxonomic experience is available and the Q-collect project will lead to a major and necessary improvement, integration and streamlining of reference phytoplasma collections. After a thorough inventory, the knowledge on existing and missing reference material as well as on taxonomic expertise attached to it and guidelines for quality, access will be presented in a user-friendly way via the info-portal. A strong basis for the infrastructure of reference collections for plant health is produced by the project after review of the currently used quarantine standards for establishment and maintenance of a sustainable European network of reference collections.

Results

The phytoplasma identification process includes: DNA extraction; PCR amplification; nested PCR; sequencing; sequence analysis and assembly, and finally online identification. Through this method identification of phytoplasma strains is achieved, however when mixed infections occur, cloning before sequencing, or deep amplicon sequencing (Contaldo *et al.*, 2012) is required. The system allows quick identification of unknown phytoplasmas in both *16S rRNA* and *tuf* genes, but also cross testing with sequences of other quarantine organisms.

The Q-bank phytoplasma database (http://www.qbank.eu/ Phytoplasmas/) contains barcode DNA sequences of more than 100 strains from worldwide phytoplasma-associated diseases. As part of Q-BOL project two sets of primers amplifying a fragment of the Tuf gene and a fragment of the *16S rRNA* gene, respectively, were designed and the potential of these fragments as DNA barcodes was experimentally validated (Makarova *et al.*, 2012; 2013).

Successful amplification and sequencing of phytoplasma strains, and ability to separate various phytoplasmas to '*Candidatus* species' level (IRPCM, 2004), 16S ribosomal group and sub-group level (Lee *et al.*, 1998) show that these barcodes are efficient detection tools, and until now identification of the following phytoplasma groups at group and in several cases subgroup levels has been achieved: 16SrI, 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXII, 16SrXIV, 16SrXV, 16SrXVIII, 16SrXX, 16SrXXI.

Discussion

Collections of pest and pathogen specimens, and the archive information which accompanies them, are valuable resources of information for taxonomic research and identification. They are widely used by scientists to resolve taxonomic and nomenclatural problems and provide critical reference material for identification and classification of the pests and pathogens under study. They are also used for the validation of diagnostic methods, as positive/negative controls in diagnostic assays and in test performance studies. Q-collect is identifying the major challenges in the development of a network of relevant phytosanitary collections, collectively harbouring quarantine organisms and their closely related species. For phytoplasmas the aim is to develop guidelines and reference criteria for the quality, access and maintenance for the phytosanitary collections available to be used by any plant diagnostic centre, national reference organisation and authority freely using the internet, to underpin the implementation of Council Directive 2000/29/EC and follow-up legislative EU regulations.

Acknowledgements

The work was carried out as part of the project "Coordination and Collaboration between reference collections of plant pests and diseases for EU Plant Health Policy" FP7-KBBE-2013-7-single-stage.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S35-S37

doi: 10.5958/2249-4677.2015.00015.8

European interlaboratory comparison of detection methods for "flavescence dorée" phytoplasma: preliminary results

The EUPHRESCO GRAFDEPI GROUP

Abstract

A working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of "flavescence dorée" phytoplasma in grapevine. Seven molecular protocols were compared in interlaboratory trials starting from extracted DNAs prepared in one laboratory. The tested molecular protocols consisted of universal and group-specific real-time and conventional nested PCR assays. Real-time PCR protocols, generally, revealed higher values of diagnostic sensitivity and diagnostic specificity than conventional PCR protocols. These preliminary results require a statistical analysis to confirm the performances of the protocols.

Keywords: ringtest, "flavescence dorée" phytoplasma, diagnostic protocols

Introduction

In the competitiveness of agricultural products the phytosanitary quality is of increasing importance and harmonized protocols for the detection of pathogens had taken an active role in the agricultural food chain. In this context, a working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of "flavescence dorée" (FD) phytoplasma in grapevine. FD is one of the main yellows diseases of grapevine in Europe included in European legislation as a quarantine pest (directive 2000/29 EC). Protocols based on conventional and real-time PCR assays for the detection of this phytoplasma were selected and submitted to interlaboratory trials performed in 14 European laboratories. Validation data was calculated according to UNI CEI EN ISO/IEC 17025 and preliminary results of the collaborative study are reported.

Materials and Methods

Design of the study. The 14 participant laboratories (Table 1) analysed an identical series of blind samples following the provided working protocols.

Samples. The tested samples consisted of DNAs to avoid problems of homogeneity and stability (Table 2). To evaluate analytical specificity, the 24 blind samples consisted of 11 grapevine samples positive for FD phytoplasma, 4 samples positive for other phytoplasmas of the 16SrV group and 9 samples containing phytoplasmas not related to FD. To evaluate the analytical sensitivity and the reproducibility, 5-fold serial dilutions for 3 samples were analysed 5 times each. Only five laboratories participated in this step of the evaluation.

Tested protocols. Seven molecular protocols were submitted to the interlaboratory trials: 1) a universal nested-PCR assay followed by RFLP analysis with *Taq*I (Martini *et*

Table 1. List of participants involved in the interlaboratory trials.

Institution	Contact	Country
CRA-PAV, Plant Pathology Research Centre	G. Pasquini	Italy
AGES, Austrian Agency for Health and Food Safety	H. Reisenzein	Austria
CRA-W, Walloon Agricultural Research Centre	S. Steyer	Belgium
PPRS, Plant Protection Research Station	N. Ustun	Turkey
INIAV National Institute of Agrarian and Veterinary	E. Sousa, E. Silva	Portugal
ACW, Agroscope Changins-Wädenswil	S. Schaerer	Switzerland
ILVO, Institute for Agricultural and Fisheries Research	K. De Jonghe	Belgium
Alma Mater Studiorum, University of Bologna	A. Bertaccini, S. Paltrinieri	Italy
Dipartimento di Produzione Vegetale, University of Milan	P. Bianco, P. Casati	Italy
IPEP, Institute of Pesticides and Environmental Protection	B. Duduk, J. Mitrovic	Serbia
NIB National Institute of Biology	M. Dermastia	Slovenia
IRTA, Institut de Recerca i Tecnologia Agroalimentaries	A. Battle	Spain
ANSES	M. Loiseau	France
CRA-VIT, Centro di Ricerca per la Viticoltura	E. Angelini	Italy

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al., 1999); 2) two group specific nested-PCR assays (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Lee *et al.*, 1994; Clair *et al.*, 2003); 3) two real-time PCR assays for specific detection of 16SrV group phytoplasmas (Angelini *et al.*, 2007; Hren *et al.*, 2007); 4) two real-time PCR assays for co-detection of 16SrV and 16SrXII group phytoplasmas, and internal grapevine control (Pelletier *et al.*, 2009 and oligonucleotides under patent IPADLAB).

Processing of the results. The following parameters were calculated: diagnostic sensitivity (SE) – an estimation of the ability of the method to detect the target; diagnostic specificity (SP) – an estimation of the ability of the method not to detect the non-target; last level at 100% positive results; reproducibility: the percentage chance of finding the same result for two identical samples analyzed in two different laboratories. The reproducibility was the percentage of all pairing giving the same results for all possible pairings of data.

Table 2. List of tested samples and their origin.

Provider	strain origin	Details
JKI	Germany	Palatinate grapevine yellows 16SrV-C
DipSA	USA	Aster yellows 16SrI-B
ANSES	France	"Stolbur" 16SrXII-A
CRA-PAV	Italy	healthy grapevine
DipSA	USA	Ash yellows 16SrVII-A
DipSA	Italy	FD-C
AGES	Austria	FD-C
ANSES	France	"Stolbur" 16SrXII-A
INIAV	Portugal	FD-D
DipSA	Italy	Rubus stunt 16SrV-E
ANSES	France	healthy grapevine
NIB	Slovenia	FD-D
ANSES	France	FD 1/2 into healthy grapevine
ANSES	France	FD + "bois noir"
ANSES	France	FD
ACW	Switzerland	mix of FD infected samples
NIB	Slovenia	healthy grapevine
DipSA	Italy	Grapevine yellows GY-U 16SrIII-B
DipSA	China	Jujube witches' broom 16SrV-B
ANSES	France	FD+ 1/5 into healthy grapevine
DipSA	Europe	Elm yellows strain ULW 16SrV-A
ANSES	France	mix of healthy grapevine
IPEP	Serbia	FD
ANSES	France	FD

Results

None of the methods was able to distinguish the FD phytoplasma subgroups except universal nested PCR + RFLP, even if this method showed a low reproducibility. Therefore, performances of methods were calculated regarding their ability to detect all 16SrV phytoplasmas and distinguish them from phytoplasmas in other ribosomal groups. Diagnostic sensitivity, specificity, last level at 100% positive results and reproducibility were calculated for each protocol and for each laboratory and mean values are reported in Table 3. The sensitivity ranged from 83.7% to 100% and the specificity ranged from 66.1% to 100%. For all conventional PCR and for two of the real-time PCR protocols, none of the tested level of dilution displayed 100% positive results. For the two triplex real time protocols, the last level at 100% positive results ranged from 1/10 to 2,700. The reproducibility ranged from 60.2% to 93.3%.

Discussion

A first and general view of the results obtained in the interlaboratory trials showed that real time PCR protocols developed by Hren *et al.* (2007), Pelletier *et al.* (2009) and under patent oligonucleotides had a diagnostic sensitivity and a diagnostic specificity higher than 90%. Generally, conventional PCR protocols resulted in less sensitivity and/or specificity. Nevertheless, the results presented should be interpreted with precautions because no statistical analysis of the data was conducted in order to underline outliers and demonstrate statistical performances of protocols.

Acknowledgements

This work was carried out in the frame of EUPHRESCO project. The Group would like to thank Michael Maixner JKI, Germany for providing DNA extracts of Palatinate Grapevine Yellows.

Table 3. Performances of methods for the detection of the 16SrV phytoplasma group.

	Universal nested PCR + RFLP	Group specific nested PCR	Duplex group specific nested PCR	Real-time PCR (Angelini *et al.*, 2007)	Real-time PCR (Hren *et al.*, 2007)	Triplex real-time PCR (Pelletier *et al.*, 2009)	Triplex real-time PCR (oligonucleotides under patent)
Targeted area of the genome	16S rDNA	16S rDNA	SecY gene	16S rDNA	SecY gene	map gene	unknown
No. of laboratories considered	5	13	12	7	10	6	7
Mean SE	88.9%	91.4%	83.7%	86.7%	97.3%	97.7%	100%
Mean SP	93.2%	88.3%	92.4%	66.1%	94.1%	93.3%	100%
Last level at 100% positive results	< 1/10	< 1/10	< 1/10	< 1/10	< 1/10	1/100 - 1/2700	1/10 - 1/300
Reproducibility	67.7%	73.8%	60.2%	75.6%	84.9%	93.3%	86.7%

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Insect vectors of phytoplasmas and phytoplasma-vector interaction

Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S41-S42

doi: 10.5958/2249-4677.2015.00016.X

First insights into the influence of aster yellows phytoplasmas on the behaviour of the leafhopper *Mgenia fuscovaria*

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Abstract

Phytoplasmas can alter the behaviour of their insect vectors by modifying host plant and insect characteristics. In a dual-choice cage experiment, field-collected adult *Mgenia fuscovaria* were preferentially attracted by grapevine (cultivar Colombard) branches infected with aster yellows phytoplasma compared with uninfected branches. The influence of colour as a visual stimulus for *M. fuscovaria* was determined in a field trial. More adults were trapped on yellow and lime green than red, blue, white, and dark grey sticky colour cards. Within the green-yellow wavelength range more adults were attracted by the colour with the highest peak reflectance. Yellow is suitable for monitoring *M. fuscovaria*.

Keywords: colour, Cicadellidae, insect vector, preference

Introduction

Aster yellows phytoplasma (AY), '*Candidatus* Phytoplasma asteris' (16SrI-B group), was recorded for the first time in grapevine in South Africa in 2006 (Engelbrecht *et al*., 2010) and it is of phytosanitary concern in this country. The indigenous leafhopper *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae) has been identified as a vector of this South African strain (Krüger *et al*., 2011).

Phytoplasmas can influence the behaviour of vectors by modifying plant and insect characteristics (Hogenhout *et al*., 2008; Mayer *et al*., 2008). For example, they can alter the volatiles emitted by their host plant and the behaviour of their insect vector by being more attractive to the vector (Mayer *et al*., 2008). There is little information on the role of visual stimuli in attracting leafhoppers to host plants. Patt and Sétamou (2007) suggested that the cicadellid *Homalodisca coagulata* Say responded to chemical cues only as an enhancement of its response to visual cues. Many insect species are attracted to yellow, and AY symptoms in white grape varieties include yellowing of leaves. This suggests that the colour of AY-infected leaves may be more attractive to insect vectors compared with uninfected leaves. Thus far, the effect of AY-infected grapevine on the behaviour of *M. fuscovaria* has not been studied.

Materials and Methods

Experiments were carried out in Vredendal in the Western Cape, South Africa, during the austral summer in 2012 and 2013. Field-collected adult *M. fuscovaria* were given a choice between AY-infected and uninfected branches (cultivar Colombard) in a dual-choice cage experiment. Grapevine branches were collected from vines not treated with insecticides in an AY-infected vineyard. Choice experiments were carried out using wooden cages (30 x 30 x 42 cm) painted white and consisting of a glass top and a white gauze back for ventilation. Adults were starved for 30 minutes before experiments. A single leafhopper was released on top of an aluminium foil-covered podium (14.5 cm height) placed at a distance of 12 cm to the closest leaves of the AY-infected and uninfected branches. Each individual was observed for 15 minutes. To prevent bias, the side (left or right) of AY-infected and uninfected branches was changed after each leafhopper. Branches were replaced after release of five individuals. Each leafhopper was used once. Adult *M. fuscovaria* were sexed and their age determined (young, light green or light brown adults; mature, dark brown adults). In addition, the presence of AY in individual *M. fuscovaria* and leaves was determined with real-time PCR (Angelini *et al*., 2007). The leaf area was measured using ImageJ 1.45s (Wayne Rasband, National Institutes of Health, U.S.A., http://imagej.nih.gov/ij).

The role of colour on the choice of adults was evaluated in a field trial. Blue, green, lime green, yellow, red and, as controls, dark grey and white colour models in the form of laminated sticky colour cardboards were used to trap adults. The coloured sticky traps were placed in a randomized design with 10 rows of the seven colours each in the same block in which adult *M. fuscovaria* were collected for the cage choice experiment. The sticky traps were left in the field for 7 days. Spectral reflectance of the laminated colour cards with glue and of AY-infected and uninfected leaves was measured with an Avantes spectrophotometer (AvaSpec-3648).

Identification of *M. fuscovaria* was confirmed by M. Stiller (ARC-Plant Protection Research Institute, South Africa).

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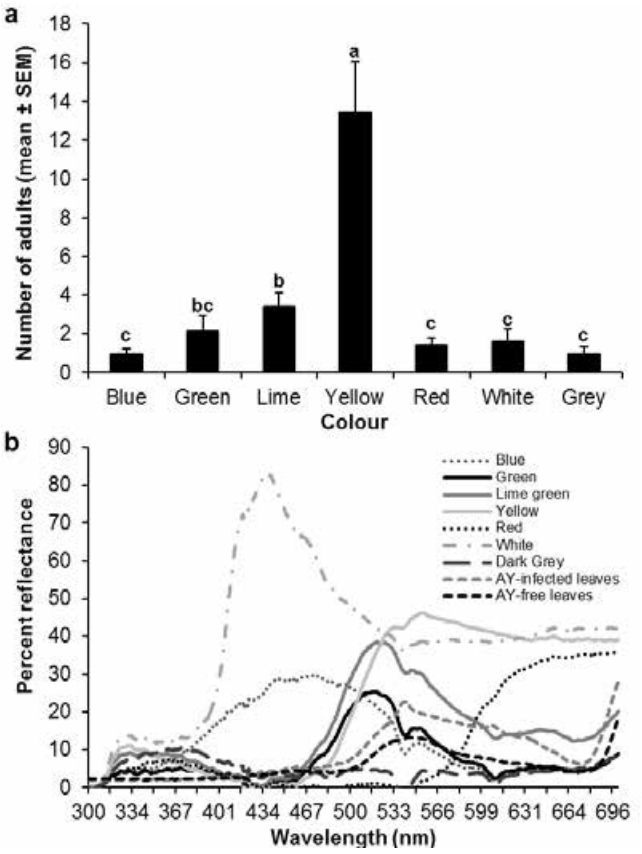

Figure 1. Number of adult *M. fuscovaria* caught on coloured traps (a) and reflectance spectra of coloured traps and AY-infected and AY-free grapevine leaves (b). Different letter above bars indicate significant differences ($P < 0.05$).

Results

Out of the 101 individual adults tested in the dual-choice cage trial, 91 (90%) responded and 74 made a choice between AY-infected and uninfected branches. Out of the 74 leafhoppers, 15 were infected with AY (2 males and 13 females). Adults of *M. fuscovaria* were significantly more attracted to AY-infected than uninfected branches; 45 (62%) chose AY-infected over AY-free branches. Neither position of the branches (left, right) in the cage, sex (male, female), age (young, mature adult), time (morning, afternoon) nor AY-infection status influenced leafhopper choice (logistic regression, $P > 0.05$). There was no significant difference between the leaf area between pairs of AY-infected and uninfected branches used in the experiment (Wilcoxon matched pairs test; $z = 0.597$, $P > 0.05$).

Colour affected the number of adult *M. fuscovaria* recorded on the traps. Significantly more adults were recorded on yellow than any other colour and more adults were recorded on lime green than blue, red, white, and dark grey sticky traps. The number of adults recorded did not differ significantly between blue, green, red, white, and dark grey traps analysis of variance (ANOVA) followed by Fisher's LSD test; $F_{6,63} = 11.802$, $P < 0.001$ (Figure 1a).

The most preferred colour traps showed the highest reflection in the 520 nm (green region) to 550 nm (yellow region) range, which is also the peak reflective region of AY-infected and uninfected grapevine leaves (Figure 1b). Within this wavelength range adults were trapped in higher numbers on the colour targets with the highest light reflectance.

Discussion

Adult *M. fuscovaria* preferred AY-infected to uninfected grapevine, suggesting that AY-infection rendered leaves of infected plants more attractive to the insect vector. It is not clear whether changes in the volatile profile or colour, or both, are responsible for the observed difference. The colour trial confirmed that yellow, which had the highest percentage reflectance in the green – yellow region, is superior to the other colours tested. The peak reflectance in AY-infected leaves was higher compared with uninfected leaves. This might in part explain the higher attractiveness of AY-infected over uninfected leaves in the current study. Additional experiments will be carried out to determine if AY-infected and uninfected leaves differ in attraction based on colour alone. In addition, experiments on the influence of olfactory cues of AY-infected grapevine are underway.

Acknowledgements

We thank Jeff Joubert (Vinpro) and growers in the Vredendal region for assistance with field trials, Piet and Sarah Coetzee for providing facilities, and the Technology and Human Resources for Industry Programme (THRIP) and the University of Pretoria for financial support.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S43-S44

doi: 10.5958/2249-4677.2015.00017.1

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Transmission of 16SrIII-J phytoplasma by *Paratanus exitiosus* (Beamer) leafhopper in grapevine

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Abstract

The most common insect in Chilean phytoplasma-infected vineyards, belonging to the family Cicadellidae, is *Paratanus exitiosus* (Beamer). This leafhopper was proved to be able to transmit the 16SrIII-J phytoplasma to periwinkle plants. In the present work we demonstrate that *P. exitiosus* transmits the same phytoplasma to grapevine plants too.

Keywords: auchenorrhyncha, transmission trials, grapevine yellows, *tuf* gene, *16S rRNA* gene

Introduction

Yellows symptoms on Chilean grapevines (*Vitis vinifera* L.) are due to phytoplasmas belonging to ribosomal subgroups 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'), 16SrIII-J (X-disease group), 16SrV-A ('*Ca.* P. ulmi'), 16SrVII-A ('*Ca.* P. fraxini'), 16SrXII-A ("stolbur" or "bois noir") (Gajardo *et al.*, 2009; González *et al.*, 2010). The presence of these pathogens depends on both propagation of infected plants and spreading by different insect species which feed on grapevine, and also on the weeds growing near and/or in vineyards. In symptomatic vineyards several leafhoppers (Cicadellidae) were found infected with phytoplasmas. The most common was *Paratanus exitiosus* (Beamer) in which phytoplasmas of 16SrI-B, 16SrIII-J, 16SrVII-A, and 16SrXII-A subgroups were detected (Longone *et al.*, 2011; Fiore *et al.*, 2012). This hemipteran insect, belonging to the suborder Auchenorrhyncha, is able to transmit 16SrIII-J phytoplasma to periwinkle plants (Fiore *et al.*, 2012). In this work, the phytoplasma transmission capability of *P. exitiosus* also to grapevine plants was verified.

Materials and Methods

During 2011 (September - December) and 2012 (January - May) a *P. exitiosus* survey was carried out in Chilean vineyards infected by phytoplasmas, two located in Metropolitana Region (Buin and Pirque) and one in Valparaiso Region (Casablanca). The insects were captured by means of an entomological sweeping net. During the sampling period adults field captured *P. exitiosus* were released into entomological cages to let them feed on three plants of grapevine (variety Cabernet Sauvignon) grown from seed and previously tested to ascertain the absence of phytoplasmas (Table 1). A total of 81 plants were used. Grapevine plants were tested starting ten months after transmission trials, and dead leafhoppers were tested after maintenance in 70% ethanol in order to detect phytoplasma presence. Total nucleic acids were extracted with chloroform/phenol, dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/µl of nucleic acid were used for amplification. Direct and nested PCR for amplification of the *tuf* gene were carried out according to Makarova *et al.* (2012). Further, direct PCR with P1/P7 and nested PCR with R16F2n/R2 primers (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Gundersen and Lee, 1996) were performed. Amplicons from nested PCRs for both genes were purified using Concert Rapid PCR Purification System and DNA fragments were cloned. Putative recombinant clones were analyzed by colony PCR. Selected fragments were sequenced in both directions using the BIG DYE sequencing terminator kit. The sequences were then aligned with those of reference phytoplasma strains deposited in GenBank and Q-Bank. Identification was done using *in silico* RFLP analyses with *Mse*I, *Nde*II, *Hha*I, *Bst*UI, and *Rsa*I restriction enzymes (Zhao *et al.*, 2009). The first two enzymes were used for *tuf* gene and the last three for *16S rRNA* gene sequences.

Results

P. exitiosus survived 3-4 days on grapevine plants. Five out of 81 plants used for transmission trials were positive to phytoplasmas in nested PCR using primers for *tuf* and *16S rRNA* genes. Two plants (V47 and V78A) corresponded to the

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Table 1. Number of *P. exitiosus*, captured in the three vineyards, that were released per month in cages to feed on grapevine plants variety Cabernet Sauvignon in transmission trials.

Month	Vineyard		
	Buin	Pirque	Casablanca
September 2011	33	6	2
October 2011	25	17	20
November 2011	34	8	22
December 2011	36	18	9
January 2012	33	20	21
February 2012	31	14	30
March 2012	34	10	24
April 2012	18	5	21
May 2012	33	3	23

transmission trials carried out with insects captured in Buin in two different months (October 2011 and May 2012 respectively), two plants (V43 and V61) were infected from insects captured in Pirque during September and December 2011 respectively. The last plant (V76B) was infected from specimens of *P. exitiosus* captured in Casablanca during April 2012. The transmission rates were 7.5% with insects captured in Buin and Pirque, and 3.7% with those from Casablanca. Cloned nested PCR fragments from both genes were sequenced and there was no sequence differences between the cloned fragments from the five grapevines in *tuf* gene sequence (438 bp), while sequence identity was 99.9 to 100% in 16S rDN*A* sequences (1,250 bp). In 16S rDNA the similarity percentages of phytoplasmas found in the five grapevines, showed a close correlation (99.8%) with the strain Ch10 (AF147706), corresponding to chayote witches' broom phytoplasmas (16SrIII-J) from Brazil. In *tuf* gene the closer similarity percentages of phytoplasmas found, was 96.7%, with the strain QPh20, corresponding to 16SrIII-D phytoplasma from USA. In Q-Bank no sequences of phytoplasmas belonging to 16SrIII-J subgroup are available so far. The tuf and 16S rDNA amplicons were also subjected to *in silico* RFLP analysis that confirmed the assignment of phytoplasmas to the ribosomal subgroups 16SrIII-J (X-disease group). The phytoplasma 16SrIII-J was also detected in *P. exitiosus* specimens used for transmission assays. Two out of five grapevines infected with 16SrIII-J were asymptomatic (V43 and V61), while the other three plants showed short internodes, and leaves with downward rolling, deformation, yellowing and necrosis.

Discussion

P. exitiosus transmitted the phytoplasma 16SrIII-J to periwinkle (Fiore *et al*., 2012). This study showed that the insect is able to transmit the same phytoplasma to grapevine as well. *P. exitiosus* lives on weeds and only occasionally feed on grapevine or other crops. The phytoplasma 16SrIII-J and its vector are largely distributed in Chile, on different weed species and crops of agronomic interest (Castro *et al*., 2000; Hepp and Vargas, 2002; González *et al*., 2010; González *et al*., 2011; Longone *et al*., 2011; Fiore *et al*., 2012). Considering the *P. exitiosus* transmission rates observed, if environmental conditions are favorable, there is a high likelihood to expect an outbreak of grapevine yellows in Chile due to 16SrIII-J phytoplasma.

Acknowledgements

Research supported by National Fund for Scientific and Technological Development (FONDECYT), Chile, project N° 1140883.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S45-S46

doi: 10.5958/2249-4677.2015.00018.3

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Molecular characterization of phytoplasma strains in leafhoppers inhabiting the vineyard agroecosystem in Southern Switzerland

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Abstract

In 2011 the insect species were surveyed in 48 vineyards in Southern Switzerland to determine potential vectors for the spread of "flavescence dorée" and "bois noir" diseases. Overall, 367 insect samples were analysed by nested PCR and RFLP analyses. Sixteen samples, from 8 vineyards, were positive for phytoplasmas. *Orientus ishidae* and *Scaphoideus titanus* harboured "flavescence dorée"-related phytoplasmas. *Hyalesthes obsoletus* and *Reptalus* species harboured "stolbur" phytoplasmas. This screening allowed to gain more insight into species potentially involved in the epidemiology of these grapevine diseases in Southern Switzerland.

Keywords: grapevine yellows, Auchenorrhyncha vectors, molecular detection

Introduction

Grapevine yellows (GY) are responsible for severe diseases associated with phytoplasmas producing serious damages in viticulture and wine industry. The diseases occurring in Europe are "flavescence dorée" (FD) and "bois noir" (BN), transmitted by the leafhopper *Scaphoideus titanus* and the cixiid *Hyalesthes obsoletus*, respectively.

These two diseases are associated with phytoplasmas belonging to different ribosomal groups, in particular 16SrV-C and -D subgroups for FD, 16SrXII-A for BN. Several molecularly differentiable strains of FD and BN phytoplasmas were reported in literature, some of them associated with specific host plants and vectors and others present in specific geographic areas (Martini *et al*., 2002 Filippin *et al*., 2009; Foissac *et al*, 2013).

In Switzerland FD and BN diseases have been recorded in 2004 (Schaerer *et al*., 2007) and 2001 (Schmid and Emery, 2001) respectively, but the epidemiologic insect aspects are poorly understood. The aim of this work was to monitor leafhoppers inhabiting Southern Swiss vineyards infected by GY, to identify known and potential vectors and to characterize the phytoplasmas present in the vineyard ecosystem, in order to gain more insight into the epidemiology of these diseases in Switzerland.

Materials and Methods

The whole wine-growing area of Southern Switzerland was surveyed and 48 study sites were selected based on slope and the dominant land use type surrounding the vineyard. Out of 48 investigated vineyards, 5 were affected by FD, 13 by BN, 10 by both and in 20 no symptoms were reported in the last decade (Jermini *et al*., 2014). Three trap sites were placed: two within the vineyard and one in the ecotonal zone between forest and vineyard. Leafhoppers were sampled using D-Vac sampler, sweep net and yellow sticky traps for a total of 8 sampling periods. All specimens were identified to species level and preserved in 70% alcohol. Insect samples were constituted by a pool of one to 20 specimens.

DNA was extracted according to a previously reported protocol (Angelini *et al*., 2001). Real time PCR analyses on ribosomal genes were carried out in order to identify the presence of phytoplasmas of the 16SrXII and 16SrV groups (Angelini *et al*., 2007). Samples that tested positive were amplified by nested PCR on three genes for strain characterization. The BN positive samples were analysed in *16SrRNA*, *tuf* and *secY* genes. For the FD-related strains *16SrRNA*, *rplV-rpsC* and *secY* genes were amplified. All amplicons were digested with different restriction enzymes: *Taq*I for 16SrDNA, *Hpa*II for tuf, and *Tru9*I for secY. Amplicons from *16SrRNA* gene were sequenced both strands and their sequences were compared with those from other phytoplasmas in public databases.

Results

In total, 55,546 Auchenorrhyncha specimens were collected and identified to species level. Among all collected specimens, 3,505 individuals belonging to 27 species and 24 genus, were selected for molecular analyses and pooled in a total of 367 samples. Sixteen samples (collected from 8 vineyards) were positive for FD and BN phytoplasmas.

Molecular analyses showed that four samples were infected with FD-related phytoplasmas: three out of 19 collected *Orientus ishidae* samples and one out of 148 collected *S. titanus* samples (Table 1).

The RFLP analyses on ribosomal gene revealed that *O. ishidae* and *S. titanus* harboured FD-related phytoplasmas belonging to 16SrV-C and 16SrV-D subgroup, respectively.

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Table 1. Known and putative phytoplasma insect vectors identified from vineyards in Southern Switzerland.

Ribosomal subgroup	Tuf-type	Insect species	Positive samples/ total tested
16SrV-D	-	*Scaphoideus titanus*	1/148
16SrV-C	-	*Orientus ishidae*	3/19
16SrXII-A	a	*Hyalesthes obsoletus*	7/39
16SrXII-A	b	*Hyalesthes obsoletus*	1/39
16SrXII-A	b	*Reptalus panzeri*	1/7
16SrXII-A	b	*Reptalus cuspidatus*	3/25

Digestions of *rplV-rpsC* and *secY* amplicons confirmed the possible FD-D like pattern of the sample from *S. titanus*; in parallel, the profile analyses on the same genes distinguished samples from *O. ishidae* in two types, one similar to FD-C and the other to alder yellows (ALY) phytoplasmas.

The BN phytoplasma was detected in eight *H. obsoletus* out of the 39 collected specimens, in three *Reptalus cuspidatus* out of 25 and in one *Reptalus panzeri* out of seven. *Hpa*II digestion of tuf amplicons showed a prevalence of tuf-type a in phytoplasmas from *H. obsoletus* (7 of 8), while one phytoplasma from *H. obsoletus* and all those from *Reptalus* spp. were tuf-type b (Table 1). The sequence data of 16SrRNA fragments confirmed the RFLP patterns. Sequencing of the other genes is ongoing and preliminary comparisons suggested peculiarities in the Swiss strains.

Discussion

Despite of mandatory control using insecticides, individuals of *S. titanus* are still captured in vineyards of the Southern Switzerland. It is interesting to notice that only 1 out of 148 *S. titanus* tested samples was FD-positive. Nevertheless, disease outbreaks are still observed, but no clear relationship with *S. titanus* population levels was observed. In Europe, *O. ishidae* was first reported in Switzerland in 2002 (Gunthart and Muhlethaler, 2002) and in 2010 specimens infected by FD were detected for the first time in Slovenia (Mehle *et al.*, 2010). In this study, samples of *O. ishidae* were positive for FD. However further research is needed to shed light on the role of *O. ishidae* in the possible transmission of different FD-phytoplasma strains to grapevine.

The results of the survey confirm the presence of both BN tuf-type a and b in *H. obsoletus* as already observed by Maniyar *et al.* (2013). In this study high population levels of *R. cuspidatus* were observed and the tested samples were infected by tuf-type b only. The recently reported BN vector *R. panzeri* (Cvrkovic *et al.*, 2014) seems to be less important in the investigated region, as it was rarely collected within vineyards. Although detection of the phytoplasma in the insect body does not necessarily prove its vector status, this screening has allowed to identify some insect species possible candidate as phytoplasma vector to be proved in transmission assays. To clarify the important issue of epidemiological cycle of both FD and BN diseases additional analysis of genomic sequencing of phytoplasma strains are in progress.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S47-S48

doi: 10.5958/2249-4677.2015.00019.5

Transmission of 16SrIII-J phytoplasma by *Bergallia valdiviana* Berg 1881 leafhopper

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Abstract

One of the most common insects in vineyards infected with phytoplasmas belonging to the family Cicadellidae, is *Bergallia valdiviana* Berg 1881 This leafhopper has not yet been described as a phytoplasma vector. The present work demonstrates that *B. valdiviana* is able to transmit phytoplasmas to periwinkle plants.

Keywords: Auchenorrhyncha, transmission trials, nested-PCR, *tuf* gene, *16S rRNA* gene, RFLP, sequencing

Introduction

Yellows symptoms on Chilean grapevines (*Vitis vinifera* L.) are due to phytoplasmas belonging to the ribosomal subgroups 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'), 16SrIII-J (X-disease group), 16SrV-A ('*Ca.* P. ulmi'), 16SrVII-A ('*Ca.* P. fraxini'), 16SrXII-A ("stolbur" or "bois noir") (Gajardo *et al.*, 2009; González *et al.*, 2010). The presence of these pathogens in the plants depends on both propagation of infected plants and spreading by different insect species which feed on grapevine, and also on the weeds growing near and/or in vineyards. In symptomatic vineyards several leafhoppers (Hemiptera, Auchenorryncha, Cicadellidae) were found positive to phytoplasmas. Among them, the third more common leafhopper species is *Bergallia valdiviana* Berg 1881, although it was not described as a vector of phytoplasmas. In the present work, evidence of its ability in phytoplasma transmission to *Cantharanthus roseus* G. Don. (periwinkle) is presented for the first time.

Materials and Methods

During 2012 (October-December) and 2013 (January-June) surveys on *B. valdiviana* presence were carried out in a phytoplasma-infected vineyard variety Pinot noir in the Valparaíso Region (Casablanca, Chile). The insects were captured by means of an entomological sweeping net. During the sampling period adults of *B. valdiviana* captured were released into entomological cages to let them feed on three plants of periwinkle grown from seed and previously tested to ascertain the absence of phytoplasmas (Table 1). A total of 27 plants were used. Periwinkle plants were tested starting one year after transmission trials, and dead leafhoppers were tested (four to six leafhoppers per test) after maintenance in 70% ethanol in order to detect phytoplasmas presence. Total nucleic acids were extracted with chloroform/phenol methods, dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/µl of nucleic acid were used for amplification. Direct and nested PCR for amplification of the *tuf* gene were carried out according to the protocol of Makarova *et al.* (2012). Further, direct PCR with primer pair P1/P7 and nested PCR with R16F2n/R2 primers on the *16SrRNA* gene (Gundersen and Lee, 1996) were performed following a published protocol (Schaff *et al.*, 1992). Amplicons from nested PCRs for both genes were purified using Concert Rapid PCR Purification System and DNA fragments were cloned. Putative recombinant clones were analyzed by colony PCR. Selected fragments from cloned DNAs were sequenced in both directions in MacrogenUSA Corp (Rockville, MD, USA). The sequences were then aligned with those of reference strains deposited in GenBank and Q-Bank, using

Table 1. Number of individuals of *B. valdiviana*, captured in the Casablanca vineyard, released per month in cages on periwinkle plants for transmission trials.

Month	Number of insects
October 2012	4
November 2012	16
December 2012	22
January 2013	25
February 2013	33
March 2013	30
April 2013	21
May 2013	19
June 2013	23

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BLAST engine for local alignment (version Blast N 2.2.12). Identification was done using *in silico* restriction fragment length polymorphism (RFLP) analyses with *Mse*I, *Nde*II, *Hha*I, *Bst*UI, and *Rsa*I restriction enzymes (Zhao *et al.*, 2009). The first two enzymes were used for *tuf* gene and the last three for *16S rRNA* gene sequences.

Results

B. valdiviana survived 6-7 days on periwinkle plants. One out of 27 plants used for transmission trials was positive to phytoplasmas in nested PCR assays using primers for *tuf* and *16S rRNA* genes. This plant (V62B) corresponds to the transmission trials performed with insects captured in April 2013. The transmission rate was 3.7%. Three cloned nested PCR fragments from both genes were sequenced and there was no sequence difference between them in *tuf* (438 bp) and *16S rRNA* (1,250 bp) genes. In *16S rRNA* gene the similarity percentage of phytoplasma found in periwinkle, showed a close correlation (99.7%) with the strain TomRed16 (GenBank accession number KC412031), corresponding to *Solanum lycopersicum* phytoplasma (16SrIII-J) from Argentina. In *tuf* gene the closer similarity percentage of phytoplasma was 96.7%, with the strain QPh20, corresponding to 16SrIII-D phytoplasma from USA. In Q-Bank are not available sequences of phytoplasmas belonging to 16SrIII-J subgroup. The tuf and 16S rDNA amplicons were also subjected to *in silico* RFLP analysis that confirmed the assignment of phytoplasmas to the ribosomal subgroups 16SrIII-J (X-disease group). The phytoplasma 16SrIII-J, with identical nucleotide sequence, was also detected in ten of the *B. valdiviana* specimens used for transmission assay in V62B. Infected periwinkle with 16SrIII-J showed leaf deformation and severe yellowing.

Discussion

B. valdiviana transmitted the phytoplasma 16SrIII-J to periwinkle. This leafhopper lives on weeds and only occasionally feed on grapevine or other crops. The phytoplasma 16SrIII-J is also transmitted by *Paratanus exitiosus* (Beamer), another leafhopper largely distributed in Chile, on different weed species and crops of agronomic interest (Castro *et al.*, 2000; Hepp and Vargas, 2002; González *et al.*, 2010; 2011; Longone *et al.*, 2011; Fiore *et al.*, 2012). This is the first report of 16SrIII-J phytoplasma transmission by *B. valdiviana*. Transmission trials are currently in progress to grapevine plants.

Acknowledgements

Research supported by National Fund for Scientific and Technological Development (FONDECYT), Chile, project N° 1140883.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S49-S50

doi: 10.5958/2249-4677.2015.00020.1

Potential Hemipteran vectors of "stolbur" phytoplasma in potato fields in Serbia

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Abstract

During 2014 composition of planthoppers and leaf hoppers populations was studied in potato fields showing symptoms of "stolbur" phytoplasma infection in several regions in Serbia. Overall, 18 localitites have been inspected for the hemipteran specimens presence, and 19 species belonging to eight families could be identified. Nine species belong to the family Cicadellidae, three to Cixiidae and two to Delphacidae, whereas the families Aphrophoridae, Tettigometridae, Issidae, Cercopidae and Membracidae were present with only a single species. The most common was *Psammotettix alienus* (Dahlbom) collected on 16 sites, followed by *Hyalesthes obsoletus* Signoret , *Reptalus quinquecostatus* (Dufour), *Reptalus panzeri* (Löw) and *Euscelis incisus* (Kirschbaum). All five dominant species captured in potato fields are known to harbour or vectoring "stolbur" phytoplasma, which gives new lights to possible pathways of potato "stolbur" transmission and propagation.

Keywords: "stolbur", Hemiptera, phytoplasma, insect vectors, '*Ca.* P. solani'

Introduction

Potato "stolbur" has become an increasingly important disease in Europe during the past decade, with significant impact on potato production. Although still having a quarantine status in the European Union, geographic spread of this phytoplasma is evident, being reported in Germany, Czech Republic, Switzerland, Turkey, Bulgaria, Romania, Russia, Serbia (EPPO 1996; Ember *et al.*, 2011; Jovic *et al.*, 2011). However, epidemiology of this severe disease is still unknown. One of the main sources for permanent infection in the field are diverse native plants as reservoirs of the phytoplasma. In addition, many hemipteran species have been found to be carriers of "stolbur" phytoplasmas. The aim of this survey was to monitor the presence of potential vectors within Auchenorrhyncha species in potato fields in Serbia expressing typical symptoms of "stolbur" infection.

Materials and Methods

The insects were collected from 17 localities in the north and north east, and one locality in central Serbia (Vojvodina). All inspected potato fields had fully developed symptoms of "stolbur" infection in form of upward rolling and yellowing of the top leaves, shorten internodes and aerial tubers (Figure 1). Insect sampling was carried out in 2014, from June to the end of August. Leafhoppers and planthoppers were collected using sweep nets and mouth-aspirators directly from symptomatic potato plants including the weeds in the very surrounding of symptomatic potato fields. Collected insects were stored in 96% ethanol for identification using the taxonomic keys described by Holzinger *et al.* (2003) and Biedermann and Niedringhaus (2004).

Results

The captured hemipteran specimens belonged to 19 different species as shown in Table 1. *Psammotettix alienus* (Dahlbom) was the species with the widest dispersal, being collected from 16 out of 18 inspected potato fields.

Figure 1. Symptoms of potato "stolbur" disease in form of aerial tubers and shortened internodes in potato fields in Serbia inspected for diversity of Auchenorrhyncha species.

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Table 1. List of insect species collected in the "stolbur" affected potato fields in Serbia.

Auchenorrhyncha	No. of localities
Cixiidae/Cixiinae	
Hyalesthes obsoletus Signoret, 1865	14
Reptalus quinquecostatus (Dufour, 1833)	11
Reptalus panzeri (Löw, 1883)	10
Delphacidae/ Asiracinae	
Asiraca clavicornis (Fabricius 1794)	1
Delphacidae/ Delphacinae	
Dicranotropis hamata (Boheman, 1847)	7
Tettigometridae	
Tettigometra atra Hagenbach 1825	1
Issidae/ Issinae	
Issus coleoptratus (Fabricius, 1781)	1
Cercopidae/ Cercopinae	
Lepyronia coleoptrata (Linnaeus, 1758)	1
Aphrophoridae/ Aphrophorinae	
Aphrophora sp.	2
Membracidae/ Smiliinae	
Stictocephala bisonia Kopp & Yonke, 1977	3
Cicadellidae/ Agalliinae	
Anaceratagallia ribauti (Ossiannilsson 1938)	4
Cicadellidae/ Cicadellinae	
Cicadella viridis (Linnaeus, 1758)	1
Cicadellidae/Typhlocybinae	
Typhlocybina sp.	5
Eupteryx atropunctata (Goeze, 1778)	5
Cicadellidae/Deltocephalinae	
Psammotettix alienus (Dahlbom, 1850)	16
Euscelis incisus (Kirschbaum, 1858)	10
Doratura impudica Horváth, 1897	3
Macrosteles sp.	2
Errastunus ocellaris (Fallén, 1806)	4

Presence of *Hyalesthes obsoletus* Signoret was monitored on 14 sites, *Reptalus quinquecostatus* (Dufour) was found on 11 sites, while *Reptalus panzeri* (Löw) and *Euscelis incisus* (Kirschbaum) were present on 10 localitites.

Discussion

In the past decade, increasing incidence of "stolbur" phytoplasma was registered in different crops in Europe. Epidemiology of "stolbur" disease is highly dependent on the host plant/insect vector associations, which may narrow or widen the transmission range of the phytoplasma in the field. Inspection of the "stolbur" affected potato fields in Serbia revealed the presence and wide distribution of the polyphagous hemipteran species which have been previously reported in association with "stolbur" phytoplasmas. Well documented vectors of "stolbur" -associated disease are *H. obsoletus* in vineyards (Maixner *et al*., 1995), and *R. panzeri* in maize fields and vineyards (Jovic *et al*., 2007; Cvrkovic *et al*., 2013). *R. quinquecostatus* could play a role as a natural vector (Trivellone *et al*., 2005), as well as *Anaceratagallia ribauti* which was reported to transmit "stolbur" to *Vicia faba* (Riedle-Bauer *et al*., 2008). Dominance of the vectors and natural carriers of "stolbur" phytoplasma among Auchenorrhyncha specimens captured in the infected potato fields in Serbia clearly reflects the rather complex epidemiological situation. Moreover, it corresponds to the progressive spread of potato "stolbur" disease observed over the past five years in Serbia, indicating several distinct transmission pathways *in situ* and which might complicate the development of an effective disease management program.

Acknowledgements

We thank the Swiss National Science Foundation for financial support during this study through the SCOPES grant IZ73Z0_152414 and Ministry of Education and Science of Republic of Serbia through grant III43001.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S51-S52

doi: 10.5958/2249-4677.2015.00021.3

Exitianus indicus (Distant): a putative vector for '*Candidatus* Phytoplasma cynodontis' in India

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Abstract

Chlorotic little leaves, witches' broom and shortened stolons/rhizomes on *Cynodon dactylon* was observed in and around sugarcane fields at Sugarcane Research Institute, Shahjahanpur, Uttar Pradesh, India during August–September 2013. The association of phytoplasmas with symptomatic *C. dactylon* was confirmed by direct and nested PCR amplification of phytoplasma *16Sr* gene. Out of the different prevalent species of leafhoppers on Bermuda grass, only *Exitianus indicus* tested positive for phytoplasma presence. Phylogenetic analysis of *16Sr* gene sequence suggested that the identified phytoplasmas from *C. dactylon* and *E. indicus* in the present study were 99% similar and belonged to 16SrXIV group. The study suggests that, *E. indicus* may be a putative vector for '*Candidatus* Phytoplasma cynodontis' and may play a role in transmitting 16SrXIV group phytoplasmas in nature.

Keywords: leafhopper, phytoplasma, 16Sr XIV group, natural infection

Introduction

Bermuda grass white leaf (BGWL) is a destructive phytoplasma disease of Bermuda grass (*Cynodon dactylon*), which is known to occur in several countries including India, Sudan, Italy, Cuba and Australia (Jung *et al*., 2003). The associated agent is a member of the BGWL group (16SrXIV), '*Candidatus* Phytoplasma cynodontis' (Marcone *et al*., 2004). Several weed species have been reported to act as reservoir hosts of many phytoplasmas in India and abroad (Alhudaib *et al.,* 2009; Mall *et al.,* 2011), but limited information is available on vector transmission of these phytoplasmas. *C. dactylon* is an important weed species found affected with 16SrXIV group phytoplasmas worldwide including India (Mall *et al*., 2011; Win and Jung, 2012). With the present study the possible insect vector responsible to transmit '*Ca.* P. cynodontis' in nature was identified.

Materials and Methods

Survey was made around sugarcane fields at Sugarcane Research Institute, Shahajahanpur, India in the month of August-September 2013 to verify phytoplasma association with white leaf disease of *C. dactylon*. The leafhopper species feeding on symptomatic *C. dactylon* were collected using sweep net method in the early morning hours and were identified from Department of Entomology, Indian Agricultural Research Institute, New Delhi, India. The nucleic acid from collected leafhopper and from symptomatic and asymptomatic *C. dactylon* plants was extracted as reported (Ahrens and Seemüller, 1992). Amplification of phytoplasma DNA was performed with the universal primer pair P1/P6 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and the nested primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). The DNA extracted from asymptomatic *C. dactylon* and sugarcane was used as negative control. The amplicons of 1.2 kb obtained from nested PCR assays were gel purified using the Pure Link gel extraction kit (Invitrogen, Germany) and directly sequenced in both directions. A search of homologous sequences was performed by BLAST analysis at NCBI. 16S rDNA sequences of *C. dactylon* and leafhopper amplicons were aligned with phytoplasma group/subgroup representatives available in GenBank by Clustal W (Thompson *et al.,* 1994) and used to construct a phylogenetic tree through MEGA 5.0 (Tamura *et al.,* 2011).

Results

Strong leaf chlorosis, shortened stolons/rhizomes, stunting and witches' broom symptoms were recorded on *C. dactylon*. Important populations of different leafhopper species were also recorded on Bermuda grass in and around the sugarcane field. The major species of planthopper/leafhoppers identified belonged to *Cofana unimaculata* Signoret, *Exitianus indicus* (Distant), *Empoasca motti* Pruthi and *Sogatella kolophon* Kirkaldy. Out of these predominant species, *C. unimaculata* and *E. indicus*, were recorded as nearly 70% of the total leafhopper population. Universal phytoplasma specific primer pair P1/P6, yielded an amplicon

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of about 1,500 bp both in symptomatic *C. dactylon* and positive control (sugarcane grassy shoot, phytoplasma) in direct PCR assay, while no amplification was observed with any leafhopper species (data not shown). Nested PCR assays yielded bands of about 1,200 bp in *E. indicus*, symptomatic *C. dactylon* and positive control (data not shown). No amplification was observed with the DNA extracted from asymptomatic Bermuda grass and other tested leafhopper/planthopper species (*C. unimaculata*, *E. motti* and *S. kolophon*). These results confirmed the association of phytoplasmas with *E. indicus* and *C. dactylon* white leaf.

The 16S rDNA sequences of phytoplasma from symptomatic *C. dactylon* (Acc. No. KM220615) and *E. indicus* (Acc. No. KM220614) were compared and found 99% similar. Comparison of 1.2 kb of R16F2n/R16R2 primed sequences from symptomatic *C. dactylon* and *E. indicus* showed 99% similarity with strains of '*Ca.* P. cynodontis' (KF234570), BGWL (AF248961), golden beard grass white leaf (AB642601) and *Brachiaria* grass white leaf phytoplasma all belonging to 16SrXIV group. Phylogenetic analysis of 16S rDNA sequence from *C. dactylon* and *E. indicus* and comparison with available sequences of 16SrXIV phytoplasma strains in GenBank also revealed their closest relationship with '*Ca.* P. cynodontis' (16SrXIV) group (Figure 1) already reported India in *C. dactylon* (Snehi *et al.,* 2008).

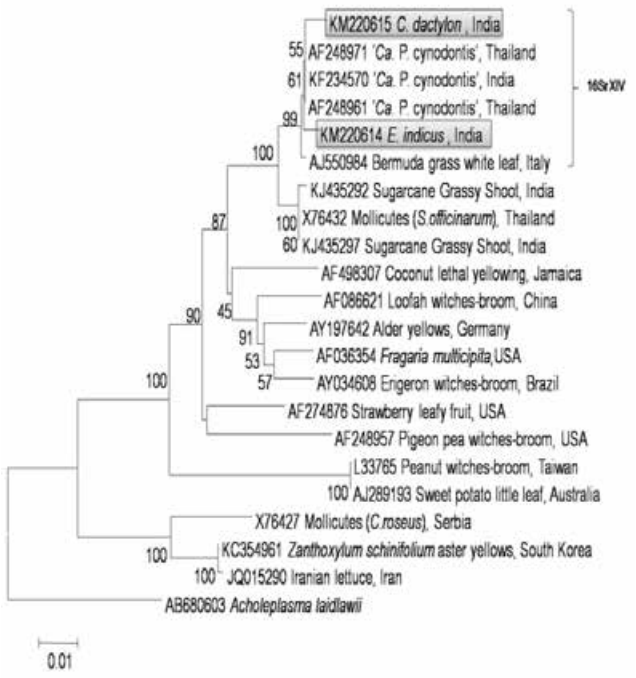

Figure 1. Phylogenetic relationship of phytoplasmas associated with Bermuda grass white leaf and *E. indicus* (highlighted) to sequences of other phytoplasmas. Bootstrap values (1,000 replications) are shown as percentages at the branch points of the phylogenetic tree.

The identification of '*Ca.* P. cynodontis' and 99% 16Sr DNA sequence identity between *C. dactylon* and *E. indicus* phytoplasmas, suggested that the latter may be a putative vector for 16SrXIV group phytoplasmas and may play a role in the spread of '*Ca.* P. cynodontis' strains in nature.

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Phytoplasma diseases worldwide

Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S55-S56

doi: 10.5958/2249-4677.2015.00022.5

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Phytoplasma diseases in date palms in Saudi Arabia

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Abstract

The date palm is one of the most important cash crops in Saudi Arabia and in the world. A disease called "Alwijam" associated with phytoplasma presence has lately been affecting date palms in Saudi Arabia. Date palm samples, both with and without the symptoms were collected from different locations and the DNA was extracted and indexed by a nested PCR reaction using different sets of phytoplasma generic primers to amplify the 16S rDNA region. The sequences of 16S rDNA obtained were compared with those of other phytoplasmas in GenBank and the results obtained indicated that there are two phytoplasma groups in symptomatic date palm: 16SrI in Alhasa and 16SrII in other locations in Saudi Arabia.

Keywords: phytoplasma, date palm, 16SrI, 16SrII, Saudi Arabia

Introduction

The date palm, (*Phoenix dactylifera* L.), is one of the most important cash crops in Saudi Arabia (SA). The area planted with date palms is 155,118 ha, in which more than 23 million trees produce more than 991,546 t of dates annually (Anonymous, 2011). "Alwijam" disease has been investigated by very few scientists. The first record of the disease is recorded in a book by Badawi (1945). The disease was recorded in SA by Elbaker (1952) and Nixon (1954). Later, Elarosi *et al.* (1983) reported that two species of *Fusarium* were always associated with the root of the Alwijam date palm. The symptoms are characterised by a stunting and yellow streaking of the leaves (Figure 1), with fruits and fruit stalks reduced in size by around 30%. A phytoplasma pathogen was suspected to cause Alwijam affected palms, following histopathology and antibiotic therapy studies (Abdusalam *et al.*, 1993). This was further supported by El-Zayat *et al.* (2000), who reported a phytoplasma associated with lethal yellow coconut palms in Florida. However, Alhudaib *et al.* (2007) reported the association of a phytoplasma of the 16SrI group, '*Candidatus* Phytoplasma asteris', with Alwijam in Alhasa, Saudi Arabia. In this work the association of phytoplasmas of the 16SrI and the 16SrII groups are associated with Alwijam in Saudi Arabia.

Materials and Methods

A survey was done during 2011-2013 in different locations in Saudi Arabia (Alhasa, Alkharj, Almadinah, Jouf, Qassim and Riyadh). More than 900 leaf samples were collected from date palms, including symptomatic and asymptomatic samples. A date palm tissue culture shoot was used in all experiments as healthy negative control. The total DNA was extracted from the collected samples using CTAB. Aliquots of final DNA preparations were used as templates for a nested PCR assay with phytoplasma 16S rDNA using a set of primers (R16mF2/R16mR1) (Gundersen and Lee, 1996) for direct PCR followed by fU5/rU3 (Lorenz *et al.*, 1995) for nested PCR. To determine in which ribosomal group this phytoplasma was classified, the PCR products in each location were purified from agarose gel and cloned into a pGEM-T easy vector (Promega, USA). An Agincourt CleanSEQ™ kit was used to clean up the PCR sequencing reaction. The 16S rDNA sequences of phytoplasmas identified in this study were compared with others in Genbank by BLAST (Altschul *et al.*,

Figure 1. Typical yellow streak in infected (I) midrib of date palm leaf; healthy leaf (H).

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1990). The sequences were aligned and a phylogenetic tree was constructed by the programme MEGA version 5 (Tamura *et al.*, 2011) using 1,000 bootstrap datasets to support the branch values. *Acholeplasma palmae* was used as the out-group to root the phylogenetic tree.

Results and Discussion

Phytoplasma rDNA was amplified in more than 900 leaf samples. Samples produced the 876 bp expected product from nested PCR amplification. No PCR products were obtained from asymptomatic palms. Data showed that some tested samples such as L4-L10 in Figure 2 were positive. Also these data showed that some plants were symptomless but gave positive reactions to phytoplasma primers that were confirmed by the nucleotide sequence analysis. The obtained data showed that the high percentage of infection with phytoplasma was in Alhasa and the infection percentage was near to 12%, however the percentages of infection in Alkharj, Riyadh and Qassim were 5.5%, 3.4% and 7.6% respectively. On the other hand, there was no infected date palm samples in Almadinah and Jouf. Positive samples from different locations (Alhasa, Alkharj, Qassim and Riyadh) were sequenced and submitted to GenBank under the accession numbers JQ045567, JQ045570, JQ045571, KC252994, and KC252995. Blast data indicates that the phytoplasmas detected in Alhasa samples may be in a different ribosomal group. Therefore, a comparative analysis of the obtained sequences and other sequences that were available in the Genebank was carried out. To determine the evolutionary relationships between those sequences, 15 phytoplasma sequences belonging to 16SrI (group I) and to 16SrII (group II) were selected. The data of the phylogeny tree indicate that the sequences JQ045570, JQ045571, KC252994, KC252995 are in the same cluster in which 16SrII phytoplasmas; only the sequence JQ045567 (phytoplasma detected in *Phoenix dactylifera* from Alhasa) clustered with 16SrI phytoplasmas. These results explained why the similarity of sequence JQ045567 to the others was 87%, because the others belonged to a different group from the one reported by Alhudaib *et al.* (2007). Taken all together, these results indicated that phytoplasma group II (16SrII) was detected in date palms in different locations in Saudi Arabia (Alkharj, Riyadh and Qassim), while the only location where the phytoplasma group I (16SrI) was detected was in Alhasa. Two types of date palm: 16SrI in Alhasa and 16SrII in other locations in Saudi Arabia. Further studies of phytoplasma transmission will be conducted to confirm that insect vectors like leafhopper and natural weeds as host plants might play a role in the etiology of this disease. These information will allow the development of more efficient control measures, and reveal new insights into the epidemiology of the "Alwijam" disease.

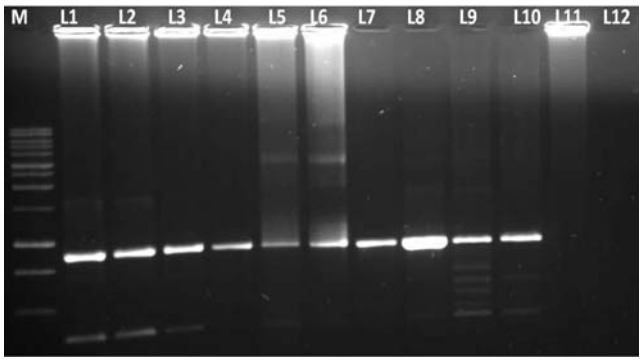

Figure 2. Agarose gel electrophoresis of nested PCR amplification products using primers fU5/rU3. M: 1kb DNA ladder, L1 to L3: positive control, L4 to L10: random date palm samples, L11: negative control and L12: dH_2O.

Acknowledgements

This work was supported by King Abdullaziz City for Science and Technologies (grant no. AT-28-111) and King Faisal University. Also we would like to thank Wael Alarby and Zake Abohamd at the Plant Protection Department and Ministry of Agriculture, Saudi Arabia for assistance during sampling.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S57-S58

doi: 10.5958/2249-4677.2015.00023.7

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Analyses based on the *16S rRNA* and *secA* genes identify a new phytoplasma subgroup associated with a lethal yellowing-type disease of coconut in Côte d'Ivoire

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Abstract

A lethal yellowing-like disease named as Côte d'Ivoire Lethal Yellowing (CILY) has been spreading throughout the Ivoirian coastal coconut plantations of the Grand-Lahou department in Côte d'Ivoire causing losses of about 12,000 tons of copra/year, and has also become a threat for the coconut Genebank. Leaf, stem apex, heart and inflorescence samples of coconut palms exhibiting CILY symptoms that resemble those associated with Cape Saint Paul Wilt Disease (CSPWD) in Ghana, were sampled and nested PCR-tested for phytoplasma presence. The phytoplasma identified was further characterized based on the sequences of the *16S rRNA* and *secA* genes. Phytoplasma universal primers yielded expected amplification products from 61 out of 84 samples from symptomatic trees tested, while samples from symptomless palms yielded no DNA amplification. Both the 16S rDNA and secA sequences of the CILY phytoplasma showed a 99% sequence identity with that of the CSPWD phytoplasma from Ghana, and clustered with previously identified West Africa phytoplasma strains of group 16SrXXII that includes the Ghanaian CSPWD strain. The phytoplasma was assigned to the sub-group 16SrXXII-B based on virtual RFLP of the 16S rDNA sequences. Results support possibility of disease spread from the neighbouring Ghana, posing a threat for the Ivorian coconut industry.

Keywords: 16SrXXII phytoplasma, coconut, RFLP, Cape Saint Paul wilt disease, '*Candidatus* Phytoplasma'

Introduction

Côte d'Ivoire is among the first 20 out of 92 world coconut-producing countries (UNCTAD, 2012), and is the top African, Caribbean and Pacific exporter of coconut oil (from copra), that nowadays accounts for 2.5% of the world vegetable oil production. Coconut palm is cultivated on approximately 50,000 hectares (1 to 5 ha/farms), and produces an average of 45,000 tons of copra/year (Allou *et al*., 2012) that is the main source of income for people living in the coastal region.

Côte d'Ivoire Lethal Yellowing (CILY) has destroyed 350 ha and is currently threatening over 7,000 ha throughout the coastal coconut plantations of the Grand-Lahou Department. CILY has also become a phytosanitary risk for the Ivorian multisite International Coconut Genebank that provides service for Africa and the Indian Ocean region. CILY symptoms include leaf yellowing starting in the old leaves quickly moving to the young ones, drying of spikelet progressing to blackening of the whole inflorescence, rotting of heart, immature fruit drop, and crown death of the palm after six months of initial symptoms appearance leaving a scenery of bare trunks, known as "telephone pole" (Figure 1), which resembled symptoms caused by the Cape Saint Paul Wilt Disease (CSPWD) phytoplasma in Ghana.

The present study aimed to characterize the CILY phytoplasma based on the *16S rRNA* and *secA* genes through PCR, sequencing, and RFLP analyses. Phylogenetic relationships were determined by comparisons with

Figure 1. Coconut palms from Grand-Lahou department in Côte d'Ivoire showing bare trunks, known as 'telephone poles', corresponding to the final stage of the disease.

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phytoplasmas associated with Lethal Yellowing-like and Lethal Disease-like from the Caribbean and West/East Africa. Results provide tools to determine the possible origin of the disease, and to develop further effective control strategies.

Materials and Methods

Total DNA was extracted from leaf, stem apex, heart and inflorescence samples of 84 coconut palms exhibiting CILY symptoms (N'nan, 2004). Total DNA was used as a template for nested PCR assays with universal primers that target the phytoplasma *16S rRNA* gene (Gundersen and Lee, 1996), R16mF2/mR1 for the direct PCR reaction, and R16F2n/R2 for the nested reaction. Primers SecAFor1/rev3 and SecA For5/rev2 were used in nested PCR reactions to amplify the *secA* gene (Dickinson and Hodgetts, 2013).

Representative R16F2n/R2 and secA amplicons were purified on spin columns (Omega Bio-Tek, USA), cloned (pGEM-T Easy Vector, Promega), and sequenced bi-directionally. Consensus sequences were compared with GenBank reference sequences and aligned using Clustal W (Thompson *et al*., 1994). Phylogenetic trees were constructed using the neighbor-joining method with MEGA4.0 (Tamura *et al*., 2007) with default values and 1,000 replicates for bootstrap analysis. *In silico* restriction analysis and virtual gel plotting was conducted using pDRAW32 (http://www.acaclone.com).

Results

Phytoplasma DNA was amplified from 61 out of 84 CILY symptom-bearing samples. No PCR amplicons were obtained from symptomless palms. Unique *Alu*I, *Bfa*I, *Hae*III, *Hpa*I, *Hpa*II, *Mse*I, *Taq*I, and *Tsp509*I RFLP patterns were obtained for the CILY phytoplasma that clearly differentiated it from 16SrIV and 16SrXXII-A strains. The R16F2n/R2 and secA sequences of the CILY phytoplasma exhibited a 99% identity with those of the Ghana CSPWD phytoplasma. Phylogenetic analysis based on the 16S rDNA and *secA* gene sequences and virtual RFLP support the CILY phytoplasma, as a member of group 16SrXXII.

Discussion

The fact that the CILY phytoplasma identified in the Grand-Lahou is very closely related to the Ghanaian strain CSPWD, highlights the complex epidemiology of two very closely related phytoplasmas that affect the same plant host in two different and nearby geographic locations.

Results supports previous suspicions of CILY phytoplasma spreading from the neighbouring Ghana, and colonizing the most susceptible local coconut varieties and hybrids by its possible adaptation to a new bio-ecological niche with a different epidemic capacity.

The identification of the CILY phytoplasma and its designation in a new 16SrXXII subgroup contribute to the knowledge of the biodiversity of coconut LY-associated phytoplasmas in West Africa, as well as, to enhance the study about the epidemic aspects of the disease. CILY phytoplasma possesses a great threat for the survival of the Genebank coconut germplasm, since it is located to just 120 kilometers from the CILY outbreak.

The presence of CILY phytoplasma also becomes a threat for the entire Ivorian coconut industry and prompts to urgently assess the phytosanitary situation of the new 16SrXXII-B subgroup in the coconut production areas of the Grand-Lahou towards identifying potential sources of CILY resistance, and providing new tools to develop effective management strategies to prevent disease spread.

Acknowledgements

This research was supported by the Côte d'Ivoire FIRCA (Fonds Interprofessionnel pour la Recherche et le Conseil Agricoles)/Cocotier CNRA – COC No. 588.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S59-S60

doi: 10.5958/2249-4677.2015.00024.9

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Potential novel '*Candidatus* Phytoplasma pini'-related strain associated with coconut lethal yellowing in Mozambique

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Abstract

In this study, phytoplasmas associated with coconut lethal yellowing disease in Mozambique were characterized. For phylogenetic analysis the *16S rRNA* genes were sequenced. Apart from '*Candidatus* Phytoplasma palmicola' 16SrXXII-A, phylogenetic analysis revealed the presence of a double infection of '*Ca.* P. palmicola' and a '*Ca.* P. pini'-related phytoplasmas. The second strain potentially represents a novel '*Candidatus* species' and is closely related to '*Ca.* P. pini'.

Keywords: Mozambique, coconut lethal yellowing phytoplasma, '*Ca.* P. palmicola', '*Ca.* P. pini'

Introduction

The coconut palm (*Cocos nucifera*) is a major cash crop that is widely grown in coastal tropical regions of the world including Mozambique and contributes to the economy, livelihood and food security of millions of rural inhabitants. Outbreak of coconut lethal yellowing disease (CLYD) is now threatening the industry and the livelihood of over three million people in Mozambique. Previous studies (Mpunani, *et al*., 1999; Harrison *et al.,* 2014) observed that CLYD in Mozambique was associated with the phytoplasmas of lethal yellowing disease in West Africa (16SrXXII-A and 16SrXXII-B). Scheneider *et al.* (2005) observed that three pine phytoplasma strains form a distinct branch in the phytoplasma phylogenetic tree and are only distantly related to other phytoplasmas, and, the closest relatives of these three strains are '*Candidatus* Phytoplasma castaneae' (AB054986, Jung *et al*., 2002) and pathogens associated with palm lethal yellowing. In this work, the detection, characterization and tentative taxonomic affiliation of a '*Ca.* P. pini'-related' strain from coconut palm in Mozambique are reported.

Materials and methods

A survey was made in Zambezia province in March 2012. Samples were collected from palms with CLYD symptoms. Sampling was performed by boring into the trunk using a 10-cm-long drill bit that was 10 mm in diameter. At the sampling site, the collected trunk tissues were dried in tubes containing silica gel and maintained at room temperature until DNA extraction. The nucleic acids were extracted using the CTAB extraction procedure described by Harrison and Oropeza (2008), with minor modifications. The DNA was then resuspended in 100 µl TE (10 mM Tris, 0.1 M EDTA, pH 8) buffer and stored at 4°C until use. The phytoplasma DNA was amplified from total DNA extracts using direct and nested PCR assays. For the 16S rRNA genes, the phytoplasma-specific universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.,* 1995) was used. To test whether co-infections occurred, the direct PCR product was diluted 1,000 times (10^{-3}) and re-amplified by PCRs employing two primer pairs sets specific to the *16S rRNA* genes, namely R16F2n/R16R2 (Gundersen and Lee, 1996) or G813/Awka SR (Tymon, 1995). The PCR products were purified using spin columns (Cycle-Pure Spin PCR purification kit) and sequenced. Sequence editing and assembly of forward and reverse sequences were performed using the SeqMan Pro software (DNASTAR Lasergene 10 core suite). Phylogenetic analyses of the phytoplasma sequences were performed with the MEGA version 6.0 software using the Neighbour Joining (NJ) and Maximum Likelihood (ML) methods evaluated with 1,000 replicates for bootstrap analysis. The *Bacillus subtilis* 16S rRNA (AB042061) was employed as outroot for the phylogenetic trees.

Results

In some coastal area of Zambezia province coconut plantations are growing along with pine trees as mean of controlling soil erosion (Figure 1). Positive results were obtained after nested PCR amplification from symptomatic samples. Sequencing of three amplicons and phylogenetic analyses revealed that '*Ca.* P. palmicola' 16SrXXII-A, clustering together with the Nigerian strain (Y14175) was the prevalent phytoplasma detected in diseased palms while a '*Ca.* P. pini'-related strain was found as a mixed infection with '*Ca.* P. palmicola' (AJ632155.1) in just one palm (Figure 2). The three Mozambican sequences were deposited in GenBank under the accession numbers KJ528957, KJ528981 and KJ528982.

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Figure 1. Coconut infected with CLYD in mixed crop with pine trees in Zambezia province of Mozambique.

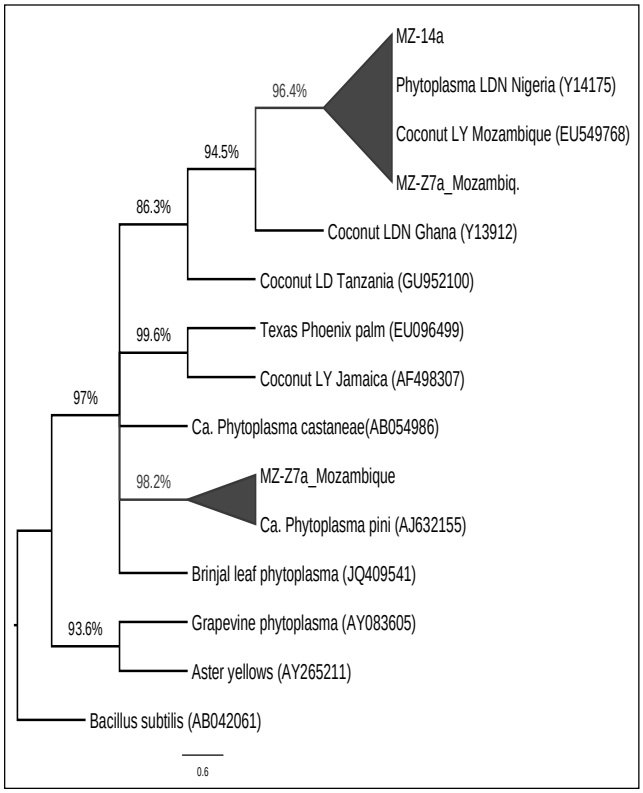

Figure 2. Dendrogram constructed by the maximum likelihood method showing the phylogenetic relationships with three phytoplasma strains from CLYD samples from Mozambique compared with strains from other 16Sr groups. The GenBank accession numbers are shown in parentheses.

Discussion

The discovery of a potential novel phytoplasma type in Mozambican coconut palms, which clustered together with '*Ca.* P. pini' suggests that these phytoplasma may switch from the pine host to coconut palms or vice versa. This is the first report associating a '*Ca.* P. pini'-related strain with CLYD. Scheneider *et al.* (2005) report that the closest relatives of '*Ca.* P. pini' is '*Ca.* P. castaneae' and phytoplasmas associated with palm lethal yellowing. However, from the topology of the phylogenetic tree it is clear that the strain detected in coconut is not '*Ca.* P. pini' but a closely related strain. Therefore, it is plausible that this strain may have a different plant host range and/or vectors than those of '*Ca.* P. pini'. Extensive sampling was conducted on pine trees and other palm species for further studies on epidemiology and possible insect vector identification.

Acknowledgements

This work was funded by the "Swedish International Development Agency" (SIDA). The authors are also thankful to the Madal Company and the small-scale coconut producers for access to samples from their coconut farms.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S61-S62

doi: 10.5958/2249-4677.2015.00025.0

Occurrence of Awka wilt disease of coconut in Nigeria for one century

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Abstract

This study was conducted to characterize phytoplasmas from coconut palms showing symptoms of Awka wilt disease in south and south-east Nigeria and determine if there is geographic differentiation among the phytoplasma strains. Stem samples were collected from symptomatic coconut palms in three region of Nigeria. For each sample, DNA was extracted and the 16S rDNA was amplified by PCR by using the P1/P7 primers. Amplicons were sequenced for five Nigerian strains. The sequences were compared with each other, with LDN (lethal decline Nigeria) sequence published 20 years ago, and with sequences from Ghana. A homology of 100% was observed among the 16S rDNA sequence published and the sequences of the five strains under investigation. Based on the known conservation of 16S rDNA sequences, this result suggests a relative stability of the lethal yellowing disease phytoplasma strains in the southern regions of Nigeria, and no introduction of the "Ghanaian strain".

Keywords: coconut, lethal yellowing disease, phytoplasma

Introduction

In West Africa, coconut lethal yellowing disease (CLYD) was reported in Nigeria, Cameroon, Togo, Ghana and Ivory Coast, where it is the most damaging disease for coconut. CLYD is still very active and spreading in Nigeria and Ghana. It is associated with the presence of a phytoplasma belonging to the 16SrXXII group (Wei Wei *et al*., 2007).

Nigeria is the first African country where the disease was described as bronze leaf wilt in 1917 in Awka district (Johnson, 1918). Recalled Awka wilt, it is only in 1993 that the disease was demonstrated to be associated with a phytoplasma in Nigeria (Tymon *et al*., 1993), and a unique 16S rDNA sequence from Nigeria (Y14175) was published (Tymon *et al*., 1998).

Almost one century after the first description of the disease, a survey was performed in Nigeria in 2012 to confirm the presence of phytoplasmas in coconut palms harboring symptoms of Awka wilt disease and to determine whether the strains of the phytoplasma detected were similar or different from the one published (Tymon *et al*., 1998).

Materials and Methods

Samples were collected in Nigeria in August 2012 by drilling the stem of coconut palms presenting typical symptoms of Awka wilt disease. DNA extraction was performed using a CTAB protocol and 16S ribosomal gene, spacer region and a portion of the 23S gene were amplified using the phytoplasma universal primers P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al*., 1995). P1/P7 PCR products presenting a band of 1,756 bp were sequenced for five samples (Beckman Coulter Genomics, UK) and the sequences were aligned with the sequence Y14175 (Tymon *et al*., 1998) and two sequences from Ghana previously obtained (Pilet *et al*., 2011) by using CLUSTAL W. Finally, the eight sequences were compared by using DNADIST under Bioedit version 7.0.9.0 program software. The origin of the eight samples used in the present study is presented Table 1.

Table 1. Names and origins of the samples used in this study.

Strain	Country	State	Year
LDN (Y14175)	Nigeria	Edo State	1993
NG12-030	Nigeria	Akwa-Ibom State	2012
NG12-036	Nigeria	Edo State	2012
NG12-037	Nigeria	Edo State	2012
NG12-043	Nigeria	Edo State	2012
NG12-044	Nigeria	Anambra State	2012
GH09-001	Ghana	Volta Region	2009
GH09-121	Ghana	Western Region	2009

Because the available sequence Y14175 (Lethal Decline Nigeria, LDN) was shorter than other sequences, all the Nigerian and Ghanaian sequences were adjusted and trimmed to a length of 1,468 bp covering almost of the full 16S rDNA gene for correct comparison.

Results

The five Nigerian P1/P7 PCR products correspond to phytoplasma sequences for all the samples. and presented 100% homology between themselves, but also with the accession Y14175 (Figure 1).

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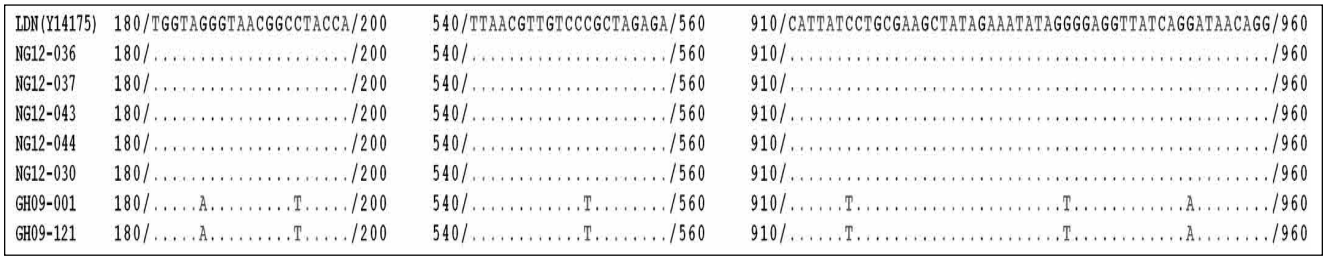

Figure 1. Alignment of the 16S rDNA sequence of the eight phytoplasma strains.

Considering the 16S rDNA gene exclusively, 6 SNPs allow the differentiation of the strains from Nigeria from the ones from Ghana and a homology of 0.995 was calculated. Considering the full 16S rDNA operon (including the tRNA-Ile gene and the intergenic spacer region), a total of 10 SNPs were observed between the Nigerian and Ghanaian sequences.

Discussion

Sequences of the 16S rDNA of the five Nigerian samples collected in 2012 confirm phytoplasma presence in coconut trees showing Awka wilt disease symptoms in Nigeria one century after the first description of the disease in Africa.

Coconut lethal yellowing phytoplasmas of the 16SrXXII group, previously known as '*Candidatus* Phytoplasma cocosnigeria', have been renamed '*Candidatus* Phytoplasma palmicola' very recently (Harrison *et al.*, 2014). Analyses of the 16S rDNA sequences confirm that two different phytoplasmas are responsible for CLYD in Ghana and Nigeria. Harrison *et al.* (2014) classified the phytoplasma responsible for CLYD in Nigeria (based on the sequence Y14175) and Mozambique in the 16SrXXII-A group, while the phytoplasma responsible for CLYD in Ghana is in the 16SrXXII-B group. Based on the sequences of the 16S rDNA gene, similarity of the five Nigerian sequences with the Y14175 obtained 20 years ago suggest a relative stability of the LYD phytoplasma populations in the southern regions of Nigeria, and no introduction of the "Ghanaian strain" in this part of the country. Because of its proximity with Ghana, a survey for Awka wilt disease covering the south-western states of Nigeria is necessary. Use of marker genes less stable than the 16S rDNA gene could detect diversity among the different populations of the phytoplasma of the 16SrXXII-A group in Nigeria.

Acknowledgements

The authors are grateful to the French Embassy in Nigeria for funding this study and to the Executive Director of NIFOR for financial support.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S63-S64

doi: 10.5958/2249-4677.2015.00026.2

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Phytoplasmas associated with yellow wilt disease of sugar beet in Chile

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Abstract

Due to the sporadic occurrence of yellow wilt of sugar beet disease throughout the last three years, a survey was carried out in two regions where the Chilean sugar beet production is concentrated. The *tuf* and *16S rRNA* genes were used for phytoplasma detection and identification. Virtual restriction fragment length polymorphism analyses, cloning, and sequencing identified the presence of a phytoplasma belonging to the ribosomal subgroup 16SrIII-J.

Keywords: yellow wilt of sugar beet, nested-PCR, *tuf* gene, *16S rRNA* gene, RFLP, sequencing

Introduction

The presence of yellow wilt disease of sugar beet (*Beta vulgaris* L.) in Chile was reported almost 40 years ago (Arentsen and Ehrenfeld, 1973). The disease may cause 100% yield loss and seemed to have disappeared from 2001 to 2012 (IANSAGRO, 2012). The first laboratory evidence for the presence of phytoplasmas in sugar beet was obtained 15 years ago (Hepp and Sandoval, 1999). Afterwards, a phytoplasma belonging to ribosomal group 16SrIII was identified (Castro *et al*., 2000). Due to the sporadic occurrence of the disease throughout the last three years, a survey was carried out in the two regions where the Chilean sugar beet production is concentrated. Two genes were used for phytoplasmas identification.

Materials and Methods

Eighteen plants were sampled during April 2014 (Table 1). Total nucleic acid was extracted from 1 g of main leaf midribs with a chloroform/phenol method, dissolved in Tris-EDTA pH 8.0 buffer, and stored at 4°C; 20 ng/µl of nucleic acid were used for amplification. Direct and nested PCR assays for amplification of the *tuf* gene were carried out according to the protocol of Makarova *et al.* (2012). Direct PCR assays with primer pair P1/P7 and nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) were performed following a published protocol (Schaff *et al*., 1992). Amplicons from nested PCRs for both genes were purified using the Concert Rapid PCR Purification System and DNA fragments were cloned. Putative recombinant clones were analyzed by colony PCR. Selected fragments from cloned DNAs were sequenced in both directions using the BIG DYE sequencing terminator kit. The sequences were then aligned with reference sequences deposited in GenBank and Q-Bank, using the BLAST engine for local alignment (version Blast N 2.2.12). Identification was done using *in silico* restriction fragment length polymorphism (RFLP) analyses with *Mse*I, *Nde*II, *Hha*I, *Bst*UI, and *Rsa*I restriction enzymes (Zhao *et al*., 2009). The first two enzymes were used for the *tuf* gene and the last three for the *16S rRNA* gene sequences.

Results

Fourteen out of the 18 sugar beet plants tested showed typical symptoms associated with phytoplasma presence (rolled up leaves with necrosis of the edge and yellowing) and were positive for phytoplasma presence in nested PCR assays using primers for the *tuf* and *16S rRNA* genes (Table 1). Of the four PCR negative plants, three (HORT 76, 77, 84) showed chlorotic ringspots and yellowing and one (HORT 83) was asymptomatic (Table 1). Cloned nested PCR fragments from both genes were sequenced and no sequence difference was observed among the cloned fragments from the 14 sugar beet plants in the *tuf* gene (438 bp, GenBank Accession No. KM658259), while sequence identity was 99.9 to 100% in the *16S rRNA* gene (1,250 bp, GenBank Accession Numbers KM658257 and KM658258). In the *16S rRNA* gene the similarity percentages of phytoplasmas found in the 14 samples showed a close correlation of 99.9% to the sequence of a phytoplasma detected in the leafhopper *Amplicephalus curtulus* (GenBank Accession No. KC834073) and 99.8% to Ch10 strain (GenBank Accession No. AF147706), corresponding to chayote witches' broom phytoplasma (16SrIII-J) from Brazil. In the *tuf* gene analysis, the higher similarity percentage found was 96.7%, with strain QPh20, corresponding to 16SrIII-D phytoplasma from USA. In Q-Bank sequences of phytoplasmas belonging to 16SrIII-J subgroup are not available. Amplicons from the *tuf* and *16S rRNA* genes were also subjected to *in silico* RFLP analysis that confirmed the assignment of the phytoplasmas to the ribosomal subgroups 16SrIII-J (X-disease group).

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Table 1. Sugar beet plants: variety, location and symptoms. Phytoplasma detection was achieved by nested PCR using primers for *tuf* and *16S rRNA* genes.

Plant	Variety	Province (Region)	Symptoms*	Nested PCR result*
HORT 71	Belleza KWS	Los Angeles (Biobío)	Ph.	+
HORT 72	Belleza KWS	Los Angeles (Biobío)	Ph.	+
HORT 73	Belleza KWS	Los Angeles (Biobío)	Ph.	+
HORT 74	Belleza KWS	Los Angeles (Biobío)	Ph.	+
HORT 75	Belleza KWS	Los Angeles (Biobío)	Ph.	+
HORT 76	Belleza KWS	Los Angeles (Biobío)	CR, Y	-
HORT 77	Magnolia	Parral (Maule)	CR, Y	-
HORT 78	Magnolia	Parral (Maule)	Ph.	+
HORT 79	Magnolia	Parral (Maule)	Ph.	+
HORT 80	Magnolia	Parral (Maule)	Ph.	+
HORT 81	Magnolia	Parral (Maule)	Ph.	+
HORT 82	Magnolia	Parral (Maule)	Ph.	+
HORT 83	Magnolia	Linares (Maule)	A	-
HORT 84	Magnolia	Linares (Maule)	CR, Y	-
HORT 85	Magnolia	Linares (Maule)	Ph.	+
HORT 86	Magnolia	Linares (Maule)	Ph.	+
HORT 87	Magnolia	Linares (Maule)	Ph.	+
HORT 88	Magnolia	Linares (Maule)	Ph.	+

*Ph. = typical phytoplasma symptoms: rolled up leaves with necrosis of the edge and yellowing; CR = chlorotic ringspot; Y = yellowing; A = asymptomatic; + = sample positive; - = sample negative.

Discussion

This study confirmed that 16SrIII-J phytoplasmas are associated with yellow wilt of sugar beet in Chile. This phytoplasma has been detected in Chile on different weed species and crops of agronomic interest and is transmitted by the leafhopper (Cicadellidae) *Paratanus exitiosus* (Beamer) (Hepp and Vargas, 2002; González *et al*., 2010; González *et al.,* 2011; Longone *et al*., 2011; Fiore *et al*., 2012). The plants showing leaves with chlorotic ringspots and yellowing may be infected by one or more viruses, probably *Beet yellows virus* (BYV), *Beet western yellows virus* (BWYV), *Beet mild yellowing virus* (BMYV) and *Beet chlorosis virus* (BChV), all present in Chile and responsible for sugar beet yellowing virus disease (Hepp and Garrido, 1996; Stevens *et al*., 2005). Assays are in progress to detect and identify the virus(es) present.

Acknowledgements

This research was supported by the National Fund for Scientific and Technological Development (FONDECYT), Chile, project N° 1140883.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S65-S66

doi: 10.5958/2249-4677.2015.00027.4

Status of alfalfa witches' broom phytoplasma disease in Iran

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Abstract

Alfalfa witches' broom (AWB) is one of the most important and destructive diseases of alfalfa in Iran. Based on characteristic disease symptoms and direct and nested polymerase chain reactions, the status of AWB disease was evaluated in different growing areas of Iran. Restriction fragment length polymorphism was used to identify AWB disease associated phytoplasmas. Furthermore, infection rate, disease severity, death rate of infected plant in the summer and winter and overwintering of disease vector were assessed. Based on the results, AWB disease was reported on different alfalfa cultivars in Yazd, Fars, Sistan-Va-Baluchestan, Kerman, Hormozgan, Bushehr, Esfahan, Chaharmahal-Va-Bakthiari, South Khorasan and Khuzestan provinces of Iran. Phytoplasmas associated with AWB in these areas were identified as '*Candidatus* Phytoplasma aurantifolia', belonging to peanut witches' broom (16SrII) group. In Abarkooh and Ashkezar (Yazd province) and Bondarooz (Bushehr province) the recorded disease incidence was up to 100%. The highest disease severity was found in Rezvan Shahr (Ashkezar, Yazd province) in 3 years old alfalfa fields. The highest death rate of infected plants in summer and winter were recorded as 26% and 13% in Ashkezar and Abarkooh in Yazd province, respectively. Different nymph stages of the insect vector, *Orosius albicinctus*, were identified on tamarisk (*Tamarix aphylla*) and saxaul (*Haloxylon persicum* and *H. aphyllum*) in the winter. The highest population of *O. albicinctus*, observed on tamarisk plants adjacent to the infected alfalfa fields in Milleshbar (Ardakan, Yazd province), suggested this as a possible source of natural spread of AWB.

Keywords: phytoplasma, overwintering, *Orosius albicinctus*, PCR

Introduction

Alfalfa witches' broom (AWB) is one of the most important diseases of alfalfa in the world. It was observed for the first time in United States of America in 1925 (Haskel, 1926) and it was reported in many parts of the world (Graham *et al.*, 1984; Lee *et al.*, 2000). The main disease symptoms associated with AWB are small leaf, dwarfing, yellowing, excessive branching of stems, flower abnormality and plant death. In Iran for the first time AWB was reported in Juyom of Fars province (Salehi and Izadpanah, 1993) and its association with phytoplasma presence was demonstrated based on symptoms, insect vector, graft and dodder transmission, positive reaction in Dienes' staining and polymerase chain reaction using universal primers (Salehi and Izadpanah, 1993; Salehi *et al.*, 1995; Esmailzadeh Hosseini *et al.*, 2003). The aim of this work was to investigate the current status of AWB disease in Iran.

Materials and Methods

During 2009-2012, infection percentage, disease severity and death rate of infected plants were evaluated in the major alfalfa producing areas of Iran. Five fields were selected and sampling was conducted randomly at five points within a 0.5 square meters on a diagonal transect across each of the five fields. Infected samples were identified based on distinct disease symptoms and nested PCR assays. For determining disease severity, all the samples collected in 0.5 m^2 separated and numbered for severity as 0, 1, 2, 3, 4. The 0 healthy plant; 1: first stage of infection with leaf deformation, curl and chlorosis; 2: virescence, phyllody, sterility in some flowers, growth of axillary shoots on the stem; 3: proliferation of shoots, yellowing, dwarfing, flower sterility in the most flowers; 4: severe stunting and death of plant. For determining winter hosts of *Orosius albicinctus* (AWB vector) (Salehi *et al.,* 1995), specimens were collected using sweeping net from man-made in naturally grown forest trees near alfalfa fields sown as windbreak and far away alfalfa fields in Ardakan and Abarkouh in winter in Yazd province. Total DNA was extracted from midrib tissue of symptomatic alfalfa plants using Zhang *et al.* (1998) procedures. DNA samples were tested for phytoplasma presence by direct and nested PCR

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assays with P1/P7 and R16F2n/R16R2 primers. RFLP analysis of direct PCR products with *Alu*I, *Hinf*I, *Hae*III, *Mse*I and *Rsa*I was used to identify AWB phytoplasma.

Results and Discussion

Based on disease symptoms and PCR assays, AWB phytoplasma was detected in different alfalfa cultivars in Yazd, Fars, Sistan-Va-Baluchestan, Kerman, Hormozgan, Bushehr, Esfahan, Chaharmahal-Va-Bakthiari, South Khorasan and Khuzestan provinces of Iran on *Medicago sativa* cultivars Yazdi, Hamedani, Baghdadi, Nikshahri and Bami. Typical virescence and phyllody symptoms were observed in 1 to 2 year-old alfalfa fields. Three year infected plants showed proliferation of shoots, yellowing and dwarfing. Disease severity varied and it was very severe in July and August. In Abarkooh (Figure 1a), Ashkezar (Yazd province) and Bondarooz (Bushehr province) the disease incidence was recorded up to 100%. The highest severity was found in Rezvan Shahr (Ashkezar, Yazd province) in 3 year-old affected fields. The highest death rate of infected plants was observed to be 26% and 13% in Ashkezar and Abarkooh, respectively (Yazd province) (Figure 1b).

Figure 1. Left. One hundred percent AWB incidence in a 3 year old alfalfa field in Abarkooh, Yazd province, Iran. Right. Drying of AWB infected plants in winter in Abarkooh, Yazd province, Iran.

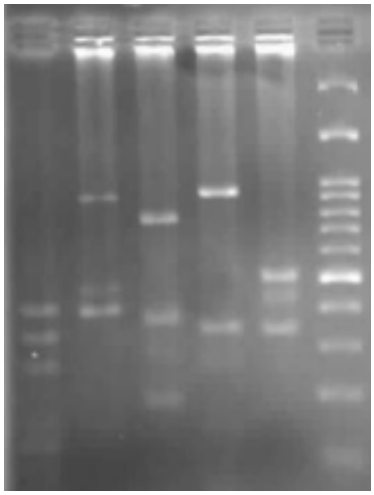

Figure 2. RFLP pattern of PCR product (1,800 bp) of AWB-Nikshahri. Wells from left to right are *Rsa*I, *Alu*I, *Mse*I, *Hin*fI, *Hae*III restriction enzymes and 100 bp Marker respectively.

Fragments of expected size (1.8 and 1.2 kbp in direct and nested PCR assays respectively) were amplified from infected alfalfa plants (data not shown). Restriction fragment length polymorphism (RFLP) analysis indicated association of a peanut witches' broom-related phytoplasma (16SrII) in infected alfalfa plants in all areas (Figure 2).

Different nymph stages of the AWB phytoplasma vector, *O. albicinctus*, were observed on tamarisk (*Tamarix aphylla*) and saxaul (*Haloxylon persicum* and *H. aphyllum*) in the winter. The highest population of insect vector were observed in man-made tamarisk adjacent to the alfalfa fields with up to 76% of AWB incidence in Milleshbar (Ardakan, Yazd province). The incidence of *O. albicinctus* on tamarisk and saxaul indicated that these plants could play important role in the overwintering the vector. Earlier, *O. albicinctus* leafhoppers were also collected on different weeds and trees during winter season (Esmailzadeh Hosseini *et al*., 2011) in the same surveyed areas, and this also supports the spread of AWB disease by these vector species in different areas of Iran.

Acknowledgements

This paper was a part of a research project No. 108 supported by Agricultural Research, Education and Extension Organization (AREEO).

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S67-S68

doi: 10.5958/2249-4677.2015.00028.6

Preliminary study on some ornamental plant phytoplasma diseases in north of Iran

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Abstract

Because of the importance of ornamental plants culture in north of Iran, and the outbreak of suspicious symptoms of phytoplasma diseases on these plants, a study was carried out in order to detect and identify phytoplasmas associated with them. The presence of phytoplasma in samples of cockscomb, tagetes and rose plants was shown by direct and nested-PCR assays using the phytoplasma-specific primers, P1/P7 and R16F2n/R2. Sequences of cockscomb, Indian marigold and rose phytoplasmas have high homology with the members of aster yellows phytoplasma group (16SrI). Phylogenetic analysis showed that cockscomb, tagetes and rose phytoplasmas are closely related to 16SrI-M the first one and 16SrI-B the latter two, respectively. To our knowledge, this is the first report of a member of 16SrI group phytoplasma infection in cockscomb in Iran. Moreover, the present study is the first report of phytoplasma disease outbreak on rose in north of Iran by subgroup classification of the associated phytoplasma.

Keywords: ornamental plant, phytoplasma, detection, identification

Introduction

Virus and phytoplasma associated diseases in ornamental crops have been reported worldwide and have caused significant economic losses. When trade in ornamentals was initiated many years ago, the threat of distributing these agents was minimized because much of the germplasm was exchanged as seed. In the last fifty years the transport of vegetatively propagated clonal material has resulted in dissemination of many viral and other graft transmissible agents (Lawson and Hsu, 2006).

In central regions of Iran, some ornamentals and weeds were tested and phytoplasma presence was proved on statice, periwinkle, tagetes and black-eyed susan (Babaie *et al*., 2007). Phytoplasmas were detected by PCR in 37 ornamental species belonging to 15 families, and RFLP analyses of *16SrRNA* gene showed that they belong to aster yellows (16SrI) and X-disease (16SrIII) groups (Samuitiene *et al*., 2007).

Based on the registered statistics of Agriculture Ministry, total cultured area for ornamental plants was 51,359,330 m^2 in Iran during 2012, including 30,944,953 m^2 as open space and 20,414,377 m^2 as greenhouse. More than 43 million pot plants and 1.5 billion cut flowers are produced each year. Pests and diseases are as the most important problems of producers in Iran. In this topic, phytoplasma disease symptoms such as witches' broom, flower deformation, or yellowing are present in ornamental plants in north of Iran. In the present work, some species showing symptoms referable to phytoplasma presence were studied to detect and identify these prokayotes.

Materials and Methods

During 2012, a survey was conducted in greenhouses and green sites growing flowers and ornamental plants in Mazandaran province (north of Iran). Totally, 50 samples with suspicious symptoms of phytoplasma diseases were collected from rose, cockscomb, dumb cane, rubber, cactus, periwinkle, tagetes, chrysanthemum and wax flower. Samples collected from asymptomatic plants from the same fields were used as negative controls. In some cases the phytoplasmas were also transmitted by *Cuscuta* sp. to periwinkle under insect-proof greenhouse conditions.

For DNA extraction, small leaves and midribs were taken from symptomatic and asymptomatic plants and extracted according to protocol of Aldaghi *et al.* (2007). The primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) was used to prime the amplification of the 16S rDNA, 16S-23S spacer region, and the start of 23S rDNA. Direct PCR products were diluted 1: 25 and used as template in nested PCR, using primer pairs R16F2n/R16R2 (Gundersen and Lee, 1996). PCR products were electrophoresed through 1% agarose gel and revealed using ethidium bromide under UV illumination.

The PCR products obtained with the R16F2n/R2 primers were purified from agarose gel and further cloned with the TA Cloning Kit (Invitrogen, Carlsbad, USA). The clones were sequenced on both strands. Each consensus sequence was deposited in the GenBank database and compared with other sequences by BLAST program. The sequences were aligned by BIOEDIT software and cluster analysis was performed by DNASTAR's Laser Gene software.

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Figure 1. Rose plant with proliferation, leaf browning and decline symptoms.

Results

Plants collected from some areas showed disease symptoms similar to those associated with phytoplasma presence. Among them, cockscomb, tagetes and rose (Figure 1) showed the most typical symptoms of phytoplasma diseases, including dwarf, yellowing, dieback, proliferation, rosette, leaf curling, phyllody and virescence. The causal agent was successfully transmitted to periwinkle and typical phytoplasma symptoms appeared (data not shown).

With universal primer pair P1/P7, target DNA fragments of approximately 1.8 kbp were amplified in some of the symptomatic plants. No signal was obtained from healthy plants and from some suspicious plants. Nested-PCR assays with primer pair R16F2n/R2 yielded 1.2 kbp DNA fragments from all cockscomb, tagetes, and rose samples containing showing possible phytoplasma symptoms (data not shown). Phytoplasma presence was not detected on asymptomatic plants of the same species nor in other symptomatic plant species.

The sequence analyses of nested-PCR products amplified from cockscomb, tagetes, and rose plants and their comparison with sequences in Genbank showed that these sequences have the higher identity (above 99.5%) to members of the aster yellows phytoplasma group (16SrI), and in particular to members of the 16SrI-M, and 16SrI-B subgroups. Moreover also phylogenetic analyses showed that cockscomb, tagetes, and rose phytoplasma strains from north of Iran clustered with strains in 16SrI-M and 16SrI-B subgroups (data not shown).

Discussion

Although the range of symptoms attributed to phytoplasma presence is indicative of detrimental effects on plants, in some cases (like poinsettia and cacti) phytoplasmas are beneficial to growers. It is remarkable to emphasize that ornamental species that are alternative hosts for phytoplasmas are common in commercial nurseries and they have a great demand because of the unusual characteristics that make them more attractive, therefore this situation may involve involuntary risk of disease transmission to crops of agronomic importance (Avina-Padilla *et al.*, 2009).

To our knowledge, this study is the first report of a member of 16SrI group infection of cockscomb (*Celosia cristata* L.) in Iran; moreover the presence of a phytoplasma from 16SrI-M subgroup was not reported from Iran. The present work is also the first report of witches' broom disease outbreak on rose in Mazandaran province and of the first subgroup identification of the associated phytoplasma.

All phytoplasmas detected in current study belong to 16SrI group. The '*Candidatus* Phytoplasma asteris' is the major phytoplasma group associated with ornamental plant species worldwide, and until 2010 more than 42 ornamental plant species were reported as infected by this phytoplasma group (Chaturvedi *et al.*, 2010). The 16SrI (aster yellows) group is also the largest, most diverse and widespread phytoplasma group not only in ornamentals, but also in all plant species worldwide (Lee *et al.*, 2004; Marcone *et al.*, 2000).

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S69-S70

doi: 10.5958/2249-4677.2015.00029.8

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Phytoplasma diseases on major crops in Vietnam

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Abstract

The nested-PCR technique was applied to detect and identify phytoplasmas associated with major crops in Vietnam including sugarcane, cassava and longan. Phylogenetic analysis confirmed that phytoplasmas associated with the sugarcane grassy shoot and white leaf diseases belong to the rice yellow dwarf group (16SrXI). In cassava showing witches' broom symptoms a mixed infection of phytoplasmas related to '*Candidatus* Phytoplasma asteris' (group 16SrI) and '*Ca.* P. aurantifolia' (group 16SrII) was detected. In samples showing longan witches' broom disease three phytoplasmas including '*Ca.* P. aurantifolia' (group 16SrII), elm yellows (group 16SrV) and "stolbur" (group 16SrXII) were identified.

Keywords: phytoplasmas, cassava witches' broom, sugarcane grassy shoot, sugarcane white leaf, longan witches' broom

Introduction

Phytoplasma are one of the most important pathogens on major crops in Vietnam in recent years. In 2006, sugarcane grassy shoot was detected in the Nghe An Tate and Lyle Sugar Mill in north-central Vietnam, where ratoon crops were severely affected. The disease has become the most important of the sugarcane industry and reduced cane yield and sucrose content by up to 60-80% (Hoat *et al*., 2012). The sugarcane white leaf disease was detected for the first time in 2006 on ROC varieties in a small spot area and by 2011, the disease was widespread in 394 ha (Hoat *et al*., 2013). In 2010, more than 60,000 ha of cassava were affected by witches' broom disease with crop losses recorded up to 80%, and reductions in yield and starch content reached 30% (Alvarez *et al*., 2013). Plants of longan (*Dimocarpus longan* Lour.) showing witches' broom symptom were detected in almost all longan-growing regions in Vietnam (Nguyen *et al*., 2012). In the present study detection and characterization of phytoplasmas associated with these diseases in Vietnam is summarized.

Materials and Methods

Symptomatic samples of sugarcane, cassava, longan with typical symptoms of grassy shoot, white leaf and witches' broom were collected from different areas, examined under electron microscope and subjected to PCR assays for phytoplasma presence detection. The symptomatic samples were examined by transmission electron microscopy as previously described by Wongkaew and Fletcher (2004) with some modification. The ultra-thin sections were stained with lead nitrate and uranyl acetate and observed on a JEOL 1010 transmission electron microscope (TEM) at 80 KV (JEOL Ltd., Tokyo, Japan). Total nucleic acids were extracted from 1 gr of infected leaves using a CTAB method. PCR was performed using P1 (Deng and Hiruki 1991) and P7 (Schneider *et al*., 1995) primers in a 25 µl reaction containing 0.4 µM of each primer, 0.2 µM of each dNTP, 1.25 U DreamTaq DNA polymerase (Fermentas, Vilnius, Lithuania) and 1 X Dream Taq polymerase buffer. The PCR conditions were 35 cycles of: 95°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes in a Mastercycler Pro (Eppendorf, Germany). For nested PCR assays, 1 µl of direct reaction was added to the PCR cocktail mix containing R16F2n and R16R2 primers (Gundersen and Lee, 1996). PCR conditions for the nested PCR assay were the same. Two negative controls, water and DNA from symptomless plants, were included. Following nested PCR, 5 µl from each reaction were electrophoresed in a 1% (w/v) agarose gel in 1XTAE buffer, stained with ethidium bromide (0.5 µg/ml) and visualized with GelDoc-It® 310 Imaging System (United Kingdom). The nested-PCR products were analyzed by single restriction endonuclease digestion with *Eco*RI, *Hha*I, *Hin*fI, *Hpa*II, *Rsa*I, and *Taq*I and the products were detected by electrophoresis in a 2% (w/v) agarose gel. These amplicons were purified using the DNA Purification Kit (QIAGEN, Singapore) and sequenced directly with

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ABI3100 by Bioneer Company, Korea. The aligned sequences were subjected to BLAST search to verify sequence homology with reference phytoplasma sequences available in GenBank (Altschul *et al.*, 1990).

Results and Discussion

The typical symptoms of green grassy shoot around the base of mature stools in the first season was observed in sugarcane (Figure 1a). Infected plants showed stunting, complete chlorosis of leaves especially in the spindle portion and the tillers. The infected stool showed shortened internodes and did not form millable canes (Figure 1b). The symptoms in the second and third ratoon crops suffered severe yield losses. In cassava-growing regions of both Northern and Southern Vietnam, shoot proliferation, short internodes, along with dark vascular tissue in the stems and roots were recorded in cassava plants (Figure 1c). The infected longan trees showed symptoms on both vegetative and floral organs. The initial symptoms observed were abnormally small and roll upwards young leaves, followed by appearance of light yellow to brown patches on younger leaves. In addition, internodes became shorter, clumped branches overgrew resembling a broom appearance. TEM observations of phloem cells of symptomatic infected sugarcane samples confirmed the presence of pleomorphic bodies ranging from 100 to 400 nm in the sieve elements of the infected tissues, but not in the healthy looking samples.

The nested-PCR amplified about 1.2 kb products from almost all symptomatic samples of sugarcane, cassava and longan plants. RFLP analyses of the nested-PCR products indicated that all the sugarcane showing grassy shoot symptoms (SCGS) were infected by molecularly undistinguishable phytoplasmas. The 16Sr RNA sequences indicated that the sugarcane grassy shoot phytoplasma strain shared 100% identity with SCGS phytoplasma from India, and 99% with SCWL from Thailand.

The phylogenetic analyses indicates that the sugarcane phytoplasmas clustered with 16SrXIV group (Hoat *et al.*, 2012). On the other hand the sugarcane white leaf phytoplasma (SCWL) sequencing and phylogenetic analyses indicates that the Vietnam strain shared 100% sequence similarity with sugarcane phytoplasmas from Thailand and Myanmar members of the 16SrXI group (rice yellow dwarf). In cassava showing witches' broom symptoms the RFLP analysis indicate the presence of 16SrI and 16SrII phytoplasma groups (Alvarez *et al.*, 2013). The nested-PCR/RFLP assays from symptomatic longan samples allowed detection of strains related to elm yellows (group 16SrV), "stolbur" (group 16SrXII) and '*Candidatus* Phytoplasma asteris' (group 16SrI). Development of rapid phytoplasma detection and identification methods and techniques for production of disease-free seedlings for farmers are necessary. Researches on insect vectors of the identified phytoplasmas together with information on resistant varieties are in progress.

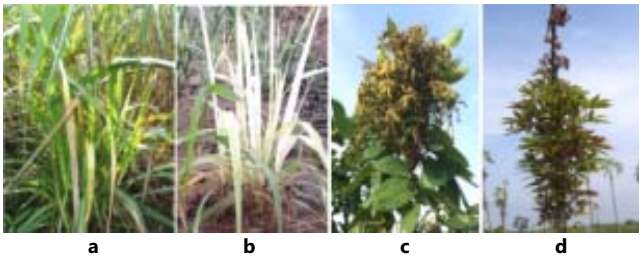

Figure 1. a) sugarcane grassy shoot disease in Nghe An province; b) sugarcane white leaf disease c) longan witches' broom disease and d) cassava witches' broom disease in Southern Vietnam.

Acknowledgements

Parts of the work of TX Hoat were supported by the World Academy of Science for the Advancement of Science in Developing Countries (TWAS), the National Foundation for Science & Technology Development (NAFOSTED) (No. 106.15-2010.07); by the Ministry of Agriculture and Rural Development (MARD) and the Ministry of Science and Technology (MOST).

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S71-S72

doi: 10.5958/2249-4677.2015.00030.4

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Report on phytoplasma new host plants in Pakistan

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Abstract

During 2012-2014 a survey was conducted in different districts of Punjab province, Pakistan and suspected phytoplasma infected plant samples of vegetables, fruits, oilseeds and weeds were collected. Molecular assays were carried out and allowed to detect phytoplasma presence in several of these samples. Nested PCR assays with universal phytoplasma specific primers (P1/P7 and R16F2n/R16R2) indicated phytoplasma presence in symptomatic tomato, *Parthenium hysterophorus* and onion in Faisalabad province. These are new phytoplasma reports for the geographic area.

Keywords: nested PCR, phytoplasma, phyllody, flower malformation, insect vectors

Introduction

Phytoplasmas have great diversity showing different symptoms on plants worldwide (Bertaccini *et al*., 2007). For the first time in 1970 in Pakistan the widespread occurrence of citrus stubborn disease symptoms associated with phytoplasmas (Chapot, 1970) was described. Nasir *et al*. (2007) reported the presence of potato purple top disease in Punjab. Akhtar *et al*. (2009) observed chickpea (*Cicer arietinum*) plants showing phytoplasma disease-like symptoms. Phytoplasmas were also identified in citrus orchards of Sahiwal (Manan *et al*., 2010). Akhtar *et al*. (2009; 2010) identified the 16SrII phytoplasma group associated with mung bean and sesame phyllody. Phytoplasmas associated with phyllody on tomato were reported to interfere in developmental process (Ahmad *et al*., 2013; 2014) but no further reports of new hosts of phytoplasmas and their natural spread sources have been reported in Pakistan. Recently different phytoplasma associated symptoms on fruits, vegetables and crops and their potential insect vectors were detected by using nested PCR assays.

Materials and Methods

Different regions from main vegetable, fruit and crop growing areas were surveyed in some districts (Rahim Yar Khan, Multan, Khanewal, DG Khan and Faisalabad) of Punjab, Pakistan. At least five symptomatic samples from plants and weeds exhibiting phytoplasma symptoms were collected from each region. Samples were from different species and locations (Table 1). DNA from plants and from putative insect vectors was extracted and amplified by nested PCR using the phytoplasma universal or group specific primers (Gundersen and Lee, 1996) and electrophoresed on 1.2% agarose gel. Nested PCR with P1/P7 followed by R16F2n/R16R2 amplified a 1.25 kb DNA fragment (Figure 1). RFLP analysis is in progress for phytoplasma group identification from the positive samples.

Table 1. Symptoms of various phytoplasma infected plants collected during the survey conducted between 2012-214 in different region of Punjab, Pakistan.

Host	Year	Region	Symptoms
Sesame	2012-2014	Faisalabad, RY Khan, Khanewal	Phyllody, virecence, whiches' broom, proliferation
Parthenium hysterophorus	2013-2014	Faisalabad, RY Khan, Khanewal	Phyllody, virescence, yellowing of leaf and proliferation, witches' broom
Chickpeas	2013-2014	RY Khan, Faisalabad	Reddening, short internodes, phyllody, proliferation
Onion	2013-2014	Faisalabad, RY Khan, DG Khan	Axillary growth, yellowing, proliferation
Radish	2013-2014	Faisalabad, RY Khan	Leaf yellowing, axillary shoots, phyllody
Cotton	2013-2014	Faisalabad, RY Khan, Multan	Stunted growth, phyllody, small leaves,
Citrus	2013-2014	Faisalabad, RY Khan, Multan	Leaf yellowing, deformed twigs, abnormal flowers
Tomato	2013-2014	Faisalabad, Multan	Phyllody, big bud, small yellow leaves
Mungbean	2012-2014	Faisalabad, RY Khan, DG Khan	Phyllody, virescence, proliferation, stunted growth

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Results

During the survey phytoplasma related symptoms such as yellowing of leaves, chlorosis, stunted growth and flower abnormalities (phyllody, virescence, proliferation) on sesame, mango, chickpea, onion, radish and cotton were detected (Table 1, Figures 1 and 2). Nested PCR assays on extracted DNA resulted in the expected size DNA bands of 1.25 kb from the symptomatic samples of sesame, chickpea, onion, radish and cotton (Figure 3). PCR amplified fragments confirmed the presence of phytoplasmas.

Figure 1. Phyllody and virescence in onion inflorescence in: A healthy, B mild symptomatology.

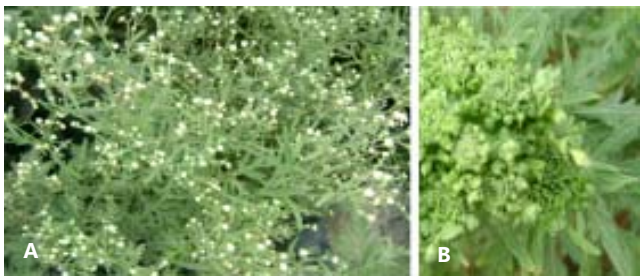

Figure 2. Symptoms of phyllody and virescence disease in star weed (*Parthenium hysterophorus)*. A healthy, B stunting, phyllody, virescence and proliferation of flowers.

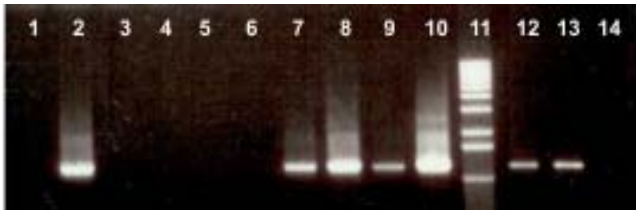

Figure 3. Agarose gel showing 1.25 kb bands obtained by nested-PCR assays with phytoplasma-specific 16S rDNA primers P1/P7 and R16F2n/R2. Lanes 1, negative control, 2. positive control ("stolbur" phytoplasma), 3-6: symptomless samples of sesame, *Parthenium hysterophorus*, chickpea, onion, 7-10: samples of symptomatic sesame, *P. hysterophorus* chickpea, and onion, 11: DNA marker (MBI Fermentas), 12-13: symptomatic tomato and mungbean, 14: water.

Discussion

These findings suggested that phytoplasma are spreading in Pakistan and may become a big threat for agriculture. Previously phytoplasma diseases in potato, sesame, citrus, chickpea, mung bean have been reported (Chapot, 1970; Nasir *et al*., 2007; Akhtar *et al*., 2009; 2010). Phytoplasma association with cotton phyllody was reported in Burkina Faso and Mali (Marzachì *et al*., 2009) and India. Mango associated phytoplasmas were reported in Egypt (Hashem *et al*., 2007). However, in the present study phytoplasma association with chickpea, onion, mungbean, tomato, star weed *(Parthenium hysterophorus)* and citrus was demonstrated. Out of these plant species, sesame, mung bean, chickpea phytoplasma symptoms were earlier reported from Pakistan. However, phytoplasma presence in onion, tomato, starweed *(Parthenium hysterophorus),* is reported for the first time. Phytoplasma symptomatic samples of mango, cotton, radish have been collected and studies on phytoplasma group and subgroup identification and putative insect vectors are in progress.

Acknowledgements

Work supported by HEC (Higher Education Commission of Pakistan) and Integrated Genomic, Cellular, Developmental and Biotechnology Laboratory, Department of Entomology, Botany and Horticulture, University of Agriculture Faisalabad, Pakistan.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S73-S74

doi: 10.5958/2249-4677.2015.00031.6

The source plant for phytoplasmas in the Israeli vineyards is still a mystery

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Abstract

The fact that phytoplasma could not be detected in *Vitex agnus-castus* which is the preferred host of the insect vector, suggested that other plant species are involved. Israeli vineyards and surroundings were surveyed. Samples from grapevines, wild and cultivated species were analysed. To confirm the presence of phytoplasma, DNA was extracted and amplified using general phytoplasma ribosomal primers and specific tub primers. Phytoplasma tuf-type was identified by PCR/RFLP analyses. The results show that "stolbur" phytoplasma tuf-type b is the only type that was detected in symptomatic grapevines but it was not detected in any other plant species that was sampled. In *V. agnus-castus*, *Convolvulus*, *Urtica* and *Crepis*, considered as potential host plants, peanut witches' broom (16SrII) or pigeon pea witches' broom (16SrIX) related phytoplasmas were detected in 2.8-23% of the samples. Consequently, the source plant for "stolbur" phytoplasma in vineyards remains unknown.

Keywords: wild plant species, host plant, "stolbur" tuf-type b, 16SrXII, 16SrII, grapevine

Introduction

Phytoplasmas are pathogens associated with grapevine yellows disease that causes heavy damage to vineyards in most growing areas around the world as well as in Israel (Weintraub *et al*., 2007). The plant-vector-phytoplasma interactions are specific. It is reported that the disease spread is through aerial infection and not from neighboring plants (Constable *et al*., 2004). *Hyalesthes obsoletus* is the known vector of "stolbur" phytoplasma to grapevines in Europe. The insect preference for plant species varies according to different geographical areas (Bressan *et al*., 2006). In Europe it can complete its life cycle on species of *Convolvulus*, *Urtica* and *Lavandula* (Langer and Maixner, 2004) while in Israel, his preferred host plant is *Vitex agnus-castus* (Sharon *et al*., 2005), however phytoplasmas could not be detected in *V. agnus castus* (unpublished data). Thus the possibility of another plant species as a source of infection was questioned. *H. obsoletus* was found to harbor "stolbur" phytoplasma tuf-type b in Europe and in Israel (Dafny Yelin *et al*., 2012), whereas tuf-type a and c were detected only in Europe. In Europe and in the Mediterranean region "bois noir" associated with "stolbur" is the most common yellows disease detected in grapevine (Langer and Maixner, 2004; Zahavi *et al*., 2013). However in Israel aster yellows and western X were also found (Orenstein *et al*., 2001). The dry summer in Israel suggest that only perennial plants that are green during the summer can serve as source for phytoplasma infection. Aims of this study were to survey the phytoplasma strains in Israeli vineyards and the plant species as potential sources for phytoplasma infection in the vineyards.

Materials and Methods

Plant material

Grapevines: symptomatic grapevine leaves were collect from 6 regions in Israel during autumn of 2012 and 2013: Northern Golan Heights; Southern Golan Heights; Galilee Mountain; Western Samaria; Inner Costal plane; Judea mountains. Wild and ornamental plants: plant samples were collected from wild plant species inside and near vineyards. The samples were collected during the spring, summer and autumn of 2011-2013 from the north, center and south of the Golan Heights (Table 1). Most plant samples were collected randomly whereas if atypical growth was recorded those were sampled as well. Some of the species are known as hosts of "stolbur" phytoplasma, *H. obsoletus* or both (Sforza *et al*., 1998; Sharon *et al*., 2005, Langer and Maixner 2004, Kosovac *et al*., 2013).

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Table 1. Phytoplasma infection in wild and cultivated plant species. Samples collected from the north (N), center (C) and south (S) of the Golan Heights. Numbers of positive samples by PCR/total samples are presented. Group assignment was performed using sequence analysis of the U3/U5 PCR product.

Species	N	C	S	Total	Group
a. plant hosts of *H. obsoletus* in Europe					
Convolvulus spp.	0/22	0/100	7/22	7/144	16SrIX
Urtica spp.	5/24	1/7	2/25	8/56	16SrII
b. potential hosts of *H. obsoletus*					
Crataegus azarolus	0/32			0/32	
Crepis spp.	2/22			2/22	16SrII
Lavandula spp.	0/15		0/4	0/19	
Myrtus spp.	0/6		0/6	0/12	
Olea europaea		0/4	2/68	2/72	
Polygonum equistiform	0/80			0/80	
Quercus spp.	1/35			1/35	16SrII
Rosa canina	0/28			0/28	
Rubus sanguine	0/20	0/31		0/51	
V. agnus-castus	0/4	18/64	11/30	28/120	16SrII
Ziziphus spp.		0/44	0/11	0/55	

PCR and RFLP analyses

Total DNA was extracted from plant tissue as described by Zahavi *et al.* (2013) and Berger *et al.* (2009). DNA from all plant samples were subjected to nested PCR assays for detection of the 16S rRNA gene using the phytoplasma universal primers P1/P7 followed by rU3/fU5 after 1: 20 dilution of the amplicons. PCR products were sequenced. *Tuf* gene was amplified from phytoplasma strains of 66 grapevines using the primers Tuf1f/r followed by TufAYf/r. The "stolbur" tuf-type in the grapevines phytoplasmas was identified with RFLP analyses according to Langer and Maixner (2004).

Results

About 70 samples from 7 grape growing regions in Israel were collected. Forty of the samples were found to harbor phytoplasmas as detected by PCR reaction with P1/P7 and rU3/fU5 primers. The same samples were found positive in the reaction with Tuf1f/r followed by TufAYf/r and RFLP analysis revealed that all had the same restriction pattern indicating that the phytoplasma is "stolbur" tuf-type b. Only five out of 13 sampled species harbored phytoplasma in rates ranging from 2.8 to 23%. *Urtica* spp., *Crepis* spp. and *V. agnus-castus* were found to harbor peanut witches' broom phytoplasmas (16SrII) whereas *Convolvulus* spp. was found to harbor pea pigeon pea witches' broom phytoplasmas (16SrIX) (Table 1).

Discussion

All tested yellows diseased grapevine were found to host tuf-type b "stolbur" phytoplasmas. This is in accordance with recent evidence from the Golan Heights (Zahavi *et al.*, 2013) but in contrast to earlier work (Orenstein *et al.*, 2001) who found "stolbur" in about 80% of the samples and aster yellows and western X in the rest. The weed survey shows that some species in the Golan-Heights harbor phytoplasmas. However, none of them harbor the "stolbur" phytoplasma that infect grapevines all over Israel. Further study is needed to find the source of the pathogenic type of phytoplasma to the grapevines.

Acknowledgements

The research was carried out in the Golan Research Institute and financed by the Israeli Ministry of Science and Technology.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S75-S76

doi: 10.5958/2249-4677.2015.00032.8

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'*Candidatus* Phytoplasma pyri' in peach orchards in Spain

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Abstract

Symptoms similar to those associated with phytoplasmas such as early reddening, curling of leaves, chlorosis, reduced vigour, abnormal fruits, irregular branches, and in some cases death of the trees, were observed in peach orchards in the area of Lleida (northeast of Spain) in 2013. The objective of this study was to carry out surveys on a larger surface area in Lleida to determine the possible spread of the disease, as well as to determine a possible transmission between peach trees through insect vectors. Results obtained after analysis for phytoplasma detection by nested PCR with generic phytoplasma rDNA primers P1/P7 and R16F2n/R16R2 and sequencing, indicated the presence of '*Candidatus* Phytoplasma pyri' in the samples and not the one of the expected '*Ca.* P. prunorum'. Results were confirmed by the study of gene *imp*. The disease is similar to the peach yellow leaf roll disease identified in California. The pull up of a great number of pear tree orchards, with new plantations of peach trees may have led to the transmission of the phytoplasma through *Cacopsylla pyri* to peach.

Keywords: Peach yellow leaf roll, phytoplasma, *Cacopsylla pruni, Prunus* spp.

Introduction

'Candidatus Phytoplasma prunorum', 16SrX-B is the main phytoplasma affecting *Prunus* spp. in Europe, and it is closely related to '*Ca.* P. mali', 16SrX-A, and '*Ca.* P. pyri', 16SrX-C, infecting apple and pear trees respectively. This group of phytoplasmas (16SrX) is naturally transmitted by different species of *Cacopsylla* spp. with a high specificity between *Cacopsylla* species, the range of hosts and the capacity to transmit a specific phytoplasma (Seemüller and Schneider, 2004). Cross infections between '*Ca.* P. prunorum', '*Ca.* P. mali' and '*Ca.* P. pyri' on *Prunus* spp. were reported, but in a very low incidence and causing no outbreaks or important losses in Europe (Cielinska and Morgas, 2011). In North America '*Ca.* P. pyri' is responsible for major losses in peach causing a disease named peach yellow leaf roll (PYLR), presenting the highest incidence in peach orchards closer to pear trees infected by '*Ca.* P. pyri'. PYLR is vectored by *Cacopsylla pyricola* from pear to peach trees (Blomquist and Kirkpatrick, 2002). Mixed phytoplasma infections have been reported, there is even evidence of inter-species recombination between different '*Candidatus* Phytoplasma' in this group (Danet *et al*., 2011). In Spain, '*Ca.* P. prunorum' is widespread on wild and cultivated *Prunus* spp. but occurrence on *P. persicae* is very low, and '*Ca.* P. pyri' is present in most of the pear orchards (Garcia-Chapa *et al*., 2003).

During the last years symptoms similar to those associated with phytoplasmas, such as early reddening, curling of leaves, chlorosis, reduced vigour, abnormal fruits, irregular branches, and in some cases death of the trees were observed in peach orchards in Lleida (northeast of Spain) (Figure 1). Lleida is a very important fruit production area with 55.000 hectares of peach crops, producing 43% pear and 49% peach of the total Spanish production. The aim of this study was to carry out surveys on a larger surface area in Lleida to determine the possible spread of the disease, as well as the possible transmission between peach trees through insect vector(s).

Materials and Methods

During the last two years, samples of symptomatic peach trees were collected in different orchards in a radius of 40 km, including a wide range of varieties and rootstocks. In each plot with symptomatic trees, five samples were taken to identify the phytoplasma associated to the symptoms. The sampled area is dedicated to intensive fruit production in the south of Lleida where *C. pruni* has not been reported. Samples of young shoots and leaves were taken from trees and analyzed. Phytoplasma DNA was extracted from phloem tissue and amplified with the universal P1/P7 (Deng and Hiruki, 1991, Schneider *et al*., 1995) primers pair, followed by nested PCR with R16F2n/R16R2 (Gundersen and Lee, 1996) and specific AP group primer pair fO1/rO1 (Lorenz *et al*., 1995). The final PCR products were digested with the enzyme R*sa*I. Amplifications with non ribosomal specific primers, Imp ESFY, ImPDA, and ImpPDB (Danet *et al*., 2011) were also carried out. Positive controls of ESFY and PD were used, as well as negative controls represented by asymptomatic peach samples. Amplified PCR products were sequenced and compared with sequences in GenBank using BLAST.

The insect capture inside the plots was also carried out using yellow sticky traps.

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Figure 1. Symptoms of PYLR in peach orchards of Lleida region in Catalonia (Spain).

Results

Phytoplasmas were detected in 70 out of the 100 samples analyzed with at least one of the specific primers employed. No phytoplasma was detected in negative peach controls. All ImpPD positive samples were also simultaneously positive with specific PD primers ImpPDA/ImpPDB. No peach sample was positive to amplification with the specific ESFY primer pair Imp ESFY. All fO1/rO1 peach samples digested showed a PD profile, no ESFY profile was detected. Sequencing of R16F2n/R16R2 and Imp PDA/B amplicons shows a stable product in all peach trees. The sequences were submitted to the European nucleotide archive (ENA) with the Acc. No. HG737345 and HG737344. Based on the 16S rDNA sequence this strain is 100% homologous to the reference strain PD1 (Genbank Acc. No. AJ542543) and 99.55% homologous to strain PD33 Lib (Genbank Acc. No. FN 600725) based on the *Imp* gene.

Results of insect capture inside plots (classification and analysis) are under processing.

Discussion

The studies of the lasts years show an increase of the phytoplasma symptomatology in peach tree in Lleida, with outbreaks, important losses, in a wide range of varieties, following an epidemic behaviour that could be similar to PYLR in North America. Genetic comparisons show that the strain is genetically closer to some European or Middle East PD strains than to North American PYLR strains (Sabaté *et al.*, 2014). These results contribute to understand the outbreak, and to implement measures to prevent its spreading, and could be useful to increase the knowledge about biologic aspects of group 16SrX of phytoplasmas.

Acknowledgements

This work was funded by grant RTA2013-00097-00-00 of the Programa Sectorial de I+D, M.A.P.A., Spain.

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New phytoplasma diseases

Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S79-S80

doi: 10.5958/2249-4677.2015.00033.X

Detection and multigene characterisation of a '*Candidatus* Phytoplasma prunorum'-related phytoplasma associated with witches' broom symptoms in pineapple fruit

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Abstract

Pineapple samples showing witches' broom symptoms in the leaves at the top of the fruit were analysed to verify phytoplasma presence. 16SrX group phytoplasmas were detected after nested PCR amplification with specific primers and RFLP analyses. Sequencing of 16Sr DNA allows verification that phytoplasmas from pineapple show a 99% shared identity with '*Candidatus* Phytoplasma prunorum'. Further characterisation of the phytoplasma on *ace*, *imp* and *secY* genes by RFLP analyses on amplicons obtained in nested PCR assays enable the differentiation of the strain infecting pineapple from those detected in fruit trees in Europe and used as reference strains. This is the first report of phytoplasmas belonging to the 16SrX-B group from a species different from pome and stone fruit worldwide.

Keywords: pineapple witches' broom, phytoplasma, multigene characterisation, sequencing

Introduction

The pineapple [*Ananas comosus* (L.) Merr.] is a perennial crop grown for its fruits and used as a fresh and processed product. It is the most economically significant plant in the Bromeliaceae family; it is the second most important harvest after bananas, contributing to over 20% of the world's production of tropical fruits. The origin of the pineapple is still uncertain, but very likely is located in the Parana-Paraguay Basin. Thailand, Brazil, the Philippines, Costa Rica and India are the main producers in the world, supplying nearly 50% of the total output. A number of diseases are reported in this species which are associated with diverse malformations. Pineapple propagation is carried out by using shoots emerging from different parts of the plant, known as bulbs, crowns and auxiliary buds. The tissue for propagation material may be the crown, from the top of the fruit. Recently a disease consisting of a witches' broom-like appearance of the leaves above the fruit was erratically observed in some of the marketed fruit from the East of Colombia and molecular tests were carried out to detect and identify possible phytoplasmas associated with this malformation.

Materials and Methods

Samples from *A. comosus* variety "Mayanes", collected in Colombia in the region of Llanos Orientales and exhibiting witches' broom on leaves at the upper part of the fruit (crown) (Figure 1), and samples from similar tissues from asymptomatic fruits were subjected to nucleic acid extraction and amplified to verify the presence of phytoplasma. After chloroform/phenol nucleic acid extraction, the universal primer pairs R16mF2/R1 and R16F2n/R2 were used in nested PCR assays under reported conditions on 20-50 ng template DNA (Gundersen and Lee, 1996). Further nested amplification was carried out on R16F2n/R2 amplicons with group specific primers R16(X)F1/R1 (Lee *et al*., 1995) and general primers 16R758f/16R1232r (=M1/M2) (Gibbs *et al.,* 1995). The amplicons obtained were subjected to RFLP analyses, with *Rsa*I, and *Tru*1I (Fermentas) respectively, following manufacturer instructions. The visualisation of the results was obtained after 6.7% polyacrylamide gels were stained with ethidium bromide and observed under a UV transilluminator. The RFLP patterns were then compared with those of real or virtually-generated published gels (Lee *et al*., 1998; Zhao *et al*., 2009). The same amplicons were directly sequenced in both directions after purification with a QIAquick PCR Purification Kit (Qiagen). Sequences were aligned using MEGA version 5 (Tamura *et al*., 2011), and compared with nucleotide sequences in the GenBank database BLASTN 2.2.18 (NCBI).

Figure 1. Typical witches' broom symptoms observed in pineapple fruits, variety "Mayanes", from Colombia (Llanos Orientales).

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Further phytoplasma characterisation was carried out on *tuf, ace, imp and secY* genes by nested-PCR amplifications with primers AceFf1/AceFr1 followed by AceFf2/AceFr2, IMPF1/IMPF2 followed by IMPF2/IMPR2, and SecYMALF1/ SecYMALR1 followed by SecMALF2/ SecMALR2 (Danet *et al*., 2011) under the cycling conditions reported for each of them. The amplification was followed by RFLP analyses with *Tru*I or *Tsp509*I, according to the amplicon, and visualised in polyacrylamide gels as described above. Reference phytoplasma strains from the collection maintained at DipSA (Bertaccini, 2010) or field collected samples were employed for RFLP profile comparison.

Results and Discussion

The nested amplification procedure allowed amplification of the fragments of the expected length from the symptomatic pineapple samples, by using primers R16(X)F1/ R1 and M1/M2. The RFLP analyses indicated that the profiles of the samples were identical to each other, and referable to the profile of the European stone fruit yellows (ESFY) used as reference, and to the 16SrX group phytoplasmas (Figure 2a and data not shown). The 16Sr DNA sequence assembly allowed a 981 bp (Genbank KM389620) fragment to be obtained that showed 100% homology with the '*Candidatus* Phytoplasma prunorum' strains available in the GenBank, and clustered with 16SrX- B strains and the RFLP analyses of the fragments of expected length obtained showed differential polymorphisms. The imp amplicons were only obtained from 16SrX-B strains and showed no polymorphism with *Tsp509*I (Figure 2b), while with the same enzyme, ace amplicons were obtained from all phytoplasmas belonging to the 16SrX group and their. restriction profiles were distinguishable from each others (Figure 2c). RFLP on SecY amplicons (Figure 2d) showed a profile that was identical.

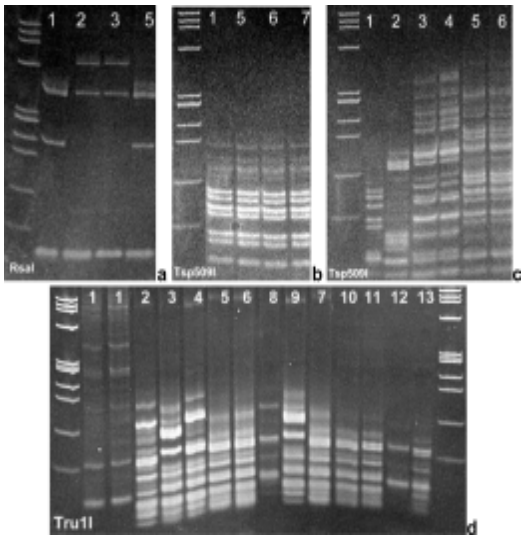

Figure 2. RFLP results of amplicons from symptomatic pineapple (sample 1) obtained respectively with primers R16(X)F1/R1 (a), IMPF2/IMPR2 (b), AceFf2/ AceFr2 (c) and SecMALF2/SecMALR2 (d). Reference strains: 2, pear decline (16SrX-C); 3, apple proliferation strain AP15 (16SrX-A); 4, apple proliferation strain AT (16SrX-A); 5, European stone fruit yellows strain ESFY2 (16SrX-B); 6, European stone fruit yellows strain GSFY (16SrX-B); 7, European stone fruit yellows strain ESFY-VR/14 (16SrX-B); 8, pear decline in pear strain PD-MO/14 (16SrX-C); 9, apple proliferation in apple strain AP-BZ/13 (16SrX-A); 10, European stone fruit yellows strain ESFY1 (16SrX-B); 11, European stone fruit yellows strain LNp (16SrX-B); 12, European stone fruit yellows strain 221-1 (16SrX-B); 13, European stone fruit yellows strain APH-1bis (16SrX-B). Marker, PhiX174 *Hae*III digested.

A pineapple proliferation phytoplasma related to 16SrXII-A was reported from Oceania (Davis *et al*., 2005; 2006). The molecular evidence showed from the diverse genes that the phytoplasma associated with pineapple fruit witches' broom belongs to group 16SrX. This is the first identification of 16SrX group phytoplasmas in a fruit tree species different from pome and stone fruit. This is the first detection on the American continent of this phytoplasma. Further studies will help to clarify the epidemiological relevance of this finding.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S81-S82

doi: 10.5958/2249-4677.2015.00034.1

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Lagenaria siceraria yellows associated with phytoplasma presence in Brazil

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Abstract

Lagenaria siceraria, the bottle gourd or calabash gourd, is distributed in Brazil and locally known as "porongo". It is used as a green vegetable and some cultivars are marketed as handicraft. In the State of Rio de Janeiro, naturally diseased plants were observed with reduced leaf size, leaf malformation and yellowing. Molecular analysis revealed the presence of a phytoplasma related to '*Candidatus* Phytoplasma pruni' that was classified as a new subgroup -Y into the 16SrIII ribosomal group. This is the first report of the genus *Lagenaria* harbouring a phytoplasma worldwide.

Keywords: Bottle gourd, calabash gourd, phytoplasma, 16SrIII, "porongo"

Introduction

Bottle gourd [*Lagenaria siceraria* (Mol.) Stand.] is a cucurbit probably native to Africa, and widely cultivated throughout the tropics (Tindall, 1983). There is diversity of cultivars and their uses, including medicinal, vessels, music instruments and folkloristic works (Teppner, 2004). In Brazil, the young fruits are eaten as a cooked vegetable and the mature fruits are used as multi-purpose containers (Tindall, 1983). It is commercially cultivated in the southern region of Brazil, mainly in the State of Rio Grande do Sul, but also in other states.

In different locations in the State of Rio de Janeiro, naturally diseased bottle gourd plants were observed exhibiting symptoms of reduced leaf size, leaf malformation and yellowing, suggestive of phytoplasma infection. Previously, the association of ChWBIII phytoplasma (group 16SrIII-J) with cucurbits in Brazil was demonstrated to chayote witches' broom disease and to witches' broom disease of *Momordica charantia* (Montano *et al*., 2000), as well as to pumpkim yellows (Montano *et al*., 2006). Moreover, phytoplasma associated with diseases of loofah (*Luffa cylindrica*) and sikana (*Sicana odorifera*) (Montano *et al*., 2011) were also reported in Brazil.

The aim of the present work was to demonstrate the presence of a phytoplasma that may be the cause of bottle gourd yellows disease in Brazil, and to carry out its preliminary identification on 16S ribosomal gene.

Materials and Methods

Samples from *L. siceraria* exhibiting reduced leaf size, malformation and yellowing (Figure 1) were subjected to nucleic acid extraction and preliminary amplified to verify phytoplasma presence as reported (Montano *et al*., 2000). Universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and R16F2n/R2 (Gundersen and Lee, 1996) were used to prime amplification of phytoplasma 16S rDNA sequences in nested PCR assays (Montano *et al*., 2000). A 16SrIII strain from "nogal cafetero" from Colombia (Mejia *et al*., 2014) was used for RFLP profile comparison together with other phytoplasma reference strains in periwinkle from the micropropagated collection maintained at DipSA (University of Bologna, Italy) (Bertaccini, 2010).

RFLP analyses of R16F2n/R2 amplicons with *Tru*I, *Hpa*II and *Hha*I (Fermentas, Vilnius, Lithuania) were then performed following the instructions of the manufacturer. The products of enzyme restriction were analyzed by electrophoresis through a 6.7% polyacrylamide gel followed by staining with ethidium bromide and visualization of DNA bands with UV transilluminator. The RFLP patterns were also compared with those of real or virtually generated gels previously published (Lee *et al*., 1998; Zhao *et al*., 2009).

Figure 1. *L. siceraria* asymptomatic (left) and with yellowing and leaf malformation (right).

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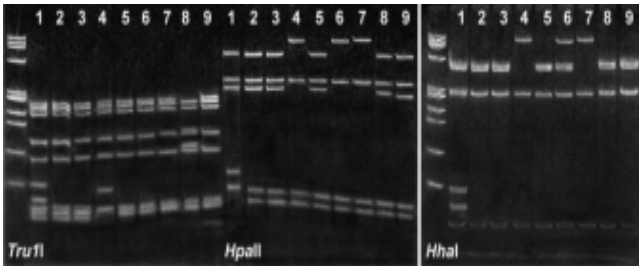

Figure 2. RFLP results obtained after digestion with *Tru1*I and *Hpa*II and *Hha*I of amplicons from symptomatic *L. siceraria* (sample 1), "nogal cafetero" from Colombia (sample 2) and reference strains in periwinkle amplified with R16F2n/R2 primers. Not marked lanes: marker Phi X174 RF *Hae*III digested. Reference strains: 3, MA (daisy yellows from Udine, 16SrIII-B); 4, WX (western X disease, 16SrIII-A); 5, SBB (*Solanum marginatum* big bud, 16SrIII-F); 6, SPI (spirea stunt, 16SrIII-E); 7, PoiB (poinsettia branch inducing, 16SrIII-H); 8, GRI (golden rod yellows; 16SrIII-D); 9, VAC (vaccinium witches' broom, 16SrIII-F).

Results

The nested amplification procedure allowed to amplify fragments of the expected lengths of about 1.2 kb from symptomatic samples of *L. siceraria* using primers R16F2n/R2. The RFLP analyses with selected restriction enzymes (Figure 2) indicated that the profiles of the symptomatic samples were referable to 16SrIII group but were distinguishable from those of reference strains, of "nogal cafetero" phytoplasma and also of other strains reported in the literature. The phytoplasma infecting *L. siceraria* in Brazil was therefore tentatively classified as new subgroup in the group 16SrIII ('*Candidatus* Phytoplasma pruni'-related strains) (Davis *et al*., 2013) and designed as 16SrIII-Y.

Discussion

The results of the present study demonstrated that a phytoplasma is associated with diseased bottle gourd in Brazil and that it belongs to group 16SrIII and tentatively to a new subgroup Y. The new subgroup affiliation of bottle gourd phytoplasma from Brazil is justified by the collective RFLP patterns of 16S rDNA that were found different from those reported previously for the ChWBIII phytoplasma (Montano *et al*., 2000), assigned to 16SrIII-J subgroup, and also from a number of phytoplasmas referable to '*Ca.* P. pruni' and belonging to the other described subgroups.

Bottle gourd is indigenous to Brazil and it is distributed in several states of the country. As earlier reports for *M. charantia*, a natural reservoir of the chayote witches' broom disease agent (Montano *et al*., 2000), bottle gourd could also be a potential natural source of phytoplasma inoculum for infection of commercial cucurbits.

To our knowledge, this is the first report of phytoplasma presence in *Lagenaria siceraria*, and it seems to be the first report in the genus *Lagenaria* worldwide.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S83-S84

doi: 10.5958/2249-4677.2015.00035.3

First report of phytoplasma associated with *Eucalyptus urophylla* showing witches' broom in Brazil

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Abstract

Eucalyptus production is of great importance to Brazil's economy. Diseases affecting the growth and the architecture of these trees lead to large losses in yield and quality of the wood. The occurrence of eucalyptus trees showing witches' broom and little leaf symptoms in different areas of Brazil is affecting seedling production. In this work, a phytoplasma was found associated with these symptomatic plants and was identified as belonging to the 16SrII group. This is the first report of a phytoplasma associated with eucalyptus in Brazil.

Keywords: '*Candidatus* Phytoplasma', little leaf disease, 16SrII group, eucalyptus, witches' broom

Introduction

Planted forests, mainly *Eucalyptus* and *Pinus* species, are very important to Brazil's economy. Minas Gerais and São Paulo are the states with the greatest production of wood. Among the cultivated forest species in Brazil, *Eucalyptus* is the main species and represents about 77% of the total wood production. The main uses of eucalyptus are in the timber, pulp and paper, charcoal and steel industries (ABRAF, 2013). The occurrence of diseases, mainly in nurseries, is a major problem in forest cultivation. The wood quality depends, in general, on the proper growth of the trees and the number of nodes and lateral branches being as small as possible, allowing the production of uniform wood. Diseases associated with phytoplasma presence usually affect the growth and shoot proliferation of the plants. The occurrence of eucalyptus plants showing symptoms similar to those induced by phytoplasmas, such as leaves of reduced size and witches' broom, in production areas in Brazil has been of concern to producers (Figure 1).

Due to the occurrence of these problems in cultivated areas of eucalyptus, and the common absence of pathogens in eucalyptus cultivation, the aim of this work was to detect the putative causal agent of this disease by testing for the presence of phytoplasmas.

Materials and methods

Twenty-two eucalyptus clones showing severe witches' broom symptoms (Figure 1), mainly in young branches, were collected from different cultivated areas in Brazil. Total DNA was isolated from these clones using the NucleoSpin Plant II kit (Macherey-Nagel), according to the manufacturer's instructions. To verify the presence of phytoplasmas in these plants, nested-PCR using universal primers for phytoplasma detection, P1 and P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by R16F2n and R16R2 (Gundersen and Lee, 1996) was carried out. These primers amplify a fragment of approximately 1,200 bp from the phytoplasma 16S rDNA that is used in sequence and restriction fragment length polymorphism (RFLP) analyses. PCR assays for phytoplasma detection were performed using the DNA amplification kit GoTaq Master Mix (Promega Corporation) and fragments generated by PCR were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions.

Figure 1. Witches' broom symptoms in *Eucalyptus urophylla*.

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The purified fragments were cloned into the pGEM-T Easy Vector System I (Promega Corporation) and transformed into *Escherichia coli* DH5á competent cells (Sambrook and Russel, 2001). Plasmid DNA was extracted and cloned fragments were sent for sequencing at Macrogen Inc.

In silico RFLP analysis of these fragments was performed using the online tool iPhyClassifier (Zhao *et al*., 2009), real RFLP with enzymes *Eco*RI, *Bst*UI and *Hpa*II, in order to confirm the *in silico* RFLP was also carried out. The cleavage reactions were visualized in polyacrylamide gel stained with GelRed (Biotium). A comparison of restriction patterns of the sequences obtained with the patterns described in the literature was made in order to identify the group to which this phytoplasma belongs.

Results

Phytoplasma presence was detected by nested-PCR assays in 14 out of the 22 eucalyptus clones analyzed, representing approximately the 64% of symptomatic plants. Amplified fragments of about 1.2 kb were cloned and sequenced (GenBank accession number: KM597065). The sequences from all positive samples showed the highest homology (99.4%) with '*Candidatus* Phytoplasma aurantifolia' (GenBank accession number: U15442). Further characterization of this phytoplasma was performed by *in silico* RFLP analyses. According to the restriction pattern and F-value obtained by the iPhyclassifier online tool, this phytoplasma belongs to the 16SrII group, subgroup C (16SrII-C) (Figure 2A). Enzymatic cleavage of three sequences from different phytoplasma clones, using three of the seventeen enzymes used in the *in silico* RFLP analysis confirmed results of virtual analysis (Figure 2B).

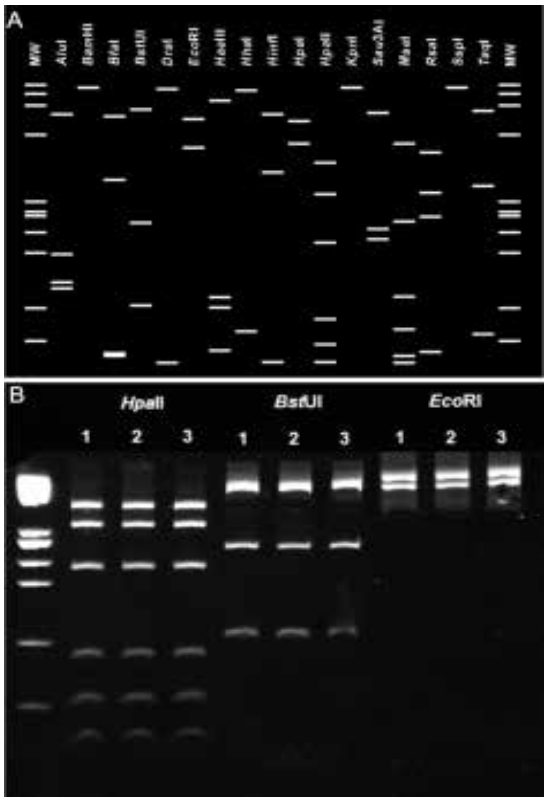

Figure 2. A) *in silico* RFLP of the amplified 16S rDNA fragment using the seventeen established enzymes for phytoplasma characterization B) RFLP of 16S rDNA fragment amplified from three different clones (1, 2 and 3) using three enzymes. MW, pattern of phiX174 digested with *Hae* III.

Discussion

The presence of phytoplasmas associated with eucalyptus trees showing reduced leaf size, excessive shoot proliferation and shortened internodes, has been reported in India, Sudan, China and Italy and the disease is known as little leaf disease (Marcone *et al*., 1996). A phytoplasma belonging to 16SrII group was already found affecting *Tabebuia pentaphylla* in Brazil, showing symptoms similar to those occurring in eucalyptus (Mafia *et al*., 2007), but until now, the occurrence of phytoplasma affecting eucalyptus in Brazil had never been described, therefore this is the first report of a phytoplasma associated with eucalyptus trees showing witches' broom and little leaf symptoms in Brazil.

Acknowledgements

We wish to thank the Suzano Papel e Celulose, for providing symptomatic eucalyptus clones from different production areas.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S85-S86

doi: 10.5958/2249-4677.2015.00036.5

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Phytoplasma identification in iberis exhibiting stunting and witches' broom symptoms

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Abstract

Iberis sempervirens samples from varieties Tahoe and Fish Back showing stunting, yellowing and witches' broom symptoms were tested to verify possible phytoplasma presence. Detection of 16SrX group phytoplasmas was obtained after nested PCR amplification and RFLP analyses in both varieties. Sequencing of 16Sr DNA allowed verification that phytoplasmas from iberis showed 99% identity with '*Candidatus* Phytoplasma mali'. Moreover, RFLP analyses also indicated the presence of aster yellows phytoplasmas (subgroups 16SrI-B and -A) in mixed infection with 16SrX phytoplasmas in the Fish Back variety. This is the first report of phytoplasma infection in iberis and also one of the few detections of phytoplasmas related to 16SrX-A group from an herbaceous species worldwide.

Keywords: phytoplasmas, PCR/RFLP analyses, sequencing, molecular characterization

Introduction

Iberis sempervirens belongs to the Brassicaceae family. The genus name originates from the fact that it mainly grows as spontaneous species in the Iberic peninsula (south west of Europe). In Italy it is cultivated in the north west part of the Ligurian Riviera (Piana of Albenga; Savona province). White varieties such as *I. sempervirens* Fish Back are flowering during winter time (December – February) and are among the few flowering plants available for the market in that period in Europe. In recent years plants of this species of different origins showed symptomatology such as stunting, yellowing and witches' broom indicating the possible presence of phytoplasmas (Figure 1); therefore, molecular analyses were carried out to verify prokaryote presence and identity.

Materials and Methods

Iberis sempervirens Tahoe and Fish Back produced by seeds showed symptoms of yellowing, stunting and witches' broom in winter/spring 2013. Samples from symptomatic and asymptomatic plants of the two varieties were subjected to molecular analyses to verify phytoplasma presence. After chloroform/phenol nucleic acid extraction (Prince *et al*., 1993) the universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) or R16F2n/R2 were used in direct PCR assays under reported conditions (Gundersen and Lee, 1996) on 20 ng template DNA. Nested amplification was carried out on P1/P7 amplicons with R16F2n/R2 and 16R758f/16R1232r (M1/M2) (Gibbs *et al.,* 1995) primers or group specific primers R16(X)F1/R1 and R16(I)F1/R1 (Lee *et al*., 1994; 1995). On R16F2n/R2 amplicons nested amplification was performed with the latter three primer pairs. The amplicons obtained were subjected to RFLP analyses with *Rsa*I, *Hha*I and *Tru1*I (Fermentas) following manufacturer instructions. The visualization of results was obtained after 6.7% polyacrylamide gels were stained with ethidium bromide and observed under UV transilluminator. The RFLP patterns were then compared with those of reference strains from the collection maintained at DipSA (University of Bologna, Italy) (Bertaccini, 2010). One of the amplicons obtained with R16(X)F1/R1 was directly sequenced in both directions after purification with a QIAquick PCR Purification Kit (Qiagen). Sequences were aligned using MEGA version 5 (Tamura *et al*., 2011) and compared with nucleotide sequences in the GenBank database.

Results

The nested amplification procedure allowed production of fragments of the expected length from the symptomatic iberis samples of both varieties using primers R16(X)F1/R1, R16(I)F1/R1 and M1/M2 (Figure 2a). The RFLP analyses indicated that the profiles of the samples from *I. sempervirens* Tahoe were referable to the profile of apple proliferation group phytoplasmas (16SrX, primers M1/M2) and to the profile of (AP) [primers R16(X)F1/R1, data not shown]. The profiles obtained with phytoplasmas from the Fish Back variety samples indicated the presence of phytoplasmas belonging to 16SrI and 16SrX groups in some cases in mixed infection (Figure 2b, samples 1, 9 and 10). Moreover, the RFLP analyses on R16(I)F1/R1 amplicons of these latter samples indicated the presence of 16SrI-B and-A subgroup phytoplasmas. The 16Sr DNA sequence assembly from the R16(X)F1/R1 amplicon allowed obtaining a 1,061 bp fragment that showed 99% homology with apple proliferation

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strains in the Genbank. Two SNPs at 662 and 873 bp allowed differentiation of the iberis strain from the '*Candidatus* Phytoplasma mali' sequences present in the Genbank. Moreover, the G-A substitution at position 662 bp is also a differential restriction site for *Bsiw*I since it is only cutting '*Ca.* P. mali' sequences (Figure 2c).

Discussion

No reports are available on phytoplasma presence in iberis. Moreover, the presence of a strain closely related to apple proliferation in mixed infection with aster yellows is puzzling. Similar mixed phytoplasma infection was reported in Poland in dahlias with proliferation symptoms (Kaminska and Sliwa 2008a), while in lilies with leaf scorch symptoms, only apple proliferation related phytoplasmas were detected (Kaminska and Sliwa, 2008b). However, it is not possible to verify if the lily and the iberis apple proliferation strains are identical since the available 16S rDNA sequence for lily (Genbank EF370450) does not cover the differential restriction site. Also, the presence of aster yellows subgroups 16SrI-A is not very common in European ornamental species. More studies should be carried out at the epidemiological level, to verify involvement of insect or seed transmission (Calari *et al.*, 2011) and avoid spreading of these phytoplasmas to other susceptible plant species.

Figure 1. Potted plants of *I. sempervirens* showing symptomatology referable to phytoplasma presence.

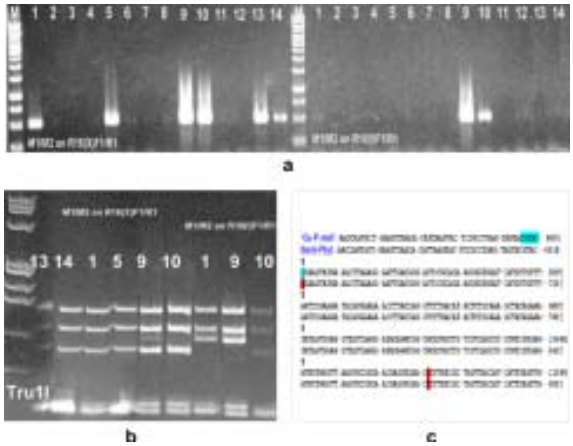

Figure 2. (a) Results of nested PCR assays carried out with M1/M2 primers. M, marker 1 kb DNA ladder, samples 1 to 11, symptomatic iberis Fish Back variety; 12, asymptomatic iberis control; 13, apple proliferation (AP-15, 16SrX-A); 14, pear decline (PD, 16SrX-C). (b) Results obtained after RFLP analyses of amplicons from symptomatic iberis and reference phytoplasma strains as indicated in (a) Marker, left lane, PhiX174 *Hae*III digested. The two different nested-PCR assays allow verification of the presence of mixed phytoplasma infection. (c) Restriction site (in light blue) in 16SrDNA sequence of '*Ca.* P. mali' that allow its differentiation from the 16SrX phytoplasma identified in symptomatic iberis samples.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S87-S88

doi: 10.5958/2249-4677.2015.00037.7

Phyllanthus niruri L.: a new host of '*Candidatus* Phytoplasma asteris' in India

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Abstract

In June 2014, *Phyllanthus niruri* plants growing wild in the university campus at Gorakhpur showed symptoms of little leaf and leaf chlorosis. Samples were collected and analyzed for phytoplasma presence through PCR assays. DNA extracted from symptomatic *P. niruri* plants produced amplicons of 1.2 kb in nested PCR using phytoplasma-specific primers. Symptomless plants did not produce amplicons. A 1.2 kb amplicon was directly sequenced. BLASTn analysis of the partial 16S rDNA sequence of *P. niruri* phytoplasma revealed 99% identity with strains from the '*Candidatus* Phytoplasma asteris' group. Phylogenetic analysis further confirmed the clustering of *P. niruri* phytoplasma with strains from the 16SrI group. This is the first report of a 16SrI group phytoplasma associated with *P. niruri*, a widespread tropical medicinal weed.

Keywords: phytoplasma, detection, plant disease, PCR, sequencing

Introduction

Phyllanthus niruri L. (fam: Euphorbiaceae) is a common rainy season weed found both in cultivated fields and wastelands. Although considered a problematic weed for farmers it is a valuable medicinal herb (Chauhan *et al*., 1977; Oudhia and Tripathi, 2002; Boim *et al*., 2010). Recently it has been proven that *P. niruri* is effective in treating liver cancer and jaundice. No effective specific therapy is available for viral hepatitis but *P. niruri* has shown clinical efficacy in viral hepatitis B (Paranjpe, 2001). Recently, lignansniranthin, nirtetralin, and phyltetralin were isolated from *P. niruri* leaves which are a major component of many popular liver tonics (Rastogi and Mehrotra, 1991). A phytoplasma disease associated with little leaf and leaf chlorosis on *P niruri* was studied.

Materials and Methods

A survey was conducted at the DDU Gorakhpur University campus at Gorakhpur in June and July 2014 for phytoplasma symptoms on weed species. *P. niruri* plants growing in the wild showing suspected phytoplasma symptoms were collected for phytoplasma analysis by PCR assays.

Fresh young stems from symptomatic and asymptomatic plant samples were used for total DNA extraction according to the described procedure of Ahrens and Seemüller (1992). Amplification of phytoplasma ribosomal DNA was performed with the universal phytoplasma primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995). Nested PCR was carried out with primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). DNA extracted from periwinkle infected with toria phyllody phytoplasma (16SrIX, pigeon pea witches' broom group) (Azadvar *et al*., 2009) was used as a positive control and DNA extracted from asymptomatic *P. niruri* plant tissues was used as a negative control. PCR reactions were performed in a Mastercycler (Eppendorf Germany) and the cycling protocol used was as reported by Rao *et al*. (2014). The about 1.25 kb nested PCR products were directly sequenced and assembled using the DNA baser V.4 program and further aligned using CLUSTALW of the Bio-Edit software. Aligned sequence was deposited in NCBI GenBank with Acc. No. KM280566 and used as query sequence in BLASTn search. The sequence generated from the present study and sequences of reference phytoplasma strains retrieved from GenBank were used to construct phylogenetic trees by neighbor joining methods with 1000 replications for each bootstrap value using MEGA 5.0 software version (Tamura *et al*., 2011). *Acholeplasma laidlawii* was used as out group to root the phylogenetic tree.

Results and Discussion

An incidence of 1-6% of phytoplasma symptoms such as leaf chlorosis and little leaves were observed (Figure 1). First round PCR amplification did not yield the expected 1.8 kb product of the 16S rDNA region from symptomatic *P. niruri* (data not shown). The 1.2 kb amplicons were obtained in nested PCR with R16F2n/R2 primers from symptomatic *P. niruri* tissues and the positive control while no DNA was amplified from asymptomatic *P. niruri* plants (data not shown). One nested PCR amplicon of *P. niruri* little leaf

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phytoplasma was directly sequenced. BLASTn analysis of 16Sr DNA sequences revealed that the phytoplasma detected shared 99% identity with strains of '*Candidatus* Phytoplasma asteris'. In phylogenetic analysis, *Phyllanthus* little leaf phytoplasma Gorakhpur strain clustered with '*Ca.* P. asteris' strains (Figure 2) confirming association of 'Ca. P. asteris' phytoplasma with little leaf and leaf chlorosis disease of *P. niruri*.

Many weed species have been reported as hosts for different groups of phytoplasmas in India and other countries (Mall *et al.*, 2011; Tran-Nguyen *et al.*, 2000). No literature is available on phytoplasma association with *P. niruri* worldwide. There is a report of phytoplasma disease on *P. amarus* in India (Samad *et al.*, 2004). However, this is the first report of a strain related to '*Ca.* P. asteris' on *P. niruri* worldwide. The potential reservoir of phytoplasmas on weed species may be larger than reported (Mall *et al.*, 2011).

Figure 1. *P. niruri* showing little leaves (A) and leaf chlorosis (B).

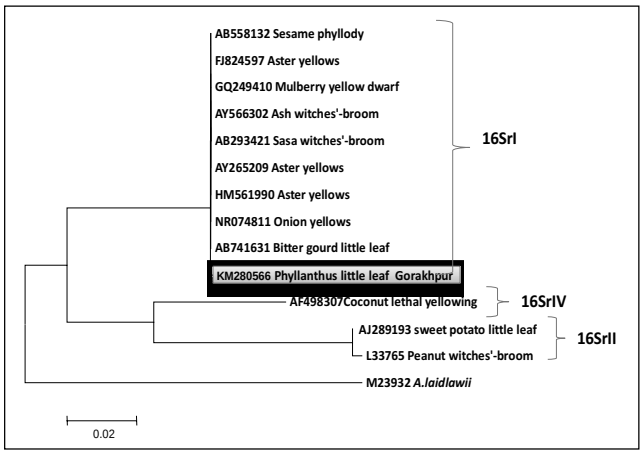

Figure 2. Phylogenetic tree constructed using the Neighbor-joining method, showing phylogenetic relationships of the *Phyllanthus* little leaf phytoplasma Gorakhpur strain with '*Ca.* P. asteris' phytoplasmas. *A. laidlawii* was used as outgroup.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S89-S90

doi: 10.5958/2249-4677.2015.00038.9

First report of oil seed rape (*Brassica napus*) associated phytoplasma diseases and their insect vector in Pakistan

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Abstract

Using direct and nested PCR technique followed by RFLP analysis, it was found that phytoplasmas were associated with *Brassica napus* plants showing symptoms such as phyllody, virescence, proliferation, flower sterility, from samples collected during a main survey carried out in different districts of Punjab, Pakistan. The samples from symptomatic plants were collected and observed by electron microscopy and tested using phytoplasma universal primer pairs R16F2n/R16R2, which confirmed the phytoplasma presence. Transmission trials by grafting and also by insects verify that the prokaryotes can be transmitted from diseased to healthy plants.

Keywords: nested PCR, phytoplasma, *Brassica napus*, phyllody, insect vectors, Pakistan

Introduction

Brassica napus is an oil seed crop, but its productivity is much lower in Pakistan as compared to other countries because of the attack of insects as well as for the presence of fungal, bacterial, and viral diseases. Phytoplasma symptoms generally include phyllody (development of floral parts into leafy structures), virescence (development of green flowers and the loss of normal pigments), witches' broom, extensive malformation of floral part, formation of bladder-like siliquae and flower sterility (Azadvar *et al*., 2009; Ahmad *et al*., 2013). The phytoplasma infecting *B. napus* are associated with phyllody symptoms. Previously, in Pakistan, phyllody phytoplasma associated diseases were found in sesame, vegetables, and in some fruits and in potential insect vectors (Akhtar *et al*., 2009). During a main survey from different districts of Punjab, samples from *B. napus* symptomatic plants and insects such as aphids, leaf hoppers and mealybugs were collected. Plant samples were observed by electron microscopy, and RFLP analysis of amplified 16S rDNA products confirms the presence of *B. napus* phyllody phytoplasma.

Materials and Methods

At least five *B. napus* symptomatic samples showing phyllody, virescence and flower sterility were collected from locations visited at University of Agriculture Faisalabad experimental fields and Multan and Rahim Yar Khan districts of Punjab, Pakistan. DNA from plants and insects collected in the same locations was extracted. In order to detect phytoplasma presence a nested PCR assay was applied by using the phytoplasma universal or group specific primers (Lee *et al*., 1994; 1995; Gundersen and Lee, 1996). The PCR amplified mixtures from DNA of healthy and diseased *B. napus* plants were electrophoresed in 1.7% agar gel in TAE buffer. Direct PCR with P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by nested PCR with R16F2n/R16R2 amplified a 1.2 kb fragment confirming the presence of phytoplasmas. First amplification was as follow: 94°C 4 minutes, 35 cycles of 94°C 1 minute, 60°C 1 minute, 72°C 1 minute and 30 seconds, with an extension at 72°C for 10 minutes. A 1:40 dilution of the first PCR product was used for the nested amplification. Cycles were as follow: 94°C 4 minutes, 35 cycles of 94°C 1 minute, 55°C 1 minute, 72°C 1 minute and 30 seconds, with an extension at 72°C for 10 minutes. Eight µl of product were analyzed on agarose gel. The PCR products were sent for sequencing in order to determine the group of the detected phytoplasma(s).

Results

Typical symptoms of phytoplasma infection such as phyllody, virescence, axillary proliferation of shoot and malformed and bladder-like siliquae and stunted growth were observed in several *B. napus* diseased plants at the time of flowering (Figure 1B). The seeds obtained from infected plants were very small and light brown in colour.

Nested PCR assays with the phytoplasma universal primer pair P1/P7 followed by primers R16F2n/R16R2 gave 1.2 kb DNA fragment from all diseased *B. napus* samples, but no amplification product was obtained from healthy plants (Figure 2). The amplified products were sent for sequencing for phytoplasma identification. The transmission trials by

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vegetative plant material and also by leafhopper verify that they can transfer the infection from diseased to healthy plants (data not shown).

Figure 1. Symptoms of phyllody and virescence in *B. napus*; A healthy and B phyllody, virescence, pod and flower malformation.

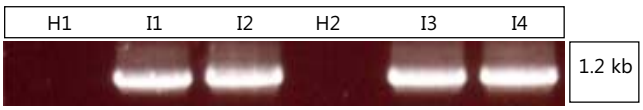

Figure 2. Agar gel electrophoresis of nested PCR products. H1-H2, Healthy *B. napus*, I1-I4, infected *B. napus*.

Discussion

Phytoplasma infection in oil seed rape/canola, was associated with the presence of 16SrI, aster yellows phytoplasmas that are the agents associated with stunting, leaf yellowing or purpling in the same species in Canada, Czech Republic, Italy and Greece (Bertaccini *et al*., 1998). The phyllody symptom in *Brassica* crop was observed for the first time in 1958 in India, and the phytoplasma associated with the disease was identified as belonging to group 16SrIX; however none of these symptoms had been observed on toria (*B. napus*) infected with the 16SrIX pigeon pea witches' broom group phytoplasma (Azadvar *et al*., 2009). *Matthiola incana*, a garden flower plant of Brassicaceae family was affected by 16SrII-A group phytoplasmas in Italy (Davino *et al*., 2007). In Canada in 2007, up to 12% of canola plants with aster yellows symptoms was recorded with a 2% of average presence. These findings suggest that phytoplasmas are spreading rapidly in Pakistan as reported for *B. napus* phyllody phytoplasma and insect vectors. Previously, phytoplasma diseases on potato, sesame, citrus, chick pea, mung bean have been reported in Pakistan (Nasir *et al*., 2007; Manan *et al*., 2010; Akhtar *et al*., 2009; 2010). Recently, phytoplasma diseases were also observed on sesame, chick peas, potato, citrus with same symptoms described before. So, there is a further need to study phytoplasma diseases and their putative insect vectors to control them properly in Pakistan.

Acknowledgements

This work was supported by the Integrated Genomic, cellular, Developmental and Biotechnology Laboratory, Department of Entomology and Department of Botany, University of Agriculture Faisalabad, Pakistan.

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Mixed infection of phytoplasmas and other pathogens

Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S93-S94

doi: 10.5958/2249-4677.2015.00039.0

'*Candidatus* Phytoplasma asteris' is associated with citrus "huanglongbing" disease in Mexico

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Abstract

"Huanglongbing" is one of the most devastating citrus diseases worldwide. '*Candidatus* Liberibacter' and '*Candidatus* Phytoplasma' species are commonly associated with the disease, although phytoplasmas were only found in citrus showing "huanglongbing"-like symptoms in Brazil and China. In Mexico samples were evaluated '*Ca.* L. asiaticus' and '*Ca.* Phytoplasma' presence by PCR assays. Fifty-four out of 86 citrus plants were positive for '*Ca.* L. asiaticus', 20 were positive for '*Ca.* Phytoplasma' and 7 were found as hosts of mixed infections with both prokaryotes. RFLP analyses of the 16S rDNA sequences enabled to identify two of the phytoplasma strains as members '*Candidatus* Phytoplasma asteris' subgroups 16SrI-B and 16SrI-S. Moreover partial '*Ca.* L. asiaticus' sequences were identical to those of strains from countries affected by "huanglongbing" disease. These results confirm the association of '*Ca.* Phytoplasma' with "huanglongbing" in citrus in Mexico, and its occurrence in mixed infections with '*Ca.* L. asiaticus'.

Keywords: "huanglongbing", phytoplasmas, '*Candidatus* Phytoplasma asteris', RFLP

Introduction

"Huanglongbing" (HLB) is one of the most devastating diseases affecting citrus production areas worldwide. It has spread rapidly to 16 of the 24 citrus-producer states of Mexico, reducing the national Mexican lime yield production to 301,314 tons (20%) from 2008 to 2013 (SAGARPA, 2014). Typical symptoms of HLB disease on Mexican lime trees include diffuse chlorosis, blotchy mottle, vein yellowing, stunted shoots and gradually death of branches as the disease progresses (Matos *et al*., 2009; Robles *et al*., 2013). These are similar to those described in citrus plants as associated with '*Candidatus* Liberibacter' species presence. Data reported in Dominican Republic in Mexican lime fruit include abscissed and lopsided fruit (Matos *et al*., 2009); however in Mexico no HLB symptoms have been observed in fruits (Robles *et al*., 2013; Arratia-Castro *et al*., 2014). Pigeon pea witches' broom phytoplasma (16SrIX) in Brazil (Texeira *et al*., 2008); and '*Candidatus* Phytoplasma asteris' (16SrI) and '*Ca.* P. aurantifolia' (16SrII) in China have been associated with HLB disease (Chen *et al*., 2009; Lou *et al*., 2014). This study aimed to determine whether in addition to '*Ca.* L. asiaticus', '*Ca.* Phytoplasma' species are also associated with HLB-like symptoms in citrus groves of Mexico.

Materials and Methods

Citrus plants showing HLB-like symptoms were collected from citrus orchards in the Mexican States of Nayarit, Colima and Sinaloa between August 2011 and September 2012. Citrus species included Mexican lime (ML) (*Citrus aurantifolia*, Christm., Swingle), Persian lime (PL) (*Citrus latifolia*, Tanaka), and Valencia sweet orange (SO) [*Citrus sinensis*, (L.) Osbeck]. Nucleic acids were extracted from lyophilized leaf midribs, using a CTAB protocol with minor modifications (Arratia-Castro *et al*., 2014). Total DNA was then evaluated for phytoplasmas and '*Ca.* L. asiaticus' presence using nested PCR (R16mF2/R16mR1 and R16F2n/R16R2) (Gundersen and Lee, 1996) and direct PCR (A2/J5) (Hocquellet *et al*., 1999), respectively. PCR products were purified, cloned into pGEM-T Easy vector, and sequenced using the Dye cycle sequencing kit. PCR phytoplasma fragments from representative citrus samples were digested with *Alu*I, *Hha*I, *Mse*I, *Hae*III, *Rsa*I, and *Taq*I restriction endonucleases and then fractionated by capillary electrophoresis using a QIAxcel system (Qiagen, Valencia, CA). Virtual RFLP analysis was performed using the virtual gel plotting program pDRAW32 (AcaClone). Phylogenetic trees were constructed with the Neighbor-Joining method, using the MEGA program (v.5.2.2). Bootstrapping with 1,000 replicates was performed to estimate branch stability and support.

Results

Phytoplasma presence was detected in symptomatic citrus infected leaves in the three states where samples were collected, in single as well as in mixed infections with '*Ca.* L. asiaticus'. Out of 86 HLB-symptomatic citrus plants, 54 were positive for '*Ca.* L. asiaticus', 20 were positive for

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phytoplasmas, 7 were found in mixed infections with both prokaryotes and 19 samples were negative for '*Ca.* L. asiaticus' and phytoplasmas. Diffuse chlorosis, blotchy mottle and vein yellowing symptoms were observed (Figure 1) in symptomatic citrus plants, however no specific symptoms were clearly associated with each prokaryote or both when occurring in mixed infections. Actual and virtual RFLP patterns allow to identify two phytoplasma strains as members of the aster yellows group (16SrI) '*Ca.* P. asteris', in particular subgroup 16SrI-B phytoplasmas were identified from Nayarit and subgroup 16SrI-S from Sinaloa and Colima. Phylogenetic analysis confirmed the clustering of the two strains. On the other hand, partial '*Ca.* L. asiaticus' sequence obtained were 100% identical to the strains isolated from several countries affected by HLB.

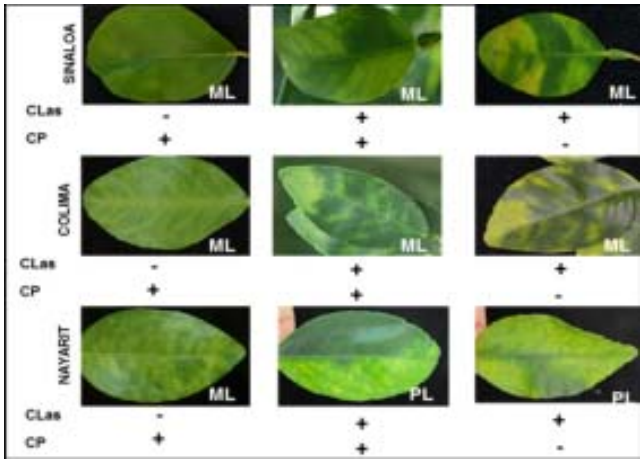

Figure 1. Diffuse chlorosis, blotchy mottle and vein yellowing symptoms observed in lime trees infected with '*Ca.* L. asiaticus' (CLas) and/or '*Ca.* P. asteris' (CP).

Discussion

RFLP analyses distinguished two phytoplasma strains of the aster yellows group, subgroups 16SrI-B and 16SrI-S detected in citrus showing HLB symptoms. Furthermore, '*Ca.* P. asteris' was detected in the 23.3% of the samples collected from the three citrus regions surveyed. '*Ca.* P. asteris' and '*Ca.* L. asiaticus' were detected in mixed infections in 8.1% of symptomatic samples, compared to 48.9% as determined for different locations in China (Chen *et al.*, 2009). However only 3.4% of the citrus samples from Brazil (Teixeira *et al.*, 2008) and 5.7% in a recent survey in China (Lou *et al.*, 2014) were infected with both '*Ca.* L. asiaticus' and '*Ca.* P. asteris'. The low incidence of '*Ca.* P. asteris' may be due to low titers of this pathogen in the leaf midribs from symptomatic leaves of the woody citrus host (Bertaccini and Duduk, 2009).

Other factors may be related to the possible irregular distribution of '*Ca.* P. asteris' throughout the plant phloem (Marcone, 2010), or even seasonal variations in the titer of phytoplasmas, or intrinsic agronomic traits of the citrus host (Marzachì 2004). Whereas only one HLB-associated phytoplasma strain has been reported in Brazil and China, we have provided evidence that two phytoplasma strains are currently present in Mexico, and occurring in mixed infection with '*Ca.* L. asiaticus' in citrus orchards.

Acknowledgements

We thank CONACYT and Instituto Politécnico Nacional (SIP 20130980 and 20131573) for financial support.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S95-S96

doi: 10.5958/2249-4677.2015.00040.7

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Detection of mixed infection of phytoplasmas and yellow leaf virus in commercial sugarcane cultivars and their impact on yield and quality parameters

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Abstract

Mixed infection of *Sugarcane yellow leaf virus* (SCYLV) and sugarcane grassy shoot (SCGS) phytoplasmas was detected in seven commercial sugarcane varieties grown at Shahjahanpur districts, Uttar Pradesh, India on the basis of RT-PCR assay with specific primers pair SCYLV and nested PCR assays using universal phytoplasma primers R16F2n/R16R2, respectively. The mixed infection reduced the sugarcane yield of about 38% in variety CoS 08272 in which 5.37% of losses in brix and 6.16% of losses in sucrose were also recorded. The sugar purity was also affected.

Keywords: sugarcane yellow leaf virus, sugarcane grassy shoot phytoplasma,mixed infection, losses

Introduction

The sugarcane (*Saccharum* spp.) crop faces several biotic and abiotic stresses, resulting in yield losses. Among the biotic stresses, sugarcane yellow leaf syndrome (YLS) is very important. It has been reported to be caused by polerovirus or phytoplasma or both (Arocha *et al*., 1999) also in India (Rao *et al*., 2012; Kumar *et al*., 2014). The polerovirus *sugarcane yellow virus* induces yellowing of the mid rib and lamina and was first reported in Hawaii, then in Brazil, Florida, and Australia, however, in South Africa, YLS was reported to be associated with the sugarcane yellow leaf phytoplasma (Rogers, 1969). YLS is widely distributed in almost all sugarcane growing countries causing losses up to 60% in susceptible cultivars (Comstock *et al*., 1994). This investigation was aimed to identify the most susceptible sugarcane varieties and to determine losses in yield and quality due to the presence of *Sugarcane yellow leaf virus* (SCYLV) and sugarcane grassy shoot phytoplasma (SCGS).

Materials and Methods

Sample collection

Ten varieties showing leaves with yellow midrib symptoms were collected from sugarcane growing areas of Shahjahnapur, Pilibhit and Faizabad districts and near by places in 2012-2013 (Table 1). The leaf samples were stored at -20°C until further processing.

Phytoplasma identification

For the molecular identification of phytoplasmas a CTAB method was followed for DNA extraction from 10 symptomatic sugarcane leaf samples. PCR assays were performed with P1/P6 universal primers, and nested PCR assay was carried out with primers R16F2n/R16R2 (Gundersen and Lee, 1996). The presence of phytoplasmas was visualized by electrophoresis of the products in 1% agarose gels in 0.5 X TBE buffer. PCR amplicons were purified by Nucleo spin kit (Gel and PCR clean up Kit, Germany), sequenced and sequences obtained were assembled using BioEdit software and used as query sequences in BLASTn search.

Sugarcane yellow leaf virus identification

Total RNA was isolated from the 10 sugarcane leaf samples by using EZ RNA kit (Biological Industries, Israel), and it was used in cDNA synthesis. RT-PCR assay with specific primers pair SCYLV-615F/615R was performed as described by Vishwanathan *et al*. (2008). A 10 µl aliquot of each amplified product was analyzed by electrophoresis on 1.5% agarose gel and photographed under UV illumination with an imaging system. Amplified products were directly sequenced and used as query sequences in BLASTn search.

Qualitative and quantitative analysis

After confirmation of the presence of SCGS phytoplasma and SCYLV in symptomatic leaf samples of variety CoS 08272 grown in SRI farm, Shahjahanpur, UP, India, phenotypic observation was carried out on 10 canes. For control, 10 canes of non symptomatic samples which resulted negative in PCR assays were used. Phenotypic observations recorded were: number of internodes, length of internodes, stalk height, cane diameter, cane weight and juice weight (Table 2). The juice quality parameters such as brix, purity and sucrose percentages were studied in 10 months old infected and healthy plants. The observations were repeated after 5 days and data presented are average values.

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Results and Discussion

The main symptom recorded in sugarcane cultivars was the yellow discolouration of midribs. The disease incidence was up to 7-10% in the variety CoS 08272 at SRI Farm. Other varieties in the present study at surveyed places were recorded with incidence of up to 8% (Table 1).

Table 1. Results of nested PCR and RT-PCR on symptomatic leaf samples.

Sample No.	Sugarcane Varieties	Location	PCR assays results		Incidence %
			SCYLV	Phyto	
1.	CoS 97264	SRI, Farm	+	+	Stray
2.	CoS 8436	SRI, Farm	+	+	Stray
3.	CoSe 96268	SRI, Farm	+	-	Stray to 5
4.	CoSe 1434	Faizabad	+	-	1 to 8
5.	UP 05125	Gola	+	-	1 to 6
6.	Co 238	Palia	+	+	Stray to 2
7.	CoS 91269	Pilibhit	+	+	Stray
8.	CoS 07250	Gola	+	+	Stray to 4
9.	CoS 08272	SRI, Farm	+	+	7-10
10.	CoS 07250	SRI, Farm	+	+	1-5

Nested amplification of DNA from leaf samples yielded about 1.2 kbp products in all the symptomatic samples except variety Gola (UP05125) and Faizabad (CoSe01434) confirming the phytoplasma presence in samples from seven varieties (Table 1). Amplified products directly sequenced shared 99% identity with described strains of the SCGS phytoplasma reported from different places. BLAST sequence results were also confirmed by phylogenetic analysis of 16Sr sequences (data not shown). In SCYLV identification, RT-PCR yielded expected 600 bp product from all the 10 symptomatic leaves and was absent in healthy samples. Sequence analysis of RT-PCR products showed maximum identity with isolates of sugarcane yellow leaf virus from India and abroad (data not shown). It was therefore confirmed that in the leaves with yellow midrib both phytoplasma and virus were present resulting in significant yield losses. The results on yield parameters showed considerable reduction in almost all yield contributing parameters due to mixed infection presence. The number of internodes was reduced to 14.1 per cane in the CoS 8272 variety against the 21.3 in healthy ones. The length of internodes was reduced of about 2.8 cm. The SCYLV and phytoplasma mixed infection reduced the cane yield of about 38% and also affected the juice extraction (Table 2). Moreover reduction in juice weight was about 4%, the brix was 17.79% in infected plants as compared to 18.80% in healthy ones (1.01% less). Sucrose percentages in juice was also reduced from 15.90% (control) to 14.92% (infected) which indicated a loss of 0.98%. Purity was also affected of about 0.64% (Table 3). SCYLV and phytoplasma has also been reported earlier in all the major sugarcane growing states of India to produce a reduction of about 40% in yield and also to reduce juice quality (Vishwanathan *et al.,* 2008; Tiwari *et al*., 2012).

It was concluded that mixed infection of SCYLV and SCGS phytoplasma were associated with YLD of sugarcane in seven commercial sugarcane varieties, which resulted in about 38% yield losses and also reduced the juice quality in variety CoS 08272. Looking to the widespread occurrence of YLD infected sugarcane crops, there is the need to monitor these important diseases. Early detection and disease free sugarcane cuttings could be the best options to reduce the further spread of the disease.

Table 2. Pathogen effect on yield in variety CoS 08272.

Yield parameters	Healthy	Infected
No of internodes	21.3	14.1
Length of internodes (cm)	11.6	8.8
Stalk height (cm)	216.2	121.9
Cane diameter (cm)	2.4	2.2
Cane weight (kg)	11.1	4.76
Juice weight (kg)	6.1	2.3

Table 3. Pathogen effect on juice quality in variety CoS 08272.

Juice quality	Healthy	Infected
Brix %	18.80	17.79
Sucrose %	15.90	14.92
Purity %	84.51	83.87

Acknowledgements

Authors are thankful to Director UPCSR, Shahjahanpur for providing lab facilities and also wish to thanks Department of Science and Technology, New Delhi, India for their financial assistance.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S97-S98

doi: 10.5958/2249-4677.2015.00041.9

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First report of mixed infection of phytoplasmas and begomoviruses in eggplant in India

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Abstract

Symptoms associated with yellows disease of eggplants were observed in fields of Meerut district of India. Total DNA extracted from symptomatic and asymptomatic plants was subjected to nested-PCR assay using phytoplasma 16S rDNA primers and generic PCR with universal begomovirus coat-protein (CP) gene primers. Amplicons of expected size were obtained from symptomatic plants in both PCR assays. No amplifications were observed from asymptomatic plants. The eggplant phytoplasma was identified as a member of group 16SrVI, while the begomovirus showed 99% of CP gene identity with other whitefly transmitted begomoviruses.

Keywords: eggplant, brinjal, phytoplasmas, begomoviruses, nested-PCR

Introduction

Eggplant (*Solanum melongena* L.) is an important vegetable grown throughout the world and India ranks third in the world in production. A disease with incidence affecting approximately 1-5% of plants was observed in eggplant fields of Horticulture Research Centre, S.V. Patel University of Agriculture and Technology, Meerut during February, 2012 to May, 2013. Symptoms included yellowing of leaf lamina with upward leaf curl as well as distortion, reduction in internode distance, new leaves size reduction, stunted growth, and drop of flower from plant before onset of fruiting. A number of eggplant diseases associated with phytoplasmas have been reported including brinjal little leaf from Bangladesh and India associated with the presence of phytoplasmas belonging to groups 16SrI and 16SrVI (Kelly *et al*., 2009; Kumar *et al*., 2012). However, similar symptoms are also induced by begomoviruses, family Geminiviridae (Pratap *et al*., 2011). The disease shows symptoms similar to those induced by phytoplasmas and causes enormous economic loss to the farmers. To identify the pathogens present in symptomatic eggplants a multiplex nested PCR method was developed for simultaneous detection of phytoplasmas and *Tomato leaf curl virus* (ToLCV).

Materials and Methods

Fifteen samples were collected from symptomatic and asymptomatic eggplant fields during February 2012 to May 2013. Total DNA was extracted from midribs with DNeasy Mini Kit, Qiagen, Germany, and used as template in PCR amplification of the begomovirus coat protein (CP) gene with primers AV1F/AV1R (Khan *et al*., 2014). All the amplifications were performed according to PCR parameters described by Singh *et al*. (2013). To test the presence of phytoplasmas, purified total DNA (8 µl with concentration of 10 ng/µl) was used for PCR amplification in a thermal cycler (Bio-Rad T100; Bio-Rad Laboratories Inc) using phytoplasma-specific 16S rDNA primers P1/P7 (Deng and Hiruki 1991; Schneider *et al*., 1995). The product was diluted 1: 25, and 2 µl of it were used in nested PCR as template with primer pair R16F2n/R16R2 (Gundersen and Lee, 1996), which covers 16S rDNA region. The PCR conditions employed were as reported by Singh *et al*. (2014) for direct and nested PCR. Duplex PCR, for detecting co-infection of begomovirus and phytoplasmas, was developed for simultaneous amplification of DNA fragments using phytoplasma primers P1/P7 and nested primer pair R16F2n/R16R2 and *Tomato leaf curl virus* specific (DNA-A region) primers AV1F/AV1R. PCR conditions for this nested multiplex have been optimized ensuring sensitive detection. Two types of template such as genomic DNA (50 ng) and amplified diluted (1: 25) PCR product of P1/P7 have been used to enable simultaneous detection of the virus and the phytoplasma. The first and second PCR products were stained with ethidium bromide and separated in 1% agarose gel electrophoresis. Three amplicons of each R16F2n/R16R2 and AV1F/AV1R were purified with PCR Purification Kit (QIAquick Gel Extraction kit, Qiagen, Germany) and sequenced at the automated DNA sequencing facility, Department of Biochemistry, Delhi University, South Campus on Applied Biosystems 3730 Genetic Analyzer. Sequences obtained were aligned using ClustalW and phylogenetic trees were constructed using the Neighbour-joining method with the program MEGA version 5.0.

Results

Amplicons of 1.8 kb and 1.2 kb were obtained by primers P1/

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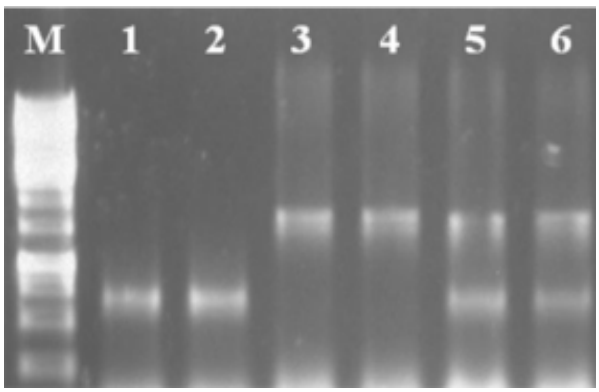

Figure 1. Detection of ToLCV and phytoplasmas in naturally infected eggplant plants. M, molecular marker (1 kb DNA ladder, MBI, Fermentas): lanes 1 and 2: 750 bp amplified product specific to cp gene obtained using AV1F/AV2R primers; lanes 3-4: amplicon from phytoplasma obtained using P1/P7 primers; lanes 5-6: 750 and 1800 bp multiplex PCR based amplification with phytoplasma specific and begomovirus coat protein primers.

P7 and R16F2n/R16R2, respectively. Amplification of about 750 bp fragments with primer pair AV1F/AV2R was obtained only from symptomatic plants. In the multiplex nested PCR assays the concentrations of the main reagents, such as primers, $MgCl_2$, dNTPs, Taq DNA polymerase and PCR parameters were optimized. Expected fragments were successfully amplified ensuring simultaneous, sensitive and specific detection of phytoplasmas and virus.

The 16S rDNA sequence and CP gene sequence were deposited in GenBank with accession No. JX104336 and KC513743, respectively. BLAST analysis showed the highest sequence identity of phytoplasmas (99%) with those of members of group 16SrVI. Phylogenetic tree constructed based on the analysis of the 16S rDNA sequences from 40 diverse phytoplasmas, showed that the eggplant phytoplasma closely clustered to those classified into subgroup 16SrVI. The partial CP gene sequence of the begomovirus showed 99% similarity with that of the begomovirus infecting *Eclipta alba* (DQ339119), and *Solanum nigrum* (DQ339123). Previously phytoplasmas have been found to be associated with eggplant diseases reported from Thailand (Green *et al*., 2003) and India (Pratap *et al*., 2011), but no reports of phytoplasma-begomovirus mixed infection have been described.

Discussion

Mixed infections of phytoplasmas and viruses have been reported in a wide range of plant species indicating that both pathogens can infect the same plant host (Arocha *et al*., 2009). There are very few reports of simultaneous detection of virus and phytoplasma by multiplex PCR (Baranwal *et al*., 2005: Matus *et al*., 2008: Biswas *et al*., 2013). The coexistence of 16SrVI phytoplasma and begomovirus in eggplant showing yellows disease symptoms need further studies to understand the mechanisms by which both are transmitted to the same plant host, and especially the insect vector involvement. Detailed analysis of epidemiology is required and potential role of non-crop species that may act as reservoirs for such plant pathogens should be studied.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S99-S100

doi: 10.5958/2249-4677.2015.00042.0

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Simultaneous transmission of phytoplasma and spiroplasma by *Dalbulus maidis* leafhopper and symptoms of infected maize

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Abstract

Two experiments were carried out to study simultaneous transmission of phytoplasmas and spiroplasmas by the leafhopper *Dalbulus maidis* from double infected popcorn-plants. The double infection was obtained through only one leafhopper from field per plant. The results indicated variability among isolates and combinations of phytoplasmas and spiroplasmas for transmission by *D. maidis* and maize leaf symptoms expression.

Keywords: Mollicutes, corn stunt, maize bushy stunt, *Zea mays*

Introduction

The corn stunt diseases are caused by two different *Mollicutes*: maize bushy stunt phytoplasma (MBS) and corn stunt spiroplasma (CSS; *Spiroplasma kunkelii*). These *Mollicutes* are transmitted by the leafhopper *Dalbulus maidis* (Nault, 1980). Knowledge about the transmission of these pathogens and predominance of each one is important to develop strategies for the disease control. The goal of this study was to verify the consistence of simultaneous transmission of phytoplasma and spiroplasma by *D. maidis*, and the leaf symptoms in infected maize.

Materials and Methods

Obtaining double-infected plants

One hundred adult *D. maidis* were collected in maize at Embrapa's experiment station in Sete Lagoas, Brazil, in June 2013 and individually confined during six days on one popcorn seedling for *Mollicutes* transmission. After 50 days, the plants presenting corn stunt symptoms and simultaneous infection by phytoplasma and spiroplasma, as detected by PCR, were used for acquisition of these *Mollicutes* and transmission to other popcorn seedlings. The new symptomatic plants, were checked for the presence of the double infection by PCR assays, and two of them were selected (plant 1 and plant 2) for the transmission experiments.

Spiroplasma and phytoplasma detection

The bacteria were detected by multiplex PCR assays (Oliveira *et al*., 2005) using primers CSSF2/CSSR6 to detect spiroplasmas (Barros *et al*., 2001) and primers R16F2/R16R2 to detect phytoplasmas following the PCR reaction conditions described by Lee *et al*. (1993).

Acquisition and inoculation

Mollicute acquisition was done using healthy leafhoppers reared in cages with healthy popcorn plants. After acquisition, the leafhoppers were feed on healthy popcorn for the latent period and, after that, used to inoculate the bacteria in other healthy popcorn seedlings. Acquisition and inoculation periods were always of six days. The latent period was three weeks after the acquisition period as reported by Nault (1980). After the first acquisition in plants infected by leafhoppers from the field, the bacteria inoculation was carried out using three leafhoppers per seedling.

Transmission experiments

Two experiments (1 and 2) were carried out to study simultaneous transmission of phytoplasmas and spiroplasmas, respectively from double infected plant 1 and plant 2, using *D. maidis* as vector. These infected plants were used for acquisition of *Mollicutes* by leafhoppers. The plant 1 had leaves that were reddish and with chlorotic stripes characteristic of spiroplasma infection. Plant 2 had only reddish leaves. The double infection of these plants by phytoplasma and spiroplasma was confirmed by PCR assays. For each experiment, 20 replicates were performed; one popcorn seedling per pot was employed with one leafhopper for bacteria inoculation, and four popcorn seedlings were used with one healthy leafhopper each, as control. The leafhoppers used for transmission were recovered, kept in freezer and analyzed by PCR for phytoplasma and spiroplasma detection, when the plant did not present corn stunt symptoms. Ten plants from the experiment 1 and ten from the experiment 2 were random sampled thirty days after sowing, and the apical leaves were collected to detect phytoplasma and spiroplasma presence. The experiments were carried out for 60 days in December and January, and plants were weekly evaluated for the disease symptoms.

Environmental conditions

The studies were carried out in a insect proof screen-house, with a roof of fiberglass, using one popcorn hybrid and pots

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with 3 kg of fertilized soil. The climatic characteristics in the Embrapa's experiment station are presented in Figure 1.

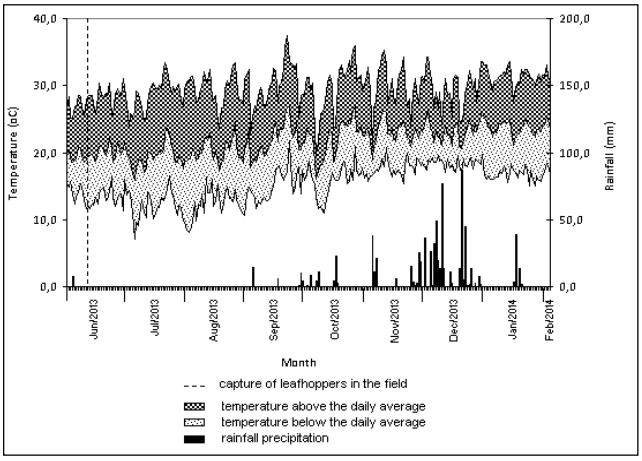

Figure 1. Climatic conditions during the experimental period from June 2013 to February 2014.

Results

Among popcorn plants subjected to leafhoppers from the field, only four showed reddish leaves and one of them also showed chlorotic stripes (plant 1). In these four plants phytoplasma and spiroplasma were detected by PCR assays. In the process to obtain the double infected plants, even using three leafhoppers for inoculation, only a maximum of 50% of new symptomatic plants presenting only reddish leaves was obtained, and never with chlorotic stripes. From the plant with chlorotic stripes, transmission using three leafhoppers resulted in 100% of new symptomatic plants with reddish leaves and chlorotic stripes. The reddish leaves appeared before the chlorotic stripes.

In experiment 1 the phytoplasma and the spiroplasma were both transmitted by each leafhopper and detected in all plants. In experiment 2 only phytoplasmas were detected in two plants, and phytoplasmas and spiroplasmas were detected in only one plant (Table 1). In experiment 1, all plants showed at least one reddish leaf, chlorotic stripes characteristics of corn stunt spiroplasma and, frequently, basal tillering. In the experiment 2, only 25% of the plants showed reddish leaves and one plant presented basal tillering (Table 2). Among the six leafhoppers recovered from plants that did not present corn stunt symptoms in experiment 2, only two showed spiroplasma bands by PCR assays.

Discussion

The results showed isolates and combinations of phytoplasma and spiroplasma variability regarding the efficiency of transmission by *D. maidis* and maize symptoms expression. The consistent transmission of both phytoplasmas and spiroplasmas from plant 1 suggest surviving adaptation of these pathogens. It is possible that different environmental conditions are differentially favorable to each one of these *Mollicutes*. Differential prevalence of each one has been reported in different maize growing seasons and also, effects of temperature on their transmission is variable according to different isolates (Moya-Raygosa and Nault, 1998; Oliveira *et al*., 2002; 2007).

Table 1. Phytoplasma and spiroplasma detected by PCR in ten plants from the experiment 1, and, ten plants from the experiment 2.

	Number of detections		
	Phyto and Spiro	Phyto	Spiro
Experiment 1	10	0	0
Experiment 2	1	2	0

Phyto = phytoplasma; Spiro = spiroplasma

Table 2. Corn stunt symptoms in plants submitted to *Mollicutes* inoculation in the transmission experiments.

	Plants with symptoms (%)			
	Total	Reddish leaves	Chlorotic stripes	Tillers
Experiment 1	100	100	100	45
Experiment 2	25	25	0	5

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S101-S102

doi: 10.5958/2249-4677.2015.00043.2

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A new disease in *Citrus aurantifolia* in Oman, "sudden decline", is associated with a pathogen complex including a 16SrII group phytoplasma

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Abstract

Witches' broom disease of lime is a devastating disease in the Sultanate of Oman. In recent years a new disease has been observed, which promotes a sudden decline in *Citrus aurantifolia* (acid lime). The etiological agent of this new disease has not yet been determined. In this study the presence of phytoplasmas and RNAs was evaluated in plants showing the sudden decline symptomatology. The results reveal the presence of a complex of at least three different pathogens: 16SrII phytoplasmas, *Citrus tristeza virus* and *Citrus exocortis viroid*.

Keywords: sudden decline, citrus, phytoplasma, CTV, CEVd

Introduction

In recent decades, lime production in Oman has been severely hit by diseases, to the point where the country, once an exporter of limes, now imports the fruit (Al-Sadi *et al*., 2004). Since the 1970's, over half a million small-fruited acid lime trees (*Citrus aurantifolia*) have been lost in Oman. The principal cause of these losses was the witches' broom disease of lime (WBDL), estimated to infect 98% of lime trees in the country. The disease is associated with the presence of '*Candidatus* Phytoplasma aurantifolia' (Zreik *et al*., 1995). Moreover a recent and increasingly important disease named sudden decline, whose etiology has yet to be elucidated appeared in lime. Symptoms are reduction in growth, leaf yellowing and necrosis followed by death of the plant (Figure 1). Due to the importance of this new disease in citrus-growing areas in Oman, the aim of this work was to search for the associated agents by testing for the presence of phytoplasmas and RNA pathogens (viruses and viroids).

Materials and Methods

Twelve citrus samples showing sudden decline symptoms (Figure 1), were collected from cultivated areas in Muscat, Oman. Total DNA extraction from these samples was performed using NucleoSpin Plant II Kit (Macherey-Nagel) according to the manufacturer's recommendations. Phytoplasma detection was carried out with a universal P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) primer set followed by nested primers R16F2n/R16R2 (Gundersen and Lee, 1996). These latter primers amplify a fragment of 1,246 bp from the phytoplasma 16S rDNA, which was used for RFLP and phylogenetic analyses. PCRs for phytoplasma detection were performed using the DNA amplification kit GoTaq Master Mix (Promega Corporation) and fragments generated were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), according to the manufacturer's instructions. The purified fragments were inserted into pGEM T Easy Vector System I (Promega Corporation) and transformed to *Escherichia coli* DH5α competent cells (Sambrook and Russel, 2001). Plasmid DNA was extracted and cloned fragments were sent for sequencing. Restriction analysis *in silico* of the obtained sequence was performed using the online tool iPhyClassifier (Zhao, *et al*., 2009), and real RFLP with enzymes *Bstu*I, *EcoR*I and *Hpa*II was also carried out on the amplicons. Additionally, real time PCR assays were performed using primers specific for phytoplasma immuno-dominant membrane protein IMP3F/IMP3R (Askari *et al*., 2011). Plants showing WBDL and healthy plants derived from seeds were used as positive and negative control, respectively.

Figure 1. Sudden decline symptoms: left, *Citrus aurantifolia* symptomatic plant; right, early symptoms of plant sudden decline.

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To verify the presence of RNA pathogens, total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. Total RNA was submitted to library construction following sequencing by 454 GS-FLX titanium platform. Reads were assembled using the software Geneious and contigs were analyzed using Blastn, Blastp and ORF Finder.

Results

The presence of phytoplasmas was confirmed by nested PCR and sequencing in samples from all 12 plants showing sudden decline. The 16S rDNA phytoplasma sequences of four samples were subjected to RFLP and phylogenetic analyses. RFLP *in silico* indicated that the phytoplasma associated with sudden decline can be classified in the 16SrII group (Figure 2). This result was confirmed with real RFLP analyses using *Bstu*I, *EcoR*I and *Hpa*II (data not shown). Phylogenetic analyses demonstrated that the phytoplasma associated with sudden decline grouped in the same clade with other representatives sequences from the 16SrII group, confirming the *in silico* and real RFLP analysis.

Quantitative real time PCR indicated that the phytoplasma associated with sudden decline has a very low concentration when compared with the positive control, with statistically significant differences by the t test at $P \leq 0.05$ (Figure 3). In addition to phytoplasma detection, deep sequencing of total RNA indicated the presence of *Citrus tristeza virus* (CTV, 7 reads) and of *Citrus exocortis viroid* (CEVd, 76 reads) in plants showing sudden decline. The presence of these pathogens was confirmed by conventional RT-PCR using specific CTV and CEVd primers.

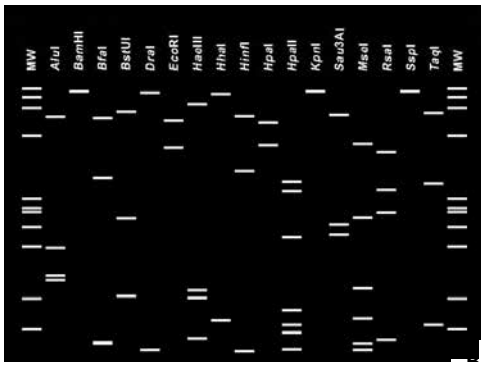

Figure 2. RFLP *in silico* of the 16S rDNA fragment amplified and sequenced using seventeen enzymes for phytoplasma characterization. MW= pattern of phiX174 digested with *Hae*III.

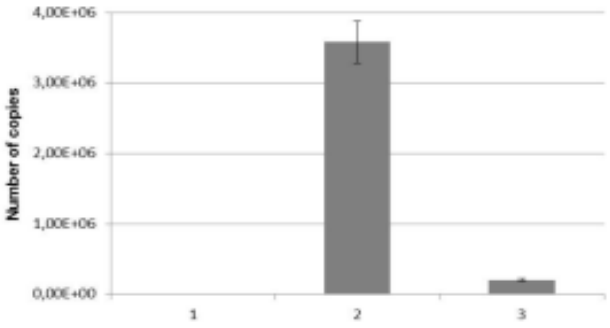

Figure 3. Quantification of phytoplasmas: 1, negative control (total DNA of *Citrus latifolia*); 2, positive control (total DNA of *C. aurantifolia* showing WBDL symptoms); 3, pool of total DNA of *C. aurantifolia* showing sudden decline symptoms. Bars represent the confidence intervals ($p \leq 0.05$).

Discussion

Although the phytoplasma detected in plants with sudden decline is classified in the same group as the phytoplasma associated with WBDL, the characteristics of the two phytoplasmas are distinct in terms of the symptoms and pathogen accumulation. The phytoplasma associated with sudden decline may represent a new strain. Additionally, the results presented here reveal a complex of pathogens that may be involved in sudden decline disease development in Oman.

Acknowledgements

This work was funded by Instituto Tecnológico Vale. FNS (first author) was an Instituto Tecnológico Vale postdoctoral fellow.

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Phytoplasma-plant interaction and control of phytoplasma diseases

Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S105-S106

doi: 10.5958/2249-4677.2015.00044.4

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Epidemiological role of spontaneous weeds in the spreading of "bois noir" phytoplasma

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Abstract

Survival of *Hyalesthes obsoletus* on some weeds was investigated to verify their possible role in "bois noir" epidemiology. Data evidenced that *H. obsoletus* survived after 14 days of forced feeding on *Urtica dioica*, *Convolvolus arvensis*, *Artemisia vulgaris*, *Plantago lanceolata* and *Taraxacum officinale*. This evidence reinforced the hypothesis of a possible involvement of such weeds, carrying the same "bois noir" phytoplasma tuf-type identified in grapevines and insects, in "bois noir" spread in north-eastern Italy.

Keywords: '*Candidatus* Phytoplasma solani', grapevine yellows, *Hyalesthes obsoletus*

Introduction

"Bois noir" (BN), a grapevine yellows (GY) disease associated with the presence of '*Candidatus* Phytoplasma solani', is a major limiting factor for wine production in European countries. BN phytoplasma strains are transmitted by the planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Cixiide), a polyphagous insect living preferentially on weeds (Quaglino *et al*., 2013). Up to now, three natural ecologies of BN have been described: - the host system *Convolvulus arvensis* - *H. obsoletus* - *Vitis vinifera* related to BN strains of tuf-type b, - the host system *Urtica dioica* - *H. obsoletus* - *V. vinifera* related to tuf-type a and - the host system *Calystegia sepium* - *H. obsoletus* - *V. vinifera* related to tuf-type c (Langer and Maixner, 2004). Moreover, alternative plant hosts (Table 1) and insect vectors have been reported (Quaglino *et al*., 2013). Recently, a multidisciplinary approach based on field surveys, molecular biology techniques and spatial data analyses was used to investigate the role of weeds in BN epidemiology within vineyards (Mori *et al*., 2014). The investigation showed that the host systems involving *C. arvensis* and *U. dioica* play the main role in BN diffusion in north-eastern Italy. However other weeds (i.e., *Amaranthus retroflexus*, *Chenopodium album*, *Malus sylvestris*, and *Plantago lanceolata*) spatially associated with symptomatic grapevines and/or insect vectors, and infected by the same tuf-type phytoplasma identified in grapevines and insects, could play a role in BN spreading. In the present study the survival of *H. obsoletus* on these weeds, reported as hosts of *H. obsoletus* and/or of BN phytoplasmas, was investigated to verify their possible role in BN disease epidemiology.

Materials and Methods

H. obsoletus was caged on the selected weeds (Table 1) and 1, 3, 7 and 14 days after caging the number of dead specimens was recorded. Four replications for each weed were used and the results were analysed using ANOVA and Tukey's post-test. Trials were carried out in a vineyard located in Ronco all'Adige, Verona (Italy). Molecular analyses on these weeds, symptomatic grapevines and collected *H. obsoletus* specimens were carried out to identify and characterize the BN strains based on PCR/RFLP assays on *tuf* gene. PCR reactions were carried out as previously reported (Langer and Maixner, 2004).

Table 1. Investigated weed species selected according to literature.

Species	Host plant for *H. obsoletus*	Host plant of BN
Amaranthus retroflexus L.		ref. 3
Artemisia vulgaris L.	ref. 1	ref. 3
Chenopodium album L.		ref. 3
Convolvolus arvensis L.	ref. 4	ref. 3
Malva sylvestris L.		ref. 3
Plantago lanceolata L.		ref. 3
Polygonum persicaria L.		ref. 3
Rumex acetosa L.		ref. 5
Taraxacum officinale W.		ref. 3
Trifolium pratense L.		ref. 6
Trifolium repens L.		ref. 5
Urtica dioica L.	ref. 2	ref. 3
Vitis vinifera L.		ref. 2

1: Alma *et al.*, 2002; 2: Belli *et al.*, 2010; 3: Kessler *et al.*, 2011; 4: Langer and Maixner, 2004; 5: Mori *et al.*, 2014; 6: Sabaté *et al.*, 2014

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Table 2. *H. obsoletus* survival rate and BN strain presence on different weeds species. Different letters on the "Days after caging" columns indicate statistical differences at ANOVA and Tukey's test per P< 0.05.

Weed species	BN tuf-type	Days after caging			
		1	3	7	14
A. retroflexus	b	56.7^{b}	6.7^{b}	3.3^{c}	0.0^{c}
A. vulgaris	b	63.2^{b}	24.0^{b}	18.0^{b}	12.0^{b}
C. album	b	55.0^{bc}	10.0^{b}	0.0^{d}	0.0^{c}
C. arvensis	b	66.7^{b}	9.0^{b}	5.0^{c}	1.2^{bc}
M. sylvestris	b	35.0^{c}	10.0^{b}	5.0^{c}	0.0^{c}
P. lanceolata	b	23.0^{c}	19.0^{b}	4.0^{c}	1.7^{bc}
P. persicaria.	b	38.3^{c}	15.0^{b}	0.0^{d}	0.0^{c}
R. acetosa	b	55.0^{bc}	0.0^{c}	0.0^{d}	0.0^{c}
T. officinale	b	43.3^{c}	20.0^{b}	5.0^{c}	3.0^{bc}
T. pratense	b	56.7^{bc}	0.0^{c}	0.0^{d}	0.0^{c}
T. repens	b	43.0^{c}	0.0^{c}	0.0^{d}	0.0^{c}
U. dioica	a	85.0^{a}	78.3^{a}	67.0^{a}	58.0^{a}
V. vinifera	a, b	37.8^{c}	15.0^{b}	0.0^{d}	0.0^{c}

Results

The best survival of *H. obsoletus* adults was obtained on host plants where the nymphs could develop, and it was clear that the cixiid survives well only on the same plants on which it was captured. After 1 day of caging, in addition to the known host plants (*U. dioica*, *C. arvensis* and *A. vulgaris*), only for *A. retroflexus*, *C. album*, *R. acetosa* and *T. pratense* survival percentages higher than 50% (Table 2) were recorded. After 7 days of forced feeding on *C. album, P. persicaria, R. acetosa, T. pretense* and *V. vinifera* the caged specimens were all dead (Table 2). After 14 day of caging, in addition to the host plants, surviving *H. obsoletus* was found only on *P. lanceolata* and *T. officinale* (Table 2).

Molecular analyses highlighted the presence of BN strains of tuf-type a only on nettle, while BN strains of tuf-type b were identified on the other weeds. Both tuf-types were identified in symptomatic grapevines and *H. obsoletus* specimens collected at the border of the same vineyards.

Discussion

BN and other plant diseases associated with phytoplasmas have a complex biological cycle, involving different host plants and/or insect vectors. Due to a lack of knowledge about the epidemiology of such diseases, it is difficult to develop efficient strategies to understand and contain their transmission.

In the present study the survival of *H. obsoletus* on weeds that could play a role in BN transmission was tested. Considering that the average of activity period of adult *H. obsoletus* is about six weeks, the possibility of longer survival on some weeds increases the possibility of *H. obsoletus* to feed, acquire and transmit BN. Consequently, the longer survival of *H. obsoletus* is strictly related to the role of the weeds in BN epidemiology. Results showing the good feeding activity of *H. obsoletus* on *U. dioica* and *C. arvensis* are fully in agreement with the results reported in previous studies, underlying the main role of these weeds in BN epidemiology in Europe (Kessler *et al.*, 2011). Moreover, the data evidenced that *H. obsoletus* survived after 14 days of forced feeding on *P. lanceolata* and *T. officinale*. This evidence, along with the presence of the same BN tuf-type in such weeds, grapevines and insects, reinforced the possible involvement of *P. lanceolata* and *T. officinale* in BN spread in north-eastern Italy.

Data obtained in this and previous studies indicate that BN epidemiology is influenced by several weed species and their distribution patterns inside and outside vineyards (Mori *et al.*, 2014), and could provide helpful indications for designing experimental plans to contain BN spreading in vineyards through weed management (Riedle-Bauer *et al.*, 2010). Given that BN-infected weeds are mainly dicotyledonous, it could be important to favour the vineyard ground cover with weeds reported as non-host of *H. obsoletus* and/or BN, or weeds associated with a low survival of *H. obsoletus*.

Acknowledgements

This study was funded by Consorzio per la Tutela del Franciacorta and University of Padua (Fondi EX 60% cod: 60A08-9409/12).

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S107-S108

doi: 10.5958/2249-4677.2015.00045.6

Trap plants reduces grapevine yellows disease incidence in commercial vineyards

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Abstract

The host plant *Vitex agnus-castus* attractiveness to the "stolbur" phytoplasma vector *Hyalesthes obsoletus* was exploited to study application methods of trap plants in order to reduce yellows disease incidence in vineyards. Following the introduction of the trap plants in vineyards, infestation level was reduced. The effect on plot infestation level diminishes with distance from trap plants and varies depending on the trap plant biomass. There is also indication of trap plant effect joined with wind direction. The grapevine yellows disease can be controlled in Israel conditions by means of trap plants that lure the vector.

Keywords: vector, trap plant, yellows disease, *Hyalesthes obsoletus*, "stolbur" phytoplasma

Introduction

"Stolbur" phytoplasma, associated with yellows disease in grapevines is transmitted by the phloem feeding vector *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae). The adults hardly survive on grapevines and their life cycle is not completed on grapevines (Sforza *et al*., 1998; Maixner *et al.,* 2007). The grapevines can recover and infection depends on repeated visitation of the vector to the vineyard. To reduce vineyards infection, the encountering of grapevines by the vector should be prevented. However, the appearance of the vector in vineyards is sporadic and conventional control methods are inefficient.

In Israel, the vector is intensely attracted to *Vitex agnus-castus* on which it completes its life cycle (Sharon *et al*., 2005). Repeated surveys revealed that "stolbur" phytoplasma can be found only in grapevines and in the vector, *H. obsoletus*. The vector's host plant, *V. agnus-castus*, does not carry the phytoplasma despite hosting infected insect vectors. Its attractiveness to the vector and its inability to acquire the pathogen pose the possibility to use it as a trap plant. We hypothesized that placing *V. agnus-castus* plants in the corner of the vineyard will lure the vector and, trapping it on the host plant, will prevent infestation of grapevines, reducing grapevine yellows incidence.

Materials and Methods

Trap plants effect on reduction of the disease

The experiment was conducted in 5 vineyards. The trial started as case study in 2009 (vineyard A; Cabernet Sauvignon; 60 plants in 2009-2011 and 30 trap plants in 2012-2014), expended with two more vineyards (B and C; Cabernet Sauvignon; 30 plants) in 2011, and with an addition of two vineyards (D and E; Chardonnay; 10 plants) in 2012. Trap plants (plant height 0.5 m) were placed in one corner of each plot. Yellow sticky traps were attached to each plant to catch the lured vectors. A distant plot (>1 km) was used as control in each of the vineyards.

Effect of wind direction

In 2014 seven Chardonnay vineyards (1-6) were added to the experiment. Potted plants (20) were placed at the corner of each vineyard. Imidacloprid was applied to the plants to prevent the vector establishment and one sticky trap was attached to each plant (Figure 1). In 3 vineyards the plants were placed upwind and in 3 downwind.

Efficacy measurements

The number of vectors/day/trap was counted. Yellows disease infected grapevines were mapped in each plot, just before harvest, each season. The number of infected

Figure 1. Trap plants placed at the corner of each vineyard with sticky trap to catch the vector.

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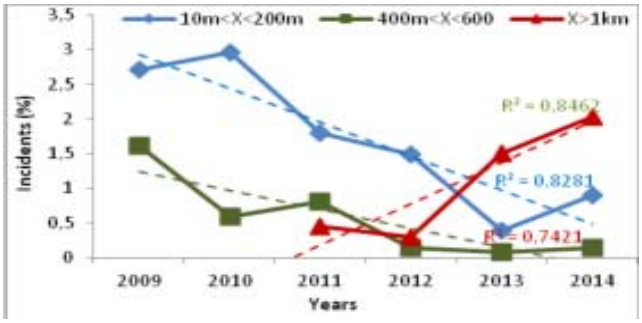

Figure 2. Infection level in vineyard A after application of trap plants (60 in 2009-2011 and 30 in 2012-2014): a plot adjacent to the trap plants (10 m<X<200 m), a plot distant more than 400 m from the trap plants (400 m<X<600 m) and a plot distant more than 1 km from trap plants (X>1 km). Dotted lines indicate linear trendline with its R^2.

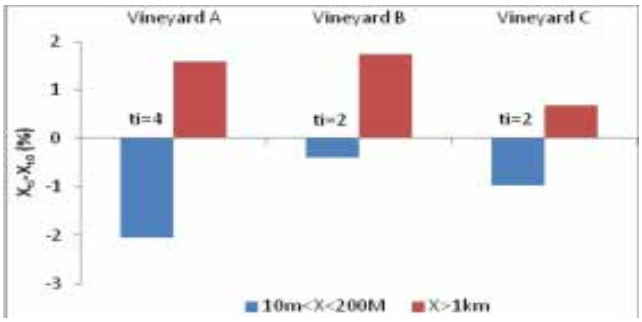

Figure 3. Infection level change [X_{ti}-X_{t0}] in vineyards A-C after application of 30 trap plants: in a plot adjacent to the trap plants (1 m-10 m<X<200 m) and in a plot more than 1 km (X>1 km) from the trap plants. t_i=time from application in years.

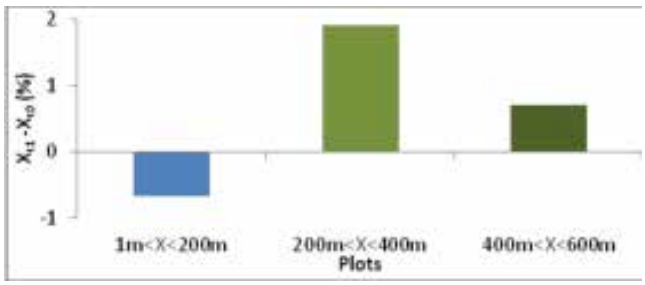

Figure 4. Infection level change [$X_{t1}X_{t0}$] in vineyard E after application of 10 trap plants: in a plot adjacent to the trap plants (1 m<X<200 m), in a plot more than 200 m from the trap plants (200 m<X<400 m) and in a plot more than 400 m away from the trap plants (400 m<X<600 m).

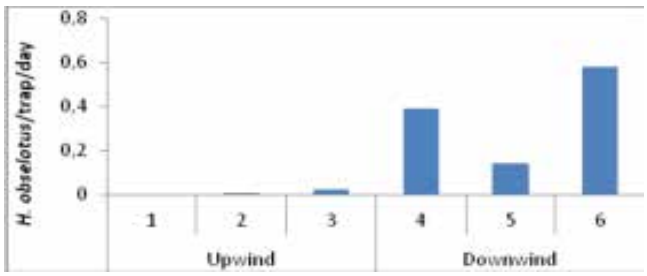

Figure 5. Number of insect vectors captured on sticky traps attached to trap plants in 6 vineyards. In 3 vineyards (vineyards 1-3) trap plants were positioned upwind and in 3 vineyards (vineyards 4-6) trap plants were positioned downwind.

grapevines was transformed to percentage of incidence in the plots (X_{ti}; X=incidence % and t_i=time from start in years). Effect was calculated by the equation [X_{ti}-X_{t0}]. Distance effect from plant traps was calculated between and along rows using ArcGIS. The intensity of the trap plant effect was measured as distance from trap plants in relation to their numbers.

Results and Discussion

When the method was applied with 60 and 30 trap plants, the percentage of infestation was reduced both in plots near the trap plants and 400 m away, but not in plots more than 1 km away from the trap plants (Figures 2 and 3). The effect accumulates over years (Figure 2). The number of vectors captured on sticky traps attached to the trap plants (individuals/year) remained the same and even increased during the studied years. Thus, infection reduction cannot be attributed to a decrease of the vector population in the area. When 10 trap plants were used, the effect on infection level was detected up to 200 m (1-10 m<X<200 m) but above that distance it diminished (Figure 4- data for vineyard E). Thus, 10 trap plants effect infection level in a 200 m radius and 30-60 trap plants increases the radius to more than 400 m but less than 1 km. Preliminary data from 6 vineyards (Figure 5) show that more vectors are captured when the main wind direction is from the fellow land through the trap plants to the vineyard (downwind) than on trap plants placed upwind. This indicates that wind direction affects the trap plant efficacy and that they should be placed downwind to the vineyard.

We conclude that the yellows disease incidence can be decreased by means of trap plants that lure the vector. The efficacy of the method depends on application duration, trap plant number and location that relate to wind direction.

Acknowledgements

This research was supported by grants from the Chief Scientist of the Ministry of Agriculture and the Wine-Grape Growers Board (Israel).

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S109-S110

doi: 10.5958/2249-4677.2015.00046.8

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Reactions of some grapevine cultivars to "bois noir" phytoplasma

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Abstract

"Bois noir" is a widespread phytoplasma disease in the vineyards of Turkey. The main syptoms are intensive reddening and inward curling in the dark colored varieties. "Bois noir" infected scions were grafted on pathogen-free grapevine varieties as rootstocks in order to investigate the response of the varieties commonly grown in Turkey to this phytoplasma. Grafting have been made in spring and the symptoms were observed in September 2012. Detection of the phytoplasma was achived by PCR and nested PCR analysis with universal and group specific primers. No symptom development was detected in the varieties Kalecik Karas, Bogazkere and Bornova Misketi that are all local varieties and were all negative in the PCR and nested-PCR tests.

Keywords: "bois noir", phytoplasma, Kalecik Karas, Bogazkere, Bornova Misketi

Introduction

In Turkey, the Northeastern part of Anatolian peninsula, located between Black sea and Caspian sea regions, is the origin of important grapevine varieties. Worldwide, several phytoplasmas are affecting grapevine: aster yellows (group 16SrI), elm yellows (group 16SrV) and "stolbur" (group 16SrXII-A), together with phytoplasmas belonging to 16SrII, 16SrIII, 16SrVII and 16SrX groups are causing serious losses (Angelini, 2010; Constable, 2010). "Bois noir" (BN) and "flavescence dorée" (FD) together with Australian and American grapevine yellows are known as grapevine yellows phytoplasmas (GY) and induce similar symptoms on the leaves such as severe redness of red varieties and irregular yellowing of white varieties, with backward curling, lack of lignification and shriveling of berries, followed by early drying of the whole cluster. "Bois noir" causes significant reduction in yields in many European countries (Angelini, 2010) and in Turkey neighboring countries (Avramov *et al*., 2008; Karimi *et al*., 2009). This research was conducted to determine the reactions of local and commonly grown grapevine varieties in Turkey to BN phytoplasma infection.

Materials and Methods

BN infected canes were obtained from the vineyards located in Thrace (Krklaeli) and Manisa provinces. Emir, Chardonnay, Syrah, Sauvignon Blanc, Alicante Bouchet were obtained from Manisa and Merlot, Malbec, Cabernet Sauvignon were obtained from Krklareli provinces. Virus and phytoplasma free rootstocks were propagated in the greenhouses of the Horticulture Department of Ankara University (Table 1). BN infected cuttings were omega grafted to a set of pathogen-free canes in April 2012 and kept under greenhouse. One bud was left alive on each part and the others were removed. Five plants were grafted for each combination. Observations of symptom expressions and nested-PCR detections of phytoplasmas were carried out by the end of September.

Nucleic acids were extracted from the grapevine leaf ribs using a chloroform/phenol protocol. Direct PCR assays with P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by nested-PCR with R16F2n/R2 (Gundersen and Lee, 1996), R16(I)F1/R1 and M1/M2 (Lee *et al*., 1994; Gibb *et al*., 1995) primer pairs were carried out. Positive controls were DNAs of "stolbur" (16SrXII-A). DNAs non-symtomatic Kalecik Karas leaves were used as negative controls.

Results and Discussion

Symptoms were evident only in the some of the combinations (Table 1). There was no symptom development on of the combinations with BN infected Chardonnay, Sauvignon Blanc, Merlot and Cabernet Sauvignon but in some of them, reddening on leaves of local and international grapevine varieties was present. Chlorosis on leaves of Emir-C. Sauvignon combination were also evident. When DNAs of those combinations were subjected to PCR and nested PCR amplification sharp 500 bp bands were obtained (Figure 1). The Turkish local varieties Sultani seedless, Öküzgözü and Çal Karası with A. Bouchet, Merlot and A. Lavallée were succeptible to phytoplasma infection and show typical BN symptoms. The other varieties, Kalecik Karas, Bogazkere, Bornova Misketi seemed resistant since no transmission and symptom occurred. It was however surprising that the well known susceptible C. Sauvignon could only be poorly infected

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Table 1. Reactions of some grapevine varieties to BN grafting.

Cane (BN infected)	Varieties	Tranmission	Symptoms
Emir	Kalecik Karasý	0/5	No symptom
	Bogazkere	0/5	No symptom
	Bornova Misketi	0/5	No symptom
	Sultani seedless	0/5	No symptom
	Öküzgözü	0/5	No symptom
	Çal Karasl	0/5	No symptom
	C. Sauvignon	2/5	Chlorosis on leaves
	Alicante Bouchet	0/5	No symptom
	Merlot	0/5	No symptom
	Alphonse Lavallée	0/5	No symptom
Syrah	Kalecik Karasl	0/5	No symptom
	Bogazkere	0/5	No symptom
	Bornova Misketi	0/5	No symptom
	Sultani seedless	0/5	No symptom
	Öküzgözü	0/5	No symptom
	Çal Karasl	0/5	No symptom
	C. Sauvignon	0/5	No symptom
	Alicante Bouchet	5/5	Severe reddening
	Merlot	5/5	Severe reddening
	Alphonse Lavallée	0/5	No symptom
A. Bouchet	Kalecik karasl	0/5	No symptom
	Bogazkere	0/5	No symptom
	Bornova Misketi	0/5	No symptom
	Sultani seedless	5/5	Severe reddening
	Öküzgözü	5/5	Severe reddening
	Çal Karasl	5/5	Severe reddening
	C. Sauvignon	0/5	No symptom
	Alicante Bouchet	5/5	Severe reddening
	Merlot	5/5	Severe reddening
	Alphonse Lavallée	0/5	No symptom
Malbec	Kalecik Karasl	0/5	No symptom
	Bogazkere	0/5	No symptom
	Bornova Misketi	0/5	No symptom
	Sultani seedless	0/5	No symptom
	Öküzgözü	0/5	No symptom
	Çal Karasl	0/5	No symptom
	C. Sauvignon	0/5	No symptom
	Alicante Bouchet	0/5	No symptom
	Merlot	0/5	No symptom
	Alphonse Lavallée	5/5	Severe reddening

Figure 1. On the left nested PCR results with M1/M2 primers. 1-2: C. Sauvignon+ C. Franc, 3-4: C. Sauvignon+Chardonnay, 5-6: C. Sauvignon+ Öküzgözü, 7: A. Bouchet+ Öküzgözü , M: 100 bp plus DNA ladder. On the right BN symptoms on Öküzgözü grafted on A. Bouchet.

in only one grafting combination. Further experiments must be conducted to confirm the resistance of Turkish varieties to BN disease.

Acknowledgements

We are gratefull to Tubitak for supporting part of this research with Project 1090005 and COST Project FA0807.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S111-S112

doi: 10.5958/2249-4677.2015.00047.X

Introduction of beneficial bacteria to grapevines as a possible control of phytoplasma associated diseases

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Abstract

The possible role of endophytes as bio-agents for controlling yellows diseases was studied in grapevines and in periwinkle plants. Bacterial isolates from grapevines and insect vector of phytoplasmas were tested. Isolates that showed inhibitory effect in a spiroplasma model system *in vitro* were identified as various *Bacilli* and *Xanthomonadaceae* and further investigated. The isolates were successfully introduced via root dip, stem injection and smeared pricked leaf into *ex vitro* plantlets and potted plants. Further study is in progress to examine whether one of these isolates can be used as a bio-control agent against phytoplasmas.

Keywords: endophytes, periwinkle, *Spiroplasma*

Introduction

The control of phloem restricted pathogens needs new strategies given that conventional application of chemical spray is inefficient. The role of endophytes as a possible control tool against these pathogens has been suggested. However, in order to proceed towards practical application, such a microorganism should be cultivable and able to penetrate and survive within the plant for a reasonable time. Since phytoplasma is transferred by phloem feeding insects, the vectors as well as the host plants, may serve as reservoirs of beneficial micro-organisms. The objectives of the study were to look for cultivable bacteria that may serve as a potential bio-control agent and examine methods of introducing them back into the plants.

Materials and Methods

Bacterial endophytes were isolated from phytoplasma infected, recovered and healthy Cabernet-Sauvignon grapevines and from desert grapevines by placing surface-sterilized discs from canes on nutrient agar medium. Bacterial endosymbionts were isolated from surface-sterilized specimens of the insect vectors. Biolog and DNA sequencing of the *16S rRNA* gene were used to identify the endophytes. Specific isolates were characterized based on colony morphology formed on potato dextrose agar and molecular pattern formed by BOX-PCR analysis (Mohapatra *et al*., 2007). The inhibition activity of the endophyte medium filtrate and medium crude extract of the various isolates was tested against a model system of *Spiroplasma melliferum* (Naor *et al*., 2011). The isolates were introduced to grapevine and periwinkle plants either healthy or infected with yellows disease. Various plant materials were used: *ex vitro* plantlets of both species, periwinkle seedlings at 3-4 leaves stage, one-node cuttings of dormant grapevine canes that were forced to sprout following vernalization for 6 weeks and 3 years old potted grapevines. Introducing methods included root dip, dip of basipetal end of stem cutting, injection to the stem and to the cluster, leaf-dip and smear of a pricked leaf (Figure 1). To confirm the presence of the isolates, 7-20 days post inoculation (PI) BOX-PCR, colony morphology and species-specific PCR analyses were used. In addition FISH staining to locate the vector symbiont within the plant tissue was employed. The changes in the plant morphology, following the isolate introduction, were monitored up to 8 weeks PI.

Results

Out of 416 endophytes that were isolated from grapevines and from the insect vector, nine (A-H and X) were chosen for further study (Figure 2). The chosen endophytes showed a stable inhibition activity *in vitro* in the *S. melliferum* model system. Furthermore, crude extracts of the growth medium from isolates B, G and X inhibited the growth of *S. melliferum* in a model system *in vitro*. The endophytes (A-H) were identified as *Bacilli* and the vector symbiont (X) was found as a member of the family *Xanthomonadaceae* (Figure 2). Isolates C, D, H and X were successfully introduced into grapevine and periwinkle plants and their presence in plant tissues was confirmed by REP-PCR or species specific PCR

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analysis, 8 to 20 days PI. Root-dip, stem injection and smearing a pricked leaf were found to be effective methods of introducing the microorganisms into the plant tissue (Figure 1). No change in plant morphology was observed when the endophytes were introduced to healthy plants. Isolate X was found to be the most effective. Morphological parameters of yellows disease in both grapevine and periwinkle *ex vitro* plantlets were markedly reduced following introduction by either root dip or *via* leaf. FISH staining detected the cells of isolate X inside the plant phloem system.

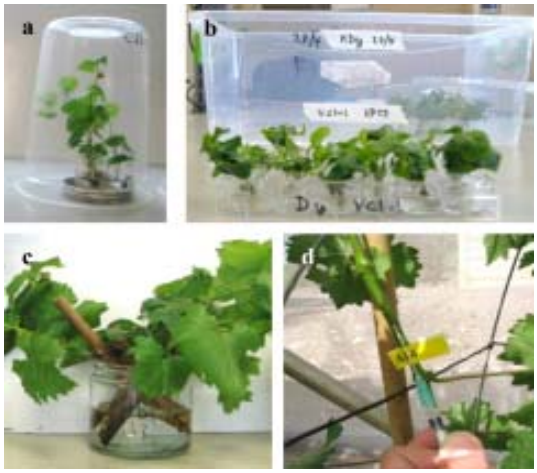

Figure 1. Methods of introduction: a. Root dip of *ex vitro* plantlets of grapevine; b. Root dip of *ex vitro* plantlet of periwinkle; c. Dip of basipetal end of sprouting stem cutting; d. Injection to 3 years old potted grapevine.

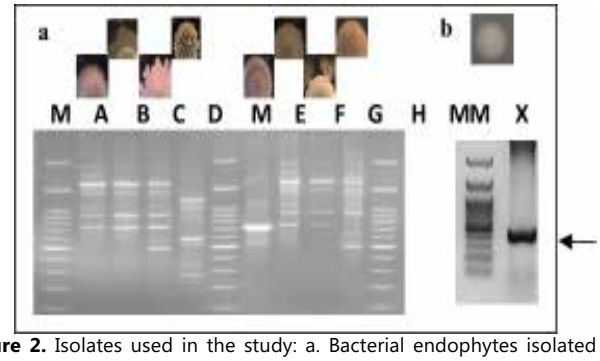

Figure 2. Isolates used in the study: a. Bacterial endophytes isolated from grapevines (A-H): colony morphology and the specific pattern revealed by BOX-PCR analysis of *16S rRNA* gene. b. Colony morphology and amplicon of 400 bp of the *16S rRNA* gene amplified by specific primers. M: molecular ladder of 100 bp.

Discussion

The fact that isolates from various biological sources show inhibitory effect in a model system provides a hint to the ability of such bacteria to affect the agent of yellows diseases and potentially other phloem-restricted plant pathogens. The results show that such bio-agents can be introduced into healthy and infected plants in various ways, which may be modified and applied based on agricultural needs. The reduction of yellows symptoms following the introduction of isolate X into phytoplasma infected plants indicates that this microorganism can be used as a bio-control agent against phytoplasma. Moreover, cells of isolate X were found inside the phloem which is not a common habitat of endophytes (Bulgari *et al.*, 2009) and its extracted medium inhibited the growth of *S. melliferum* suggesting that the mechanism of action involves the secretion of an inhibitory compound (Compant *et al.*, 2013). Since other isolates were also re-introduced into the plant, a variety of potential bio-control agents that needs to be examined were found. Further study is in progress to determine whether one of these isolates can also be used as a bio-control agent against phytoplasmas.

Acknowledgements

This research was partially funded by Research Grant No. CA-9110-09 from BARD, the United States-Israel Binational Agricultural Research and Development Fund, the Lady Davis Fellowship Trust, The Ministry of Science and the Ministry of Economy, Israel.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S113-S114

doi: 10.5958/2249-4677.2015.00048.1

Urtica dioica, main host plant of the "stolbur" phytoplasma in vineyards of La Rioja and Navarre (Spain)

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Abstract

The "bois noir" disease, associated with '*Candidatus* Phytoplasma solani' was detected in grapevine areas of La Rioja, Navarre, Aragon and Catalonia (Spain) since long time. The principal insect vector of the phytoplasma to grapevine, *Hyalesthes obsoletus*, was identified until 2010 in very low populations and always linked to the weed, *Convolvulus arvensis*. Due to the increase of the disease in Navarre and "La Rioja", during 2013 and 2014 individual *H. obsoletus* were captured from both the principal host plants of this insect, *C. arvensis* and *Urtica dioica*, in grapevine plots of "La Rioja" and Navarre. The results obtained in 2013 indicated that the populations of *H. obsoletus* in *U. dioica* were higher (7-30 individuals per aspiration) than those identified in *C. arvensis* (0.25-6 individuals per aspiration) in these regions, and that the peak of population occurred in *U. dioica* in mid-August and later in *C. arvensis*, in mid-July. The total number of individuals captured in Navarre in 2013 was 80, with 50% of the individuals being carriers of the phytoplasma. Similar results were obtained in 2014. These results indicate that *H. obsoletus* captured in *U. dioica* is very likely the main responsible of the disease transmission to the grapevines plots of "La Rioja" and Navarre.

Keywords: "bois noir" phytoplasma, epidemiology, detection, grapevine

Introduction

The "bois noir" disease of grapevine, associated with the presence of '*Candidatus* Phytoplasma solani' was identified for the first time in Spain in 1994, in vineyards of "La Rioja" Navarre, Aragon and Catalonia (Laviña *et al*., 1995). Since then different samplings have been carried out to determine the incidence of the disease and the presence of insect vectors. The incidence in the affected plots ranges between 3% and 75%, depending on the areas. The principal vector of the phytoplasma to grapevine, *Hyalesthes obsoletus*, was identified until 2010 in very low populations and always linked to *Convolvulus arvensis* presence. The average percentage of individuals carrying the phytoplasma was 55% (Sabaté *et al*., 2007; 2014).

PCR-RFLP analysis of sequences of the gene encoding the elongation factor Tu (*tuf* gene) distinguish three different "stolbur" types in *H. obsoletus* as well as in infected grapevines and wild host plants. Each type showed a specific association with an herbaceous host, tuf-type a is associated with stinging nettle (*Urtica dioica*), while tuf-type b is detected in bindweed (*C. arvensis*) as natural host and tuf-type c in *Callistegia sepium* (Langer and Maixner, 2004).

There was a high incidence of the disease in some plots of Navarre and "La Rioja" and the strain tuf-type a of "stolbur" was identified in the plants. This strain has not been previously identified in individuals of *H. obsoletus* captured, thus, new samplings were conducted in order to identify carriers of the strain tuf-type a.

Materials and Methods

During 2013 and 2014 individual *H. obsoletus* were captured in *C. arvensis* and *U. dioica*, in grapevine plots of "La Rioja", Navarre and Catalonia. The insects were captured weekly with a D-Vac aspirator from June to September. The "stolbur" genotypes in plants and insects were identified using PCR-RFLP analyses of *tuf* gene by nested-PCR with tuf1f/r primers in the first step and tuf AY primers in the second step (Langer and Maixner, 2004).

Results

The results obtained in 2013 indicated that the populations of *H. obsoletus* in *U. dioica* were higher (7-30 individuals per aspiration) than those identified in *C. arvensis* (0.25-6 individuals per aspiration) in these regions, and that the peak of population in *U. dioica* (middle of August), appeared later than in *C. arvensis* (middle of July) (Figure 1). The total number of individuals captured in Navarre in 2013 was 80, with 50% of the individuals being carriers of the phytoplasma (10% with strain tuf-type b and 90% with tuf-type a). In "La Rioja" all individuals (31) were carriers of the strain tuf-type a. Similar results were obtained in 2014. Until July a mean of 2 individuals was captured in Navarre while

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a mean of 17 individuals was captured on *U. dioica* (Figure 2). In 2014 in Catalonia the first *H. obsoletus* were obtained earlier than in 2013, due probably to the warm temperatures in spring 2014 (Figure 2).

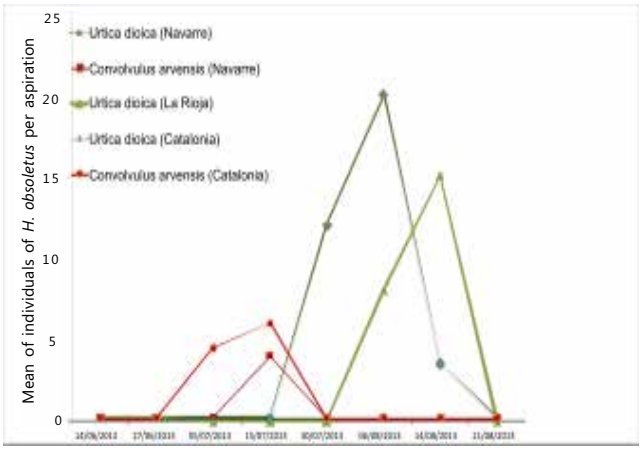

Figure 1. Population dynamics of *H. obsoletus* in *U. dioica* and in *C. arvensis* in Navarre, "La Rioja" and Catalonia in 2013.

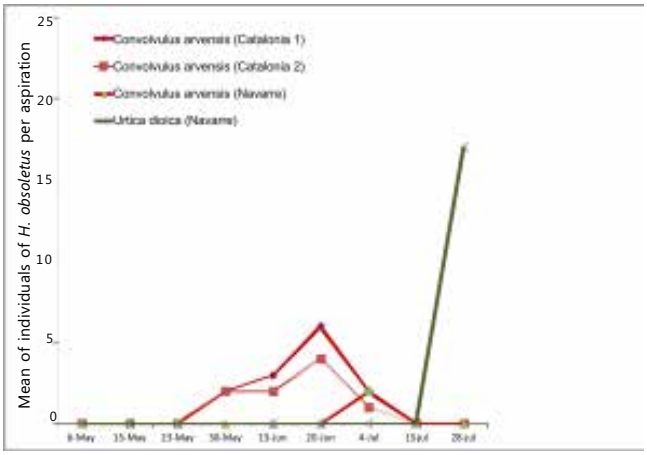

Figure 2. Population evolution of *H. obsoletus* in *U. dioica* and in *C. arvensis* in Navarre, "La Rioja" and Catalonia until July 2014.

Discussion

The results indicate that the *H. obsoletus* captured on *U. dioica* are very likely the main responsible of the transmission of the tuf-type a "stolbur" strains to grapevine and therefore from the disease to the affected grapevines of "La Rioja" and Navarre.

In Spain, the phytoplasma was detected mainly in bindweed which is one of the major natural reservoirs in vineyards and plays an important epidemiological role in insect vector maintenance in the environment. Nevertheless, the increase in the spread of this disease observed in "La Rioja" and Navarre was associated with the tuf-type a, usually detected in *U. dioica*.

Acknowledgements

We would like to thank Ana Sagües from EVENA (Navarra) for the help in conducting the surveys. This work was funded by grant RTA11-067-C4 of the Programa Sectorial de I+D, M.A.P.A., Spain.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S115-S116

doi: 10.5958/2249-4677.2015.00049.3

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Detection of 16SrIII-J phytoplasma in *Galega officinalis* L., a weed commonly associated to pome fruit orchards in Chile

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Abstract

In a pear orchard of variety d'Anjou located in Maule Region (Chile) several *Galega officinalis* L. plants exhibited yellowing leaves. Nested polymerase chain reaction assays, using phytoplasma universal primer pairs P1/P7 and R16F2n/R2 detected phytoplasmas in 100% of symptomatic samples. Restriction fragment length polymorphism analyses, cloning, and sequencing allowed identification of a phytoplasma belonging to ribosomal subgroup 16SrIII-J. This phytoplasma represents a new finding in *G. officinalis* in Chile. The leaf hopper *Paratanus exitiosus*, vector of 16SrIII-J phytoplasma, is largely distributed in the country, associated to different weed species and crops of agronomic interest confirming the potential epidemiological importance of this phytoplasma in Chile.

Keywords: *Galega officinalis*, nested-PCR, *16S rRNA* gene, RFLP, sequencing

Introduction

Pome fruits are very important crops in Chile, known to be the main apple exporter in the world (ODEPA, 2013). As every widely cultivated crop, apples and pear are under a high risk of dissemination of plant diseases. Among the wide number of pathogens that can affect pome fruit trees, phytoplasmas are of a great concern because of the absence of effective field management strategies. Pear decline (PD) and apple proliferation (AP) are severe diseases that affect pome fruits and are associated with the presence of '*Candidatus* Phytoplasma pyri' and '*Ca.* P. mali', respectively (Seemüller and Schneider, 2004). In Chile, none of these pathogens have been reported so far.

In 2011, the presence of 16SrI-B, 16SrVII-A and 16SrXII-A phytoplasmas in *Convolvulus arvensis* L., *Polygonum aviculare* L. and *Galega officinalis* L. was reported, these weeds were located in or near phytoplasma infected Chilean vineyards (Longone *et al*., 2011). Those vineyards were positive to the same phytoplasmas, so the authors concluded that these weeds are important phytoplasma reservoirs. This is a relevant finding because these weeds are also widely present in pome fruit orchards. The data reported for vineyards prompted us to investigate whether these weeds represent a risk of phytoplasma infection for pome fruit trees as well.

Materials and Methods

In the southern hemisphere, during fall of 2014, different pome fruit orchards were visited to collect samples for phytoplasma detection. On a d'Anjou pear (*Pyrus communis* L.) orchard located in Maule Region, several *G. officinalis* L. plants exhibited yellowing leaves. Five symptomatic and two asymptomatic plants were collected and employed for phytoplasma analysis. Total nucleic acids were extracted with a chloroform/phenol based method, dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/µl of nucleic acid were used for amplification. Direct PCR with primer pair P1/P7 and nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) were performed following published protocols (Schaff *et al*., 1992). Amplicons from nested PCR assays were purified using Concert Rapid PCR Purification System and DNA fragments were cloned. Putative recombinant clones were analyzed by colony PCR. Selected fragments from cloned DNAs were sequenced in both directions in MacrogenUSA Corp. (Rockville, MD, USA). The sequences were then aligned with those of reference strains deposited in GenBank using BLAST engine for local alignment (version Blast N 2.2.12). Identification was done using *in silico* restriction fragment length polymorphism (RFLP) analyses with *Hha*I, *Bst*UI, and *Rsa*I restriction enzymes (Zhao *et al*., 2009).

Results

In all tested symptomatic samples the presence and identity of phytoplasmas was confirmed by PCR, *in silico* RFLP and sequence analysis. Products of expected length were obtained in R16F2n/R2 PCR assays (1,250 bp). The samples showed a distinctive profile in RFLP analyses, corresponding to phytoplasmas belonging to 16SrIII-J ribosomal subgroup. Phytoplasma identity was also confirmed with BLAST analysis since sequences from all strains showed 99.8% of nucleotide identity with 16SrIII-J phytoplasmas found in *Amplicephalus curtulus* from Chile (KC834073), *Solanum*

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lycopersicum L. from Argentina (KC412031), and *Delphinium* sp. from the UK (EF514210). No symptoms referable to possible phytoplasma presence have been observed in pear trees of the variety d'Anjou.

Discussion

The 16SrIII-J phytoplasmas are widely distributed in South America in herbaceous hosts like garlic, tomato, squash, sunflower and eggplant (Galdeano *et al*., 2004; 2013; Amaral Mello *et al*., 2011; Guzmán *et al*., 2014) and woody hosts like sweet cherry and grapevine (Gonzalez *et al*., 2010; 2011). Moreover, Fiore *et al*., (2012) demonstrated that the leafhopper *Paratanus exitiosus* (Beamer) was able to transmit the 16SrIII-J phytoplasma. Considering that *P. exitiosus* is largely distributed in Chile, associated to different weed species and crops of agronomic interest (Castro *et al*., 2000; Hepp and Vargas, 2002; González *et al*., 2010; 2011; Longone *et al*., 2011; Fiore *et al*., 2012), there is a high risk of infection of pear trees by 16SrIII-J phytoplasma.

Acknowledgements

Research supported by National Fund for Scientific and Technological Development (FONDECYT), Chile, project N° 1140883.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S117-S118

doi: 10.5958/2249-4677.2015.00050.X

Abundance of the insect vector of two different *Mollicutes* plant pathogens in the vegetative maize cycle

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Abstract

The abundance of *Dalbulus maidis* leafhopper was evaluated in intervals of 20 days in the vegetative cycle of maize and popcorn hybrids simultaneously cultivated in three areas at four different sowing dates. The amount of leafhoppers increased from the first until the third sampling, around the flowering, and decreased after that. The highest amount of leafhoppers detected in the late sowings was attributed to effect of their concentration derived from oldest maize crops.

Keywords: *Dalbulus maidis*, phytoplasma, spiroplasma, *Zea mays*

Introduction

The maize production can be seriously damaged by diseases due to two *Mollicutes*: the maize bushy stunt phytoplasma (MBS) and the corn stunt spiroplasma (Oliveira *et al*., 2013a). The agents of these diseases, respectively, MBS phytoplasma and *Spiroplasma kunkelii*, are both transmitted by the leafhopper *Dalbulus maidis* in a persistent manner (Oliveira *et al*., 2007). In Brazil, maize is the only host of these *Mollicutes* and of their insect vector. The insect vector uses the migration as main surviving strategy (Oliveira *et al*., 2013b). It leaves the senescent maize crops and colonizes new young maize crops that are sometimes separated by large distances. It acts as storage of the *Mollicutes* during the off-maize season and when colonizing new young maize it can transmit the pathogens. There is little information about which factors are determining corn stunt disease outbreaks but, certainly, the population of the insect vector is an essential factor. Knowledge about the spreading of *D. maidis* in maize can help to develop models to forecast outbreaks of these diseases and strategies for their control. The goal of this study was to evaluate the abundance of *D. maidis* during the vegetative maize cycle, to understand aspects of the multiplication and migration of the insect vector in spreading the two *Mollicutes* and its epidemiological role.

Materials and Methods

For experimental evaluations, one maize hybrid and one popcorn hybrid were sowed four times at different dates, during two years, in three different areas (areas 1, 2, 3) at Embrapa's experimental station in Sete Lagoas, MG, Brazil. The area 1 was distant 1,600 m from area 2 that was distant 1,050 m from area 3, and the area 3 was 2,650 m distant from area 1. The areas 1 and 2 were close to other maize crops, having variable density and ages. The area 3 was always surrounded only by grasses and at least 1,000 m distant from other maize plantations. The incidence of *D. maidis* was evaluated in each maize vegetative cycle in intervals of 20 days starting from the sowing date, considering that the biological cycle of the leafhopper from egg to adult is about 20 days (Marín, 1987). For each sampling, in each area, one sample was collected from maize hybrid and another from popcorn hybrid and each sampling consisted of three insects collected with 30 sweep net movements between two 10 m long plant rows. The insects collected were transferred to plastic bags, killed by freezing and *D. maidis* were counted and preserved in 70% ethanol. The data were submitted to statistical analysis of variance (Montgomery, 2001) using the software R (R Core Team, 2014).

Results

D. maidis was detected in maize and popcorn hybrids in all areas, at all sowing dates, since the first sampling. There was no statistical difference among the three areas regarding the amount of collected leafhoppers and there was no difference between the amount of collected leafhoppers in maize and in popcorn. Thus, for each sampling date all data could be grouped. Significant differences of the amount of collected leafhoppers were observed for different sowing dates in the vegetative maize cycle, the highest ones were in the late sowing dates in February and April (Table 1).

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Table 1. Amount of *D. maidis* sampled during the vegetative maize cycle within intervals of 20 days after sowing, with two late sowing dates (February and April) and two early sowing dates (November and October), in two years. The data represent the total amount of insects captured on the respective date in three different areas on two different maize crops in Sete Lagoas, MG, Brazil.

Sowing date	Sampling								Amount
	1	2	3	4	5	6	7	8	
02/23/12	109	251	370	108	101	58	232	5	1,234 a
11/13/12	25	184	95	95	87	30	0	0	516 b
04/17/13	7	363	255	137	170	122	63	0	1117 a
10/22/13	23	201	172	103	36	10	0	0	545 b
Amount	164	998	892	443	394	220	295	5	3,411

Coefficient of variation (%) = 52.88. Averages followed by the same letter are not statistically different by the Tukey test (p value < 0.05)

Discussion

The results showed that maize age affects the multiplication and migration of *D. maidis*. In the first sampling, maize seedlings were around 15 days after emerging, which is not long enough to permit the development of *D. maidis* from eggs to adults (Marín, 1987), indicating that collected leafhoppers came from another place. The increase in the number of leafhoppers until the third sampling, when plants were around flowering, probably include leafhoppers that still were immigrating and those already borne in that areas.

This insect vector is attracted and multiplies in maize before flowering and, after that, it starts migration to young maize plants. Thus, the population density is function of the amount of young maize crops in the field and, therefore, the variation in the sowing dates of these crops have an important influence on the vector abundance. In particular the late sowing during the maize growing season can expose the maize seedlings to high populations of this insect. Maize sowing in February and April are late sowings (or sowings of the second maize growing season in Brazil) and the high populations of *D. maidis* found in these crops confirmed this hypothesis. Also, it is important to mark that, in the first sowing date in the area 1, it was observed a surprising increase in the number of collected leafhoppers at the end of the maize cycle, while the next sampling, the number of leafhoppers was very low, indicating that they migrate (Table 1). This concentration effect was due to the harvest of the maize crops close to that area. The results indicate that concentration effects of *D. maidis* leafhopper populations can occur in function of the maize sowing date, e.g. due to the increase of young maize plantations and, consequently, the increase of the number of migrating insects after flowering, or due to the maize crops close to harvest in a given area. These concentration effects can explain "peaks" of *D. maidis* populations in different months as reported by Arce and Ávila (2008). Then, it is probable that outbreaks of the corn stunt diseases is a consequence of the *D. maidis* concentration and the coincidence of favorable environmental conditions for the development of the *Mollicutes* in the insect vector and in the maize host plant. These conditions can vary among the different regions.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S119-S120

doi: 10.5958/2249-4677.2015.00051.1

Status of sesame phyllody and its control methods in Yazd, Iran

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Abstract

Phyllody is one of the most important diseases of sesame in Iran. During 2008-10, to evaluate disease status, a survey was carried out in sesame fields in Yazd province. Occurrence of sesame phyllody was observed in all the sesame growing fields in Yazd, but whole infection of fields was observed in Rezvan Shahr (Ashkezar, Yazd province). On the basis of RFLP analysis sesame phyllody phytoplasma was identified as a member of peanut witches' broom (16SrII) group. No differences were observed in plant heights of infected and healthy plants. Total seed yield was reduced by 55.07% in infected plants and 1,000 seed weight showed a 21.49% decrease in infected plants. Wrinkled seeds increased in diseased plants up to 56.07%. Results of variance analysis of control methods showed that sowing date and spraying have significant effects on infection. Mean comparison of infection percent with LSD tests showed that delay in sowing date reduced sesame phyllody up to 31%. Spraying with Confidor reduced disease incidence at the first and second sowing date by 18.8 and 7.8% respectively but no differences were observed in yields. Seed treatment with Gaucho has no effect on disease incidence. Collectively, sowing of sesame straight after wheat harvesting in May needed spraying to reduce disease incidence, but delay in the sowing date to July 5 can reduce disease incidence without any significant differences in seed yield. Delay in sowing date is dependent on weather and needs to be determined for each area.

Keywords: *Sesamum indicum* L., phyllody disease, phytoplasma, PCR, RFLP, control

Introduction

Sesame (*Sesamum indicum* L.) is cultivated in different parts of Iran especially in the tropical and subtropical regions and phyllody is one of its most important diseases. The disease incidence varies in different regions of the country and the prevalent symptoms are virescence, phyllody, yellowing, flower sterility and stem proliferation. Different leafhoppers have been reported as vectors of sesame phyllody in the world (Kersting, 1993; Klein, 1997, Schneider *et al*., 1995a). In Iran, the leafhopper *Neoaliturus haematoceps* transmits the phytoplasma associated with sesame phyllody in Fars province, Iran (Salehi and Izadpanah, 1991) but in Yazd province, *Orosiuis albicinctus* L. was reported as vector (Esmailzadeh Hosseini *et al*., 2007). Under greenhouse condition sesame phyllody was transmitted to garden beet (*Beta vulgaris* subsp. *esculenta*), sesame (*Sesamum indicum*) and garden cress (*Lepidium sativum*) (Esmailzadeh Hosseini *et al*., 2007). The aims of the present work were to investigate the status of sesame phyllody in Yazd area and the evaluation of its control methods.

Materials and Methods

During 2008-2010 a survey was carried out at a sesame plantation in Yazd province and plant height, total seed yields, 1,000 seed weight and wrinkled seeds were compared in healthy and diseased plants. To evaluate sowing date, insecticide spraying and seed treatment on sesame phyllody incidence, a field experiment was arranged as factorial based on randomized complete blocks design with three replicates in Rezvan Shahr (Ashkezar, Yazd province). The sesame were grown in 30 m plots, 1.5 m apart with three replicates and each plot contained 6 rows. Experimental factors were sowing date at two times (June 10 and July 5), spraying with insecticide Confidor SC 35% (0.3/1000) and control and seed treatment Gaucho 70% WS (3/1000) and control. To determine incidence of sesame phyllody two 1 m lines were harvested from each row.

Infected plants were separated from healthy based on characteristic disease symptoms and direct and nested PCR using P1/P7 and R16F2n/R16R2 universal primers. Samples were collected from various sesame fields.

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Figure 1. A view of infected fields (right) compared to healthy (left).

Total DNA was extracted from 1 g of midrib tissue of infected plants (Zhang *et al*., 1998) and subjected to direct PCR using P1/P7 (Schneider *et al*., 1995b) and nested PCR using primer pairs P1/P7 and R16F2n/R16R2 (Gundersen and Lee, 1996). Each 25 µl PCR reaction mix contained 100 ng of total DNA, 2.5 µl 10 X PCR buffer, 0.8 U *Taq* polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.4 µM each primer. PCR was performed for 35 cycles using the following conditions: 1 minute (2 minutes for the first cycle) denaturation step at 94°C, 2 minutes for annealing at 55°C and 3 minutes (10 minutes for the last cycle) at 72°C for primer extension. A negative control, devoid of DNA template in the reaction mix, was included in all PCR assays. The PCR products (5 µl) were electrophoresed through a 1% agarose gel, stained by RedSafe™, and visualized with a UV transilluminator. RFLP analysis of direct PCR products (1.8 kb) was carried out and comparison of RFLP profiles with published ones (Lee *et al.,* 1998) was used for identification of the phytoplasma detected. The PCR products were digested with the restriction enzymes *Alu*I, *Hin*fI, *Hae*III, *Mse*I and *Rsa*I.

Results and Discussion

Occurrence of sesame phyllody was observed in all the sesame growing fields in Yazd, but whole infection of fields was observed in Rezvan Shahr (Ashkezar, Yazd province). The main disease symptoms were virescence, phyllody, yellowing, flower sterility, stem proliferation and plants falling on the ground due to heavy weight caused by shoot proliferation (Figure 1). Direct and nested PCR assays using P1/P7 and R16F2n/R16R2 universal primer pairs amplified 1.8 and 1.2 kb fragments, respectively. RFLP profiles of 16S rDNA digested with five restriction enzymes showed that sesame phyllody phytoplasma in Yazd belonged to the 16SII group (peanut witches' broom phytoplasma). No differences were observed in plant heights of infected plant compared to healthy. Total kernel yields were reduced by 55.07% in infected plant and 1,000 kernel weight decreased by 21.49% in infected plants. Wrinkled seeds increased in diseased plants up to 56.07%. Results of analysis of variance showed that sowing date and spraying have significant effects on infection at 1% probability level. Mean comparison of infection percentage with LSD tests showed that delay in sowing date reduced sesame phyllody up to 31%. Spraying with Confidor reduced disease incidence at the first and second sowing date by 18.8% and 7.8% respectively, but no differences were observed in seed yield. Seed treatment with Gaucho had no effect on disease incidence. Collectively, sowing sesame straight after wheat harvesting in May, needed spraying to reduce disease incidence, but delay alone in sowing date reduced disease incidence without producing significant differences in seed yield. Delay in sowing date is dependent on weather and needs to be determined for each area.

Acknowledgements

This work was a part of the research project No. 88074 supported by Agricultural Research, Education and Extension Organization (AREEO) of Iran.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S121-S122

doi: 10.5958/2249-4677.2015.00052.3

Molecular study of the effect of exogenous phytohormones application in "stolbur" phytoplasma infected tomatoes on disease development

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Abstract

'*Candidatus* Phytoplasma solani' alters developmental processes in tomato and cause malformations of both vegetative and reproductive organs. The study was performed to determine the effect of application of phytohormones on "stolbur" strain PO infected tomato and to assess whether application of BTH, an analogue of salicylic acid (SA), jasmonic acid (JA) and ethylen (Et) could protect tomato against the "stolbur" disease development. Expression of SA- and JA-dependent marker genes was also studied in tomato by qRT-PCR. Results indicated that the SA-mediated defence response delay the "stolbur" phytoplasma strain PO multiplication in contrast to the JA and Et-dependent defence pathways.

Keywords: qRT-PCR, "stolbur" phytoplasma, phytohormones, marker genes, defense pathways

Introduction

Phytoplasmas are phytopathogenic wall-less bacteria restricted to phloem sieve tubes and are naturally transmitted between plants by sap-sucking insects belonging to the families Cicadellidae (leafhoppers) and Fulgoridae (planthoppers) in which they multiply. They induce plant disorders such as leaf yellowing, growth aberrations and flower malformations and are reported as associated with more than 300 different diseases in more than 700 different plant species including fruit trees, vegetables and ornamentals worldwide (Bertaccini, 2007). The "stolbur" phytoplasma infects several economically important plant species including solanaceous crops (potato, tobacco and tomato). In "stolbur" PO phytoplasma infected tomato, floral abnormalities are associated with changes in expression of flower developmental genes (Pracros *et al*., 2006, Ahmad *et al*., 2013). The antagonistic interaction and synergistic defence pathways have been described (Spoel *et al*., 2007), and the latter are generally monitored through expression of genes such as acidic PR1, basic PR1, and PIN2, which are specifically related to salicylic acid (SA)-, ethylene (ET)-, and jasmonic acid (JA)-dependent pathways, respectively (Block *et al*., 2005). However, it is not known if the plant defense pathways are activated in response to the infection. The three major plant defense pathways involve SA, ET and JA. Here, the expression of marker genes of these pathways was studied in tomato infected with "stolbur" phytoplasma strain PO. Defence stimulators BTH (analogue of SA) and MeJA were used to determine whether pre-activation of SA- or JA pathways would reduce the phytoplasma load and the disease symptoms of "stolbur" PO-infected tomato.

Materials and Methods

Tomato (*Solanum lycopersicum* cv Ailsa Craig) plants were infected with the "stolbur" phytoplasma strain PO (Stol-PO) which belongs to the 16SrXII-A subgroup. For the experiments, two-month-old tomatoes were inoculated with "stolbur" phytoplasmas PO by side-grafting. Control plants were grafted with healthy scions. RNA was extracted from tomato leaves with Tri reagent (SIGMA). RT-PCR was done with primers specific for each gene studied. For each RT tube, 1 µg of DNA, 0.55 µM of an oligodT18, 0.01 M DTT, 2.2 mM dNTP, 280 U of RNase OUT (Invitrogen) were mixed in a final volume of 19 µl. After 5 minutes at 65°C and 5 minutes at 45°C, 1 µl (200 U) of Superscript II reverse transcriptase (Invitrogen) was added and incubated for 1 hour. Five minutes at 75°C allowed the enzyme denaturation. PCR were done with 1 µl of cDNA, 0.35 mM $MgCl_2$, 0.9 mM dNTP, 2 µg/ml BSA, 4.4 µM each primer (forward and reverse) and 2.2 U Taq DNA polymerase (Promega), in a final volume of 25 µl. RT-PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The intensity of the bands was measured with a FluorS and the associated software Quantity One (Biorad). All samples were amplified in triplicate from three distinct RNA batches extracted from distinct tomato samples. DNA extracted from samples was amplified for phytoplasma detection by using universal primers for nested PCR assays according to Gundersen and Lee (1996).

Results and Discussion

The qPCR results (not shown) indicated that SA pathway induced by BTH application up-regulated PR1 and down-

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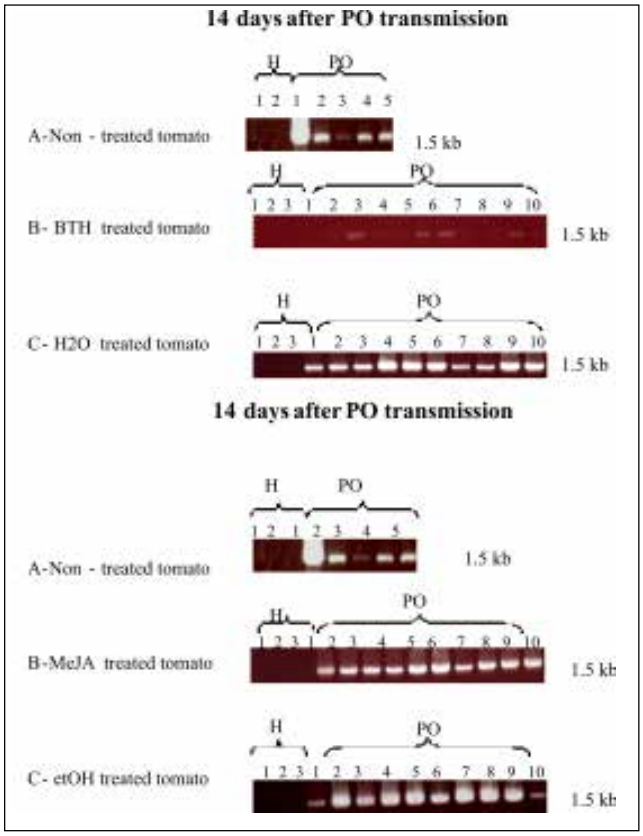

Figure 1. "Stolbur" phytoplasma detection through nested-PCR in tomato plants after treatment with BTH (analogue of SA) or MeJA (analogue of JA).

regulated PIN2 genes expression consistent with the known antagonistic interactions between SA and JA signalling pathways. This confirmed that the SA dependent defence pathway is activated in the SA or BTH-treated tomatoes and delayed the multiplication of phytoplasma, as faint amplified bands and less symptomatic plants were observed as compared to non-treated or control infected plants at 14 days post-grafting (Figure 1), however this effect is no longer observed 21 days post-grafting. Treatment of tomato with MeJA had no effect on disease symptom expression. Indeed, "stolbur" PO symptoms in the MeJA-treated plants were identical to those in the control plants and PCR detection of the phytoplasma in "stolbur" PO-inoculated plants also revealed that treatment by MeJA did not reduce multiplication of the bacteria, as DNA from the treated plants yielded strong signals similar to those of the non-treated plants. With the aim to control "stolbur" disease in tomato we showed that pre-treatment of plants with BTH before inoculation temporarily reduced the multiplication rate of the "stolbur" PO phytoplasma and delayed symptom development (Ahmad *et al.*, 2014) (Figure 2). Together with the results of Bressan and Purcell (2005) where BTH treatment reduced by approximately 50% the transmission rate of the X disease phytoplasma to *Arabidopsis* by its

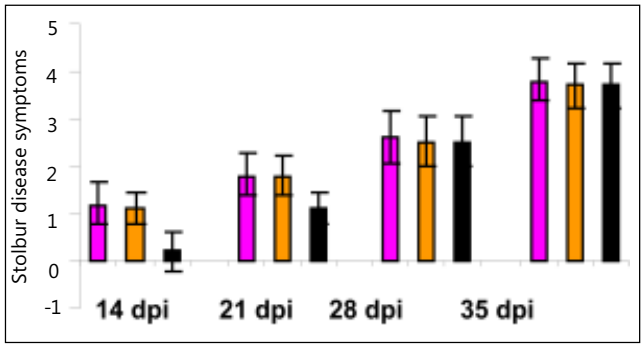

Figure 2. Symptoms observation after application of BTH (analogue of SA) on "stolbur" infected tomato. Pink: no treatment, orange: water treatment, black: BTH treatment. Symptom scale from 0 (no symptom) to 5 (dead plant).

insect vector *Colladonus montanus*, our results suggested that a combination of BTH treatment with chemical control of the leafhopper vector population by limited insecticide treatments is a promising approach to control phytoplasma disease development.

Acknowledgements

JNA received a grant from Higher Education Commission of Pakistan through SFERE. This work was done in INRA France. We thank Samina Jam Nazeer Ahmad for helping to improve the manuscript.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S123-S124

doi: 10.5958/2249-4677.2015.00053.5

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Transcriptional profiling in infected and recovered grapevine plant responses to '*Candidatus* Phytoplasma solani'

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Abstract

The infection of grapevine plants of cultivar Chardonnay with '*Candidatus* Phytoplasma solani', the agent associated with grapevine "bois noir", and the recovery from this disease were followed at the transcriptional level. The analysis showed that recovered plants behaved similar as healthy plants and revealed some, thus far unexplored, genes as important indicators of "bois noir" phytoplasma presence.

Keywords: "bois noir", gene expression, recovery

Introduction

"Bois noir" (BN), associated with the presence of phytoplasma of the "stolbur" group 16SrXII '*Candidatus* Phytoplasma solani' (Quaglino *et al*., 2013), is the most widespread grapevine yellows disease in Europe. Besides the limited knowledge about interactions between the host and the pathogen involved in BN, equally unclear remains the recovery phenomenon as a spontaneous remission of symptoms from previously symptomatic plants (Osler *et al*., 1993). Several recent studies have shown that '*Ca*. P. solani' induces significant reprogramming of grapevine genes involved in carbohydrate metabolism, photosynthesis, secondary metabolism and that their expression differs in healthy, diseased and recovered plants (Hren *et al*., 2009; Landi and Romanazzi, 2011; Santi *et al*., 2013). Here some additional changes during the development of BN disease at the transcriptional level are described.

Materials and Methods

A two year trial was carried out in an experimental vineyard of cultivar Chardonnay located in Northern Italy. Plants that were naturally infected with '*Ca*. P. solani' and uninfected plants were included. Some of the infected plants were partially uprooted at the end of the first growing season to induce recovery (Romanazzi and Murolo, 2008). Sampling (Table 1) and gene expression studies were done as described (Hren *et al*., 2009). Plants were sampled twice in the second year of the trial. Genes were selected according to a previous study and their expression was determined by qPCR in midribs of sampled leaves essentially as described (Hren *et al*., 2009). Analyzed gene were: genes involved in sugar metabolism (*VvInv2* encoding vacuolar acid invertase 2, *VvAgpL* encoding large subunit of ADP-glucose pyrophosphorylase, *VvSuSy* encoding sucrose synthase, *VvCaSy2* encoding callose synthase); genes with possible roles in signaling – *VvLox* encoding lipoxygenase, *VvSamt* encoding S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase; a gene involved in secondary metabolism – *VvLdox* encoding leucoanthoc-yanidin dioxygenase in the anthocyanin and proanthoc-yanidin biosynthetic pathways; pathogenesis-related proteins (*VvOlp* encoding osmotin protein, three genes encoding three different β-1,3-glucanases – *VvGlc1*, *VvGlc2*, *VvGlc3*); genes involved in oxidative stress (*VvGpx* encoding glutathione peroxidase, two genes encoding glutathione S-transferase *VvGst1* and *VvGst3*, *VvApx6* encoding ascorbate peroxidase); and *VvDMR6* with an unknown role.

Results

In plant response to the infection with '*Ca*. P. solani' several groups of genes were differentially expressed in uninfected, infected and recovered grapevines (Figure 1). There were no significant differences in gene expression between the samples of spontaneously recovered plants and samples of plants in which the recovery was induced by up rooting (data not shown).

Table 1. Sanitary status of plants in the second year of the trial and number of samples included in the analysis. '*Ca*. P. solani' presence was confirmed by qPCR (Hren *et al*., 2007).

Sanitary status of plant	No. of samples	Detection of phytoplasma
Healthy	3[a] / 3[b]	–[a] / –[b]
Spontaneously recovered	4[a] / 4[b]	–[a] / –[b]
Recovered upon uprooting	10[a] / 10[b]	–[a] / –[b]
Infected	10[a] / 10[b]	–[a] / +[b]

[a]spring sampling
[b]late summer sampling

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In spring sampling the only significant differences in expression were that of *VvDrm6* and *VvGlc2* genes. Their transcript abundance was significantly larger in the infected samples (data not shown). In the late summer samples, most of detected gene expressions were significantly induced in the infected samples and only the expression of *VvApx6* gene was lower. However, the gene expression of *VvCaSy2*, *VvGpx* and *VvGst3* genes was similar in healthy and infected samples. Comparison of the transcript abundances between healthy and recovered samples revealed the up-regulation of *VvDrm6*, *VvOlp* and *VvGlc2* genes. On the other hand, similar comparison between infected and recovered plants showed the pattern similar to the one obtained with the comparison between healthy and infected plants (Figure 1).

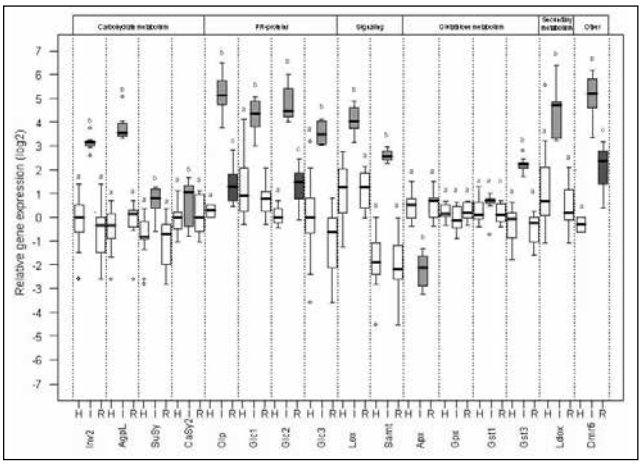

Figure 1. Boxplots showing relative expression ratios (log2 transformed) of genes normalized using 18S/Cox (geometric mean of 18S and Cox Cq values). Threshold for statistical in late summer samples from healthy (H), infected (I) and recovered (R) plants. Threshold for statistical significance between relative expression ratios of healthy and infected samples, and healthy and recovered samples is $P < 0{\cdot}05$ (Welch two sample t-test). Different letters denote significant difference.

Discussion

The expression of genes encoding vacuolar invertase, sucrose synthase, calose synthase and the large subunit of ADP-glucose pyrophosphorylase in infected and recovered samples was in agreement with other studies (Hren *et al.*, 2009; Santi *et al.*, 2013) confirming that during BN infection carbohydrate metabolism is affected. The interesting observation was the up-regulation of three *VvGlc* and *VvOlp* genes from the groups of pathogenesis related protein PR-2 and PR-5, respectively, and of *VvSamt*, which encodes S-adenosyl-L-methionine: salicylic acid carboxyl methyl-transferase in infected samples. The PR-2 and PR-5 genes are commonly used as molecular markers for salicylic acid (SA)-dependent systemic acquired resistance (SAR)-signaling and their expression is coordinately regulated by SA (Frías *et al.*, 2013). Therefore, these results suggest that '*Ca.* P. solani' induces SA-dependent SAR in leaves of infected grapevines. The recovered plants behaved more like healthy plants. The only exception was the increased gene expression of *VvDrm6*, *VvGlc2* and *VvOlp*. The first two were the only ones that were also up-regulated in infected plants in spring. *VvDrm6* encodes a 2OG-Fe(II) oxygenase and its biological role is unknown. However, it has been shown that arabidopsis plants lacking a functional *DMR6* gene have reduced susceptibility to downy mildew (van Damme *et al.*, 2008).

Acknowledgements

This work was financed by the Slovenian Research Agency (P4-0165; young researcher grant awarded to PN). Authors thank P. Ermacora for providing the grapevine samples.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S125-S126

doi: 10.5958/2249-4677.2015.00054.7

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Phytoplasma effector SAP11 altered phosphate starvation responses and root architecture in *Arabidopsis*

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Abstract

Phytoplasmas have the smallest genome among bacteria and lack many essential genes required for biosynthetic and metabolic functions, they are phloem-limited plant pathogens. In this study the transgenic arabidopsis expressing the secreted aster yellows phytoplasma strain witches' broom protein 11, shows an altered root architecture, similar to the symptoms of phytoplasma-infected plants, by forming hairy roots. This morphological change is paralleled by an accumulation of phosphate (Pi) and an increase in the expression levels of Pi starvation-induced genes and microRNAs. In addition to the Pi starvation responses, secreted aster yellows phytoplasma strain witches' broom protein 11 suppresses salicylic acid-mediated defense responses and enhances the growth of bacterial pathogens. These results contribute to an improved understanding of the role of phytoplasma effector SAP11 and provide new insights for understanding the molecular basis of plant-pathogen interactions.

Keywords: phytoplasma, SAP11, phosphate starvation response, root architecture

Introduction

Recent studies have shown that the protein 11 ($SAP11_{AYWB}$) secreted by the aster yellows phytoplasma strain witches' broom, contains a nuclear signal for targeting outside phloem cells (Bai *et al.*, 2009). Arabidopsis plants that overexpress $SAP11_{AYWB}$ display crinkled leaves that resemble transgenic plants overexpressing miR319 (Sugio *et al.*, 2011). Although $SAP11_{AYWB}$ has not been shown to interfere with microRNA (miRNA) expression, it has been reported that it can destabilize TCP transcription factors through direct interaction (Sugio *et al.*, 2011). In this study, the expression of phytoplasma effector $SAP11_{AYWB}$ in arabidopsis was shown to trigger Pi (phosphate) starvation responses. These findings advance the understanding of molecular mechanism underlying the disease symptoms elicited by the secreted effectors and provide new insights into the interaction between host plants and phytoplasmas.

Materials and Methods

Arabidopsis seeds were germinated on 1/2X MS medium with 1% (w/v) sucrose and 1% (w/v) agar. For root architecture investigation, seedlings were placed vertically to allow root growth along the surface of the agar, and the root architecture was observed at 14 days after germination. For anthocyanin measurement, seedlings were harvested at 13 days after germination, and the anthocyanin content was determined as described with modifications (Saijo *et al.*, 2009). Seedlings were homogenized in the extraction buffer [propanol: HCl: water (18: 1: 81)] and immersed into boiling water for 1.5 minutes. After centrifugation, the supernatant was collected for measuring the absorbance at 535 and 650 nm. The relative anthocyanin amount was calculated by the following equation: [A535 – (2 x A650)] / fresh weight (grams). Pi contents were measured according to the method described by Chiou *et al.* (2006) using Tecan Infinite 200 PRO at A820.

Results

To investigate whether Pi starvation responses were altered in *35S::SAP11*$_{AYWB}$ transgenic plants, the expression levels of Pi starvation-induced genes were examined using qRT-PCR. RNA samples were extracted from seedlings grown in 1/2X MS (Pi-sufficient) medium, and typical Pi starvation-induced genes were examined and all elicited in *35S::SAP11*$_{AYWB}$ transgenic plants, compared with wild-type plants (Figure 1A). To examine the levels of cellular Pi, arabidopsis seedlings grown in hydroponic solution were collected. A higher amount of Pi was detected in the aerial parts of *35S::SAP11*$_{AYWB}$ transgenic plants grown in 0.01 mM KH_2PO_4 (Pi-deficient) and 0.25 mM KH_2PO_4 (Pi-sufficient) hydroponic solutions than in wild-type plants (Figure 1B). These results indicate that Pi starvation responses and Pi homeostasis are altered in *35S::SAP11*$_{AYWB}$ transgenic plants.

Pi is a major limiting factor for the growth and development of plants. Thus, plants have evolved a series of morphological and physiological modifications triggered by Pi deficiency, such as an increase in root/shoot ratio, proliferation of lateral roots, and accumulation of anthocyanin (Rouached *et al.*, 2010). As a control, wild-type

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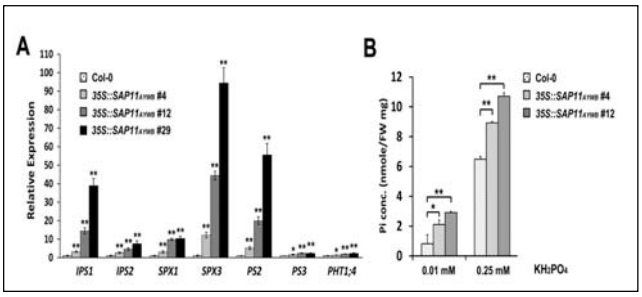

Figure 1. SAP11$_{AYWB}$ elicits the expression of Pi starvation-induced genes and increases the accumulation of Pi in arabidopsis.

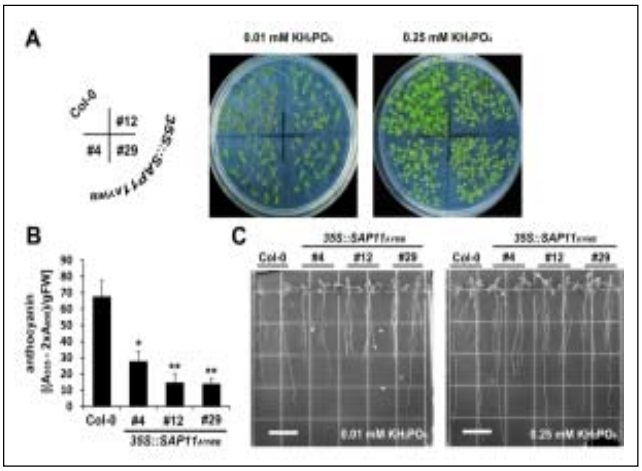

Figure 2. Expression of SAP11$_{AYWB}$ in arabidopsis alters the phenotypes in anthocyanin accumulation and root architecture.

plants showed a significant reduction in plant size and displayed red/purple color in leaf when grown on 0.01 mM KH_2PO_4 medium (Figure 2A). Compared with wild-type plants, *35S::SAP11$_{AYWB}$* transgenic plants showed a growth-inhibiting phenotype on both 0.01 and 0.25 mM KH_2PO_4 media (Figure 2A). However, the accumulation of anthocyanin was strongly reduced in *35S::SAP11$_{AYWB}$* transgenic plants grown on 0.01 mM KH_2PO_4 medium (Figure 2B). Compared with wild-type plants, *35S::SAP11$_{AYWB}$* transgenic plants grown on both 0.01 and 0.25 mM KH_2PO_4 media showed a clear inhibition of primary roots, a significant proliferation of adventitious roots, and an elongation of lateral roots (Figure 2C). Taken together, the molecular alterations associated with the expression of SAP11$_{AYWB}$ were correlated with the morphological changes observed in the *35S::SAP11$_{AYWB}$* transgenic plants.

Discussion

The importance of Pi in the development of disease symptoms could be revealed in huanglongbing disease, in which an increase in the expression levels of miR399 and Pi starvation-induced genes was observed in citrus plants infected by '*Candidatus* Liberibacter asiaticus' (Zhao *et al.*, 2013). Noticeably, the disease symptoms of '*Ca.* L. asiaticus'-infected citrus were reduced after the application of Pi. In the case of phytoplasmas, although the correlation between Pi and symptoms remains unclear, the disease symptoms in phytoplasma-infected plants could be reduced by the application of AM fungi (Sampò *et al.*, 2012). Thus, it will be interesting to explore whether the application of Pi could reduce the phytoplasma-mediated disease symptoms.

Acknowledgements

This work was supported by the National Science Council (NSC100-2321-B-005-007-MY3, NSC101-2911-I-005-301, and NSC 102-2911-I-005-301) and Ministry of Education (Aim for the Top University project), Taiwan.

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Phytopathogenic Mollicutes
Vol. 5 (1-Supplement), January 2015, S127-S128

doi: 10.5958/2249-4677.2015.00055.9

Interaction between cold atmospheric plasma and phytoplasmas in micropropagated infected periwinkle shoots

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Abstract

Plasma activated water (PAW) was employed to treat phytoplasma infected micropropagated periwinkle shoots to verify effects on symptomatology and phytoplasma presence. The preliminary results indicate that PAW do not induce shoot toxicity, and allow an improved detection of phytoplasma presence in shoots and also in liquid phytoplasma isolation medium. The reduced symptomatology and the lack of colony formation from liquid isolation medium induce to speculate that PAW interact with phytoplasmas and /or endophytes viability. Research to verify these aspects are in progress.

Keywords: cold atmospheric pressure plasma, plasma activated water, phytoplasmas, PCR/RFLP analyses

Introduction

Phytoplasmas are insect-transmitted plant pathogenic prokaryotes, associated with severe diseases in agronomic important crops. Management of these diseases has mainly focused on insect vector chemical control and on infected plant rouging. There is therefore a strong need for effective phytoplasma control strategies. The use of plasma activated water (PAW), water with chemical composition modified by exposure to cold atmospheric pressure plasma, was then investigated as innovative management approach. PAW was employed in preliminary trials, using diffusion methods, to evaluate its efficacy against phytoplasmas.

Materials and Methods

Experiments were carried out using sterile distilled water (SDW) treated by a nanopulsed dielectric barrier discharge (Kogelschatz, 2003). Ten minutes treatment with a peak voltage of 19 kV and a pulse repetition frequency of 1,000 Hz induced in SDW the formation of nitrates, nitrites and peroxides and a change in pH (Machala *et al*., 2013; Park *et al*., 2013). PAW was then used on periwinkles micropropagated shoots infected with phytoplasmas belonging to '*Candidatus* phytoplasma asteris' group, particularly HYD8 (16SrI-B) and KVE (16SrI-C), and maintained in micropropagation.

Three trials with two thesis each were performed: three infected shoots were treated with addition of 1 ml of PAW on the agar surface in the micropropagation tubes containing about 10 ml agar-solidified culture medium (Bertaccini *et al*., 1992) and three shoots were treated with 1 ml of sterile SDW as control. After 1 month all shoots were transferred to tubes with fresh medium without PAW and SDW. Three and six month after the treatments, total DNA was extracted from leaves of treated and untreated shoots HYD8- and KVE- and from healthy micropropagated periwinkle shoots using a CTAB method (Angelini *et al*., 2001). Meanwhile, phytoplasmas isolation was performed from all the treated and from the KVE untreated shoots, according to Contaldo *et al*. (2012).

DNA extraction from inoculated tubes was carried out 10 days after isolation using a phenol/chloroform based method (Contaldo *et al*., 2014). The tubes were also daily inspected for signs of a colour change from red to yellow, at this moment 100 µl of acid broth cultures were inoculated onto 6 plates containing 8 ml of solid medium and incubated under previously described conditions. The agar surface was observed every 24 hours for up to seven days with optical bifocal microscope at 40X magnification. In order to verify the presence of phytoplasmas, PCR assays were carried out on *16SrRNA* gene using primers R16F2n/R2 followed by nested PCR with 16Sr group specific primers R16(I)F1/R1 and/or 16Sr general primers 16R758f/16R1232r (M1/M2) (Duduk *et al*., 2013). The phytoplasma identification was then obtained by RFLP analyses using *TruI* on obtained amplicons as previously described (Contaldo *et al*., 2012).

Results and Discussion

Periwinkle shoots treated with PAW did not show toxicity symptoms, indicating that the treatment did not negatively affect their physiology. On the other hand these shoots

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showed increased leaf size and bushy appearance compared with healthy and untreated ones (Figure 1).

All the tested shoots gave positive results for phytoplasma presence. In particular, after direct PCR with R16F2n/R2 primer pair, amplicons of the expected lengths were obtained from all the treated shoot tested (Table 2), while only after nested-PCR with R16(I)F1/R1 group specific primers amplification was obtained from HYD8 and KVE untreated shoots (Table 1). RFLP identification verified that the HYD8 phytoplasmas belong to 16SrI-B while phytoplasmas in KVE before PAW treatment, were identified as 16SrI-C and after PAW treatment 16SrI-B (Tables 1 and 2). Acid colour change occurred in a number of isolation tubes after up to 15 days, according to previous reports (Contaldo *et al*., 2013). However, there were differences in the colour observed in the tubes, in particular it was possible to detect heavy growth, together with strong medium acidification or no colour change in some KVE-inoculated tubes (Table 2).

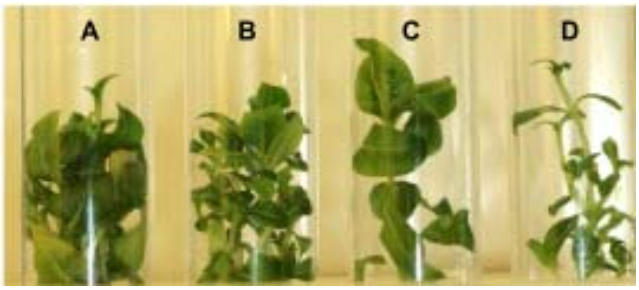

Figure 1. Periwinkle micropropagated shoots: A and B after PAW treatement on shoots HYD8 strain infected; C, healthy periwinkle shoot and D periwinkle shoot HYD8 strain infected without treatments.

Table 1. Phytoplasma detection before PAW treatment.

Phytoplasma strains	Nested PCR on shoots	Cultures colour change (<15 days)	nested PCR on DNA from isolation tubes
KVE (for PAW)	16SrI-C	Strong yellow	bacteria
KVE (for SDW)	16SrI-C	Orange	16SrI 16SrI+16SrXII 16SrI+16SrXII
HDY8 (for PAW)	16SrI-B	Red	negative
HDY8 (for SDW)	16SrI-B	Red	negative

Table 2. Phytoplasma detection after PAW treatment. *HDY8-SDW shoots were contaminated and not tested.

Phytoplasma strains	PCR on shoots	Cultures colour change (< 15 days)	nested PCR PAW/3 months	nested PCR PAW/6 months
KVE-PAW	16SrI-B	Red	negative	not tested
KVE-PAW	16SrI-B	Yellow	bacteria	not tested
KVE-PAW	16SrI-B	Orange	16SrXII-A	not tested
KVE-SDW	16SrI-B	Orange	16SrI	not tested
KVE-SDW	16SrI-B	Orange	16SrXII-A	not tested
KVE-SDW	dead	-	-	-
HDY8-PAW*	16SrI-B	Orange	16SrI-B	16SrI-B
HDY8-PAW*	16SrI-B	Orange	16SrI-B	16SrI-B
HDY8-PAW*	16SrI-B	Orange	16SrI-B	16SrI-B

After colour change and insemination in Petri dishes these were inspected for colonies formation. One week after plating, some heavy bacterial growth was observed in dishes inoculated with broth from KVE untreated shoots, while no growth was observed on dishes containing broth from PAW treated periwinkles. DNAs extracted from KVE isolation tubes after 3 months were amplified in nested PCR assays using M1/M2 primers, in one cases there was detection of bacteria presence, while in the majority of tubes the presence of 16SrXII-A or 16SrI was detected (Table 2). From all the HYD8-PAW isolation tubes 16SrI-B phytoplasmas were identified by RFLP on R16(I)F1/R1 amplicons (Table 2). The results show some interaction between PAW treatment and phytoplasma presence consisting mainly in the improvement of phytoplasma detection in both, shoots and isolation medium. The KVE results also indicate that some of the shoots can be infected by diverse phytoplasmas, and contaminated by endophytic bacteria that were isolated in the media used.

Acknowledgements

Work performed under Patent "PCT/IB2012/052965, method for culturing phytoplasma". Authors thank D.G. and H.M. Windsor for providing the media employed.

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