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Food and Agriculture COST Action FA0807

Integrated Management of Phytoplasma Epidemics
in Different Crop Systems

Phytoplasmas and phytoplasma disease management:

how to reduce their economic impact

Edited by Assunta Bertaccini

Food and
Agriculture

COST Action FA0807
Integrated Management of Phytoplasma Epidemics
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COST Action FA0807

Integrated Management of Phytoplasma Epidemics
in Different Crop Systems

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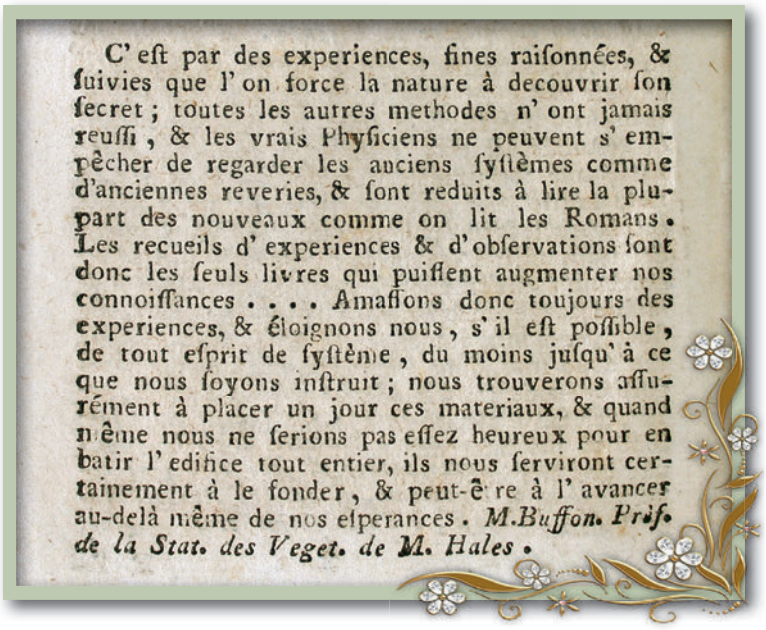
C' eſt par des experiences, fines raiſonnées, & ſuivies que l' on force la nature à decouvrir ſon ſecret ; toutes les autres methodes n' ont jamais reuſſi, & les vrais Phyſiciens ne peuvent s' empêcher de regarder les anciens ſyſtèmes comme d'anciennes reveries, & ſont reduits à lire la plupart des nouveaux comme on lit les Romans. Les recueils d' experiences & d' obſervations ſont donc les ſeuls livres qui puiſſent augmenter nos connoiſſances . . . . Amaſſons donc toujours des experiences, & éloignons nous, s' il eſt poſſible, de tout eſprit de ſyſtème, du moins juſqu' à ce que nous ſoyons inſtruit ; nous trouverons aſſurément à placer un jour ces materiaux, & quand même nous ne ſerions pas eſſez heureux pour en bâtir l' edifice tout entier, ils nous ſerviront certainement à le fonder, & peut-être à l' avancer au-delà même de nos eſperances. *M. Buffon. Préf. de la Stat. des Veget. de M. Hales.*

Phytoplasmas and COST Action FA0807

Assunta Bertaccini

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Abstract

The total number of participants involved in the Action was 367 of which 60% of females and 32% of early stage researchers. Total publications were about 800 (list available at http://www.costphytoplasma.ipwgnet.org/publications.htm).

Innovative knowledge resulting from COST networking through the Action

The comparison of diagnostic protocol for phytoplasma identification towards the achievement of harmonized protocols for rapid and reliable detection of fruit trees and grapevine phytoplasma-associated diseases, and the report of phytoplasma molecular diversity in diverse Countries, in different host plant and vector species, were obtained. Identification of molecular markers to follow insect vector population distribution, particularly for fruit tree phytoplasmas, was also achieved. A network for insect vector identification to increase the knowledge about phytoplasma disease epidemiology was established. New phytoplasma-associated diseases, new insect vectors and new phytoplasma host plants were identified. Dedicated schools were carried out to handle sampling for phytoplasma detection in plants and insects, and to identify their vectors by specific molecular tools, in some cases developed during the action. Morphological and molecular tools developed were used to improve knowledge on vector ecology and for a more precise timing of control measures application.

Information about existing management strategies for sustainable phytoplasma disease control was shared, and the application of developed tools helped to propose strategies for obtaining healthy planting material. Research conducted on genetic and induced resistance to phytoplasmas in fruit trees and grapevine, development of innovative control strategies against vectors and application of genetic resistance in apple, and model systems to study the genetic base of the resistance mechanism were established. The presence of genetic resistance to pear decline and European stone fruit yellows was screened and attempts to identify genetic resistance in grapevine were undertaken. Basic molecular and histological research on phytoplasma-recovered fruit trees and grapevine led to a better understanding of the reaction of the plant towards phytoplasma infections. Strategies proposed are to induce resistance by bioactive compounds (mainly in grapevine) or to screen for resistance induced also by endophyte presence.

Today, control of the insect vector is the most efficient way to limit the spread of phytoplasma diseases in fruit trees and grapevine. Focusing on the development of environmentally friendly strategies basic data on the identification of the vector species

or population, as well as on the biology of the insect vectors was achieved. A totally new control strategy based on plant info chemicals used either in traps or as repellents was established in apple and stone fruits. Molecular characterization of the microbial communities associated with different species and populations of leafhopper and cixiid vectors were studied and the endosymbiont '*Candidatus* Sulcia muelleri' was detected in populations of *Hyalesthes obsoletus* and *Reptalus panzeri*, both vectors of "stolbur". Whole genome sequences of multiple diverse phytoplasmas were obtained through a community-wide sequencing effort, named phytoplasma genome sequencing initiative (PGSI), which involved the collection of DNA from phytoplasma-infected plants and insects worldwide. During the genome annotation schools analyses of phytoplasma sequences were carried out. Current and future phytoplasma genome sequence information is being integrated into a single website and used to investigate the molecular basis of phytoplasma diseases in plants and insects. Some of the published work show the presence of a link between phytoplasma genomics achievements and insect vector transmission, including the discovery of phytoplasma genomic islands encoding genes that are upregulated during phytoplasma colonization of insects. The identification of some phytoplasma virulence genes that are expressed in insects and plants, and the presence of insect vector-specific diversification of variable membrane proteins of stolbur and "flavescence dorée" phytoplasmas, used as molecular markers for epidemiology, were also achieved.

Significant scientific breakthroughs

New diseases associated with phytoplasmas and differentiation of phytoplasma strains associated with quarantine phytoplasmas such as "flavescence dorée" and apple proliferation were reported. A first MLST (multilocus sequence typing) survey of fruit tree phytoplasma genetic diversity at the scale of the Euro-Mediterranean basin was published. Characterisation of 16Sr DNA of "bois noir" phytoplasma strains showed the presence of genetic variability of this phytoplasma in grapevine. Coniferous trees and shrubs were firstly described as natural host plants of diverse phytoplasmas. The Phytoplasma Genome Sequencing Initiative lead to the sequencing and annotation of the first genome drafts for the phytoplasma agents of pear decline, European stone fruit yellows, "flavescence dorée", grapevine yellows from German Palatinate, rubus stunt, decline of Nigerian coconut, yellows of African napier grass, maize bushy stunt and witches' broom disease of lime. Diverse targets of phytoplasma effectors were studied to elucidate pathogenicity mechanisms: SAP11 in Arabidopsis plants show crinkled leaves and increase in stem numbers as in the witches' broom phenotype; SAP54 transformed plants exhibit leafy flowers; SAP05 transformed plants has long slender leaves and early flowering. Amplification of *HfIB* gene of apple proliferation phytoplasma allow the differentiation of severe and mild strains and evidence that strain composition determines disease severity in multiply infected apple trees. *Actinidia* spp. was reported

as a phytoplasma host and possible interaction between phytoplasmas and the Psa (*Psudomonas syringae* pv *actinidiae*) strain(s) was proposed.

Tangible medium term socio-economic impacts

The increase of public awareness about phytoplasma diseases with emphasis on filling gaps in national awareness of phytoplasma disease threats was achieved. Specific projects were initiated and risk awareness was increased in farmers and growers; information was also added into EPPO web site, as a first step to improve sanitary conditions in nurseries and orchards. Development of a more effective integrated control of the main phytoplasma diseases is expected by the use of resistant plant material, environmentally friendly insect vector control strategies, and efficient disease management. The awareness was extended to international Countries where specific projects were initiated and new phytoplasma-associated diseases reported. Environmentally friendly insect vector control strategies, efficient disease management through early protection measures and the use of phytoplasma-free material were proposed together with innovative tools such as the use of endophytes and the practical application of some 'recovery'-related techniques to reduce the impact of diseases.

Promotion and dissemination

Current status and perspectives of phytoplasma disease research and management
Sitges, Spain: January 31 and February 1, 2010



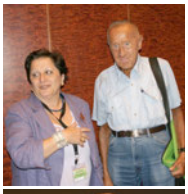

Phytoplasma classification - Chianciano Terme, Siena, Italy: July 16, 2010






Phytoplasma control in crop systems
Ancona, Italy: September 23-24, 2010

Integrated management of phytoplasma epidemics in different crop systems
Neustadt, Germany: September 15-16, 2011


Emerging phytoplasmas diseases of stone fruits and other crops and their possible impact on EU Countries
Istanbul, Turkey: December 1-2, 2011

Phytoplasmas, insect vectors, symbionts and plant endophytes
Milan, Italy: March 5, 2012



Phytoplasma and virus managements in grapevine collections for germplasm conservation, mobilization and evaluation Sofia, Bulgaria: May 8-9, 2012





Phytoplasmas in fruit trees: multidisciplinary approaches toward disease management
Rome, Italy: June 8, 2012




Perspectives of endophytes as biocontrol agents in the management of phytoplasma diseases
San Michele all'Adige, Italy: November 14, 2012

New perspectives in phytoplasma disease management
Barcelona, Spain: March 22, 2013

Psyllid school - AlPlanta, Neustadt - Germany: April 19-23, 2010
Grapevine yellows vector sampling and monitoring school
Bernkastel-Kues, Germany: July 3-9, 2010




Schools on bioinformatics of phytoplasma sequences
Vilnius, Lithuania: July 5-8, 2011 and Belgrade, Serbia: October 31-November 3, 2011

School on phytoplasma DNA extraction from plants and insects
Ankara, Turkey: 3-7 September 2012, and Ceske Budejiovice, Czech Republic: 15-18 July 2013

Phytoplasma Genome Sequencing Initiative (PGSI) annotation school
The John Innes Centre, Norwich, UK: February 20-24, 2012 and April 29-May 3, 2013


Molecular identification of psyllid vectors - Montpellier, France: October 1-5, 2012
Molecular tools to identify planthopper and leafhopper vector of phytoplasmas
Ivrea-Grugliasco, Italy: November 5-9, 2012



Final COST meeting
Carcavelos–Lisbon, Portugal: September 30 and October 1, 2013





The COST action's FA0807 web site: dissemination of phytoplasma-related knowledge

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Abstract

A web site for the COST action FA0807 was set up soon after the start of this action. This site was intended to be a tool to meet one of the most important goals of the action: to disseminate phytoplasma related knowledge and to connect researchers in the field of phytoplasma research. Two independent software programs were used to monitor the traffic on the web site. The results of these programs indicate that the FA0807 action's web site contributed importantly to the dissemination of phytoplasma related knowledge.

Key words: knowledge transfer, internet, results, publication.

Introduction

As the main objective in the COST action FA0807 was to promote information exchange in order to design integrated phytoplasma management strategies (Memorandum of Understanding, http://www.costphytoplasma.ipwgnet.org/publications.htm), an important goal was to set up a web site for this action. The web site appeared to be an outstanding tool for disseminating the COST action's output. This output comprises a list of publications written by COST action's contributors, STSM reports, minutes and reports of meetings and workshops. Publication of practical items, such as protocols, pictures of symptoms, phytoplasma vector tables, and geographical information on the occurrence of phytoplasmas and their vectors, contributed greatly to phytoplasma research updating.

Besides the dissemination of output, the web site proved to be a tool to connect people in the field of phytoplasma research. Every working group presented member lists on the web site, allowing researchers to connect to each other and to build their professional network. And last, but not least, the web site was built in such way that it was informative and attractive to the wider public interested in plant diseases, and particularly diseases associated with phytoplasma presence.

This chapter presents some comments on the web site set up and its functionality, as indicated by the results of web site usage statistics software.

Web site set up

The COST action FA0807 web site was launched in July 2009 with the Uniform Resource Locater (URL) http://www.costphytoplasma.eu. Since the COST Action FA0807 has ended, the web site was transferred on 1 January 2014 to the IPWG web site, and is now available at the URL http://www.costphytoplasma.ipwgnet.org.
The pages were organized in such a way that both general information and Working Group (WG)-specific information could be found fast and simply. From the home page, one could easily navigate to the pages for 'Objectives', 'Working groups', 'Symptom images' (Figure 1), 'Insect vectors', 'Meetings', 'News', 'Publications', 'Short-Term Scientific Missions (STSM)', 'Links' and 'Contact', and underlying pages. Also a search engine was built in to provide accurate searches within the COST FA0807 web site.

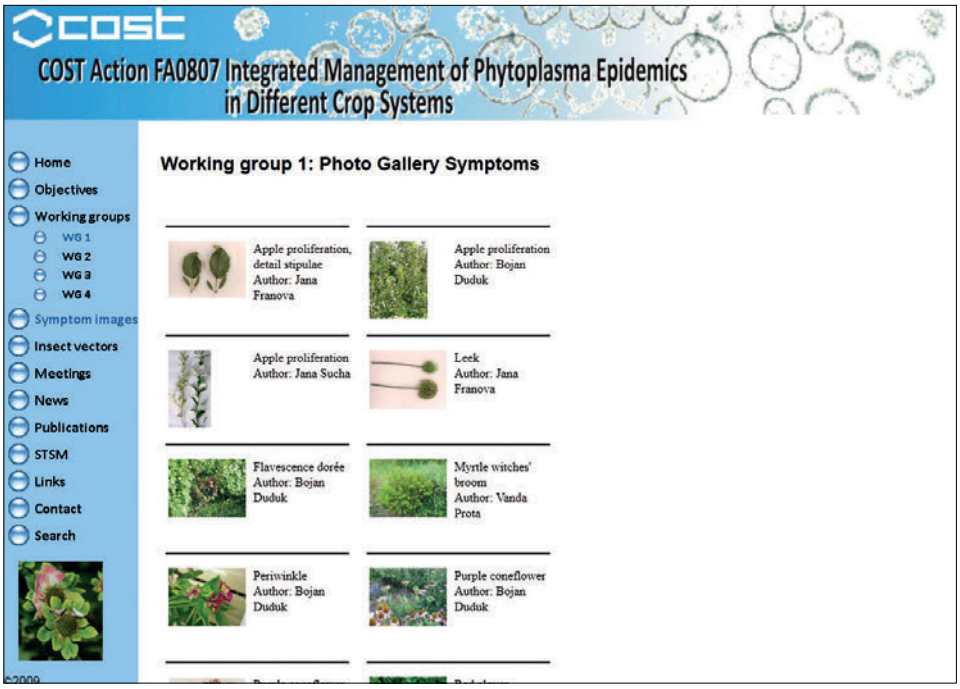


Figure 1. Example of the COST action FA0807 web page; the phytoplasma symptoms photo gallery.

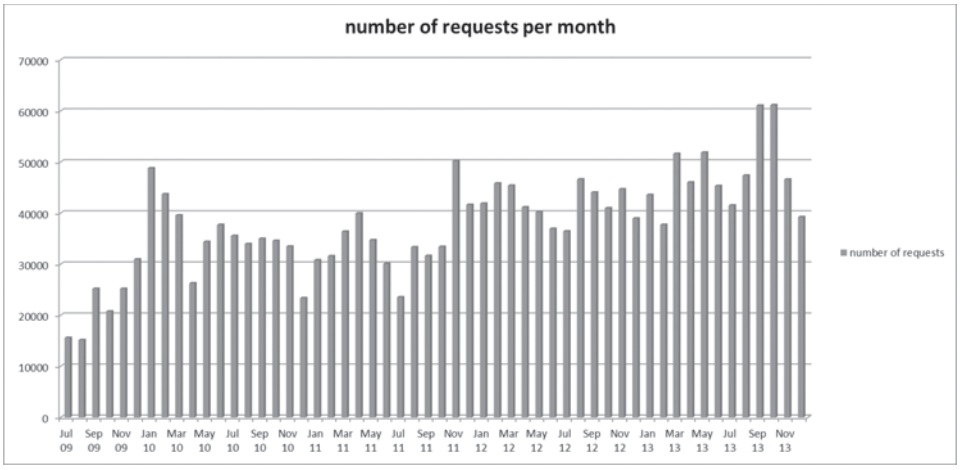
Web site usage statistics

To obtain information on the effective usage of a web site, specialized web usage statistics software is available. On the COST FA0807 web site's server, two programs were installed to monitor the web site's statistics: 1) the Analog web server statistics (http://www.analog.cx/) and 2) Webalizer Version 2.23 (http://www.webalizer.org/).

Both programs keep track of e.g. the number of requests for pages or files, IP addresses of visitors, search strings entered in Search Engines (such as Google, Bing), etc.
In this overview three examples will be presented that illustrate the success of the action's web site. These examples are: 1) the number of requests for pages or files, indicating the number of visits or hits by people requesting information on phytoplasma research, 2) the countries from which persons accessed the action's web site, and 3) the query words that were used in Search Engines to find or approach the action's web site.

Traffic on the web site (number of requests and data transfer)

From the launching of the COST Action FA0807 web site in July 2009 until the end of the COST action in December 2013, many requests (hits) were counted, indicating a great interest in the information on this site. The total successful requests during this period were over 2 million (2,053,167, with an average of 1,282 per day). The amount of data transferred during this period was 132.79 gigabytes (the average data transferred per day: 83.77 megabytes). An overview of the number of requests per month shows that the traffic just after launch was already over 15,000 hits per month, but this figure increased rapidly in the following period. Peaks in these figures were observed mostly just before meetings of the COST Action, indicating that delegates used the web site to gain information regarding the meeting. The largest peak in the number of hits was counted in the period in which the final meeting in Lisbon was organised, in October 2013. During this period the number of hits per months exceeded 60,000 (Figure 2).



(source Analog)

Figure 2. Number of requests for pages or files per month (period from July 2009 until December 2013).

The COST FA0807 action's web site: a tool to reach out to the world

One of the features of the Webalizer tool is the logging of the country belonging to the IP address of the web site visitor. Every month a top 30 of the visiting countries was displayed. Unfortunately, this software does not generate an overview for the entire period of this COST Action. The average of visiting countries during the last four months was 79. In Table 1, the top 25 countries of the month October 2013 is listed. A considerable part of the visitors has an IP address which was not linked to a certain country, and so they were listed as unresolved/unknown, commercial (.com) or as network (.net). Table 1 also shows that the web site visitors are not exclusively located in countries which are member states in this COST action, but originate from countries all over the world. This illustrates the benefit of an easily accessible web site with information useful to the general and scientific public.

Table 1. Top 25 countries of 82 (total) in October 2013 (source Webalizer).

Rank	Country	Hits	Files	Kbytes
1	Unresolved/Unknown	19504 (30.68%)	18368 (52.09%)	1707762 (30.16%)
2	Commercial (com)	13796 (21.70%)	8467 (24.01%)	1997302 (35.28%)
3	Network (net)	6017 (9.47%)	5497 (15.59%)	407873 (7.20%)
4	Italy	4737 (7.45%)	4589 (13.01%)	203182 (3.59%)
5	Bulgaria	2126 (3.34%)	2126 (6.03%)	42532 (0.75%)
6	Turkey	1282 (2.02%)	1276 (3.62%)	50391 (0.89%)
7	Russian Federation	1268 (1.99%)	859 (2.44%)	66520 (1.17%)
8	Germany	937 (1.47%)	891 (2.53%)	98567 (1.74%)
9	Czech Republic	922 (1.45%)	761 (2.16%)	76673 (1.35%)
10	Educational (edu)	917 (1.44%)	914 (2.59%)	81518 (1.44%)
11	Spain	832 (1.31%)	610 (1.73%)	45613 (0.81%)
12	Argentina	687 (1.08%)	462 (1.31%)	27805 (0.49%)
13	Japan	686 (1.08%)	672 (1.91%)	35917 (0.63%)
14	Israel	619 (0.97%)	619 (1.76%)	41680 (0.74%)
15	The Netherlands	606 (0.95%)	594 (1.68%)	21286 (0.38%)
16	India	487 (0.77%)	481 (1.36%)	50652 (0.89%)
17	Hungary	439 (0.69%)	346 (0.98%)	41110 (0.73%)
18	Serbia	437 (0.69%)	429 (1.22%)	35004 (0.62%)
19	Romania	427 (0.67%)	312 (0.88%)	12121 (0.21%)
20	Australia	425 (0.67%)	423 (1.20%)	41145 (0.73%)
21	France	424 (0.67%)	423 (1.20%)	42797 (0.76%)
22	Brazil	415 (0.65%)	413 (1.17%)	47385 (0.84%)
23	United Kingdom	352 (0.55%)	348 (0.99%)	26327 (0.47%)
24	Portugal	338 (0.53%)	336 (0.95%)	15277 (0.27%)
25	Canada	320 (0.50%)	311 (0.88%)	52914 (0.93%)

Table 2. Top 10 search strings (source Webalizer, October 2013).

Rank	Search string
1	phytoplasma
2	cost fa0807
3	cost action phytoplasma
4	cost fitoplasmi
5	pear decline phytoplasma
6	phyto-
7	phytoplasma meeting
8	phytoplasma vector
9	phytoplasmas cost
10	plytoplasma

Table 3. Top 15 query words (source Analog, *not listed: 8,885 search terms*).

Rank	Query word
1	phytoplasma
2	cost
3	pdf
4	grapevine
5	phytoplasmas
6	diseases
7	disease
8	plant
9	dna
10	classification
11	molecular
12	identification
13	tree
14	pcr
15	phylogenetic

Queries used in Search Engines

But only displaying good information is not good enough. It is significant that the web site can be found easily by the most commonly used Search Engines, with the most common query words that visitors enter in their search windows. An example of the most used search strings can be found in Table 2, and an overview of the most used query words in Table 3. It is clear that words like phytoplasma and COST would bring the web surfer to the COST actions' web site, but also words like grapevine and identification were used to navigate to the FA0807 web site.

Concluding remarks

As shown by the results of two independently operating web usage statistics programs, we can conclude that the COST FA0807 action's web site was very successful in reaching researchers and the general public who are interested in phytoplasma research. During the COST action's period, over 2 million times information was requested from the web site by visitors from all over the world. The web site has proven to be a very useful tool to meet one of the most important goals of the FA0807 action: to disseminate the knowledge on phytoplasma research and to connect researchers in the field of phytoplasma research.

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Chapter 1

The phytoplasmas and phytoplasma vectors in COST FA0807 Countries


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Phytoplasmas and phytoplasma vectors in Denmark, Finland, Lithuania and Norway

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Abstract

While Denmark resulted to be the only COST FA0807 Country where phytoplasmas were never detected, the project allow detection and/or confirmation of phytoplasma-associated disease presence as well as of potential insect vector in Norway, Finland and Lithuania. All these countries located in the Northern part of the EU are believed to be not seriously affected by phytoplasma diseases, however, during the project reports have emerged on the presence of phytoplasmas in Norway, and Finland in an important agricultural plant species such as apple. Moreover in Lithuania confirmation of phytoplasma presence and of their genetic polymorphism were reported and potential insect vectors of phytoplasmas were also found.

Key words: phytoplasmas, insect vector, molecular identification, disease, epidemic.

Introduction

The improvements on phytoplasma and vector knowledge in Finland, Norway and Lithuania in the frame of the COST FA0807 are of great importance for whole EU. The information concerns also new plant hosts of phytoplasmas, insect vectors and development of new techniques for phytoplasma detection. Also, the characterization of phytoplasmas infecting established hosts allowed to give more information concerning their epidemiology.

Denmark

No phytoplasma or phytoplasma vectors were reported from Denmark.

Finland

Phytoplasmas were detected in old reverted black currant plants and RFLP analysis showed that they belonged to the aster yellows (AY) group (16SrI). In carrot infested by *Trioza apicalis* Förster together with the bacterium '*Candidatus* Liberibacter solanacearum', aster yellows phytoplasma (16SrI-A) was detected in 20% of samples showing both leaf curling and discoloration, but no phytoplasma was detected in the carrot psyllid samples.

Results of a survey confirmed the presence of both psyllid vector species of '*Candidatus* Phytoplasma mali', *Cacopsylla picta* and *C. melanoneura*, in Finnish apple orchards.

The number of individuals of both species varied but *C. picta* prevailed in both years. Reduction in numbers of *C. melanoneura* after the hard winter in 2010 was relatively higher compared with that for *C. picta*. As *C. melanoneura* is considered to be a newcomer in Finland, it may be present at its northernmost border. Occurrence of '*Ca*. P. mali' in *C. picta* was determined in samples collected from two localities, one in which numerous imported apple varieties were planted and the other with local varieties (Lemmetty *et al*., 2011). To verify the presence of '*Ca*. P. mali' in apple trees, a survey was conducted in 17 commercial apple orchards. Two samples tested positive and were further analyzed for subtypes; they were shown to belong to apple proliferation subtype AT-1 (Lemmetty *et al*., 2013).

Lithuania

Studies on phytoplasma diseases in this country had at least a fifteen year history allowing identification of several phytoplasmas in diverse plant species. Since 2009 a few new phytoplasma-associated diseases were reported. In diseased sour cherry on the Neringa peninsula showing shoot proliferation and abnormally small leaves, phytoplasmas classified in 16SrI (aster yellows), new subgroup 16SrI-Q were identified (Valiunas *et al*., 2009). Onions plants grown for seed production in the Kaunas region exhibited mild yellowing of leaves and stems, stunting, phyllody, and proliferation of flowers. RFLP and sequence analysis of PCR-amplified 16S rRNA, ribosomal protein, and secY genes revealed the presence of phytoplasmas belonging to subgroups 16SrI-A (rpI-A) and 16SrI-L (rpIB, secYI-B). The results indicated that phytoplasma strains in subgroup16SrI-A (rpI-A) have the potential to infect onions in Europe and for the first time demonstrated onion as a host for subgroup 16SrI-L. (Jomantiene *et al*., 2010).
In diseased plants of clover exhibiting symptoms of clover phyllody or of clover dwarf, two distinct phytoplasmas were identified: clover phyllody-diseased plants were infected by a subgroup 16SrI-C, while clover dwarf-diseased plants were infected by 16SrI-C and a phytoplasma classified in subgroup 16SrIII-B. These findings extend the known geographic ranges of these subgroups. Phytoplasmas belonging to subgroups 16SrIII-P and 16SrI-C were identified in leafhoppers: strains belonging to subgroup 16SrI-C were found in *Euscelis incisus* and *Macrosteles sexnotatus* and strains belonging to subgroup 16SrIII-P were detected in *E. incisus* (Vanauskas *et al*., 2011). These results indicate that *E. incisus* and *M. sexnotatus* possibly act as vectors of strains classified in subgroups 16SrI-C and 16SrIII-P, the latter subgroup containing phytoplasma strains that have to date been found only in Lithuania.

Norway

Apple production (AP) is an important disease in specific areas in both the western and the eastern parts of the Country. AP is listed as quarantine disease and a survey in

the years 1996 and 1997 revealed 14 diseased trees in orchards throughout the country, however no conclusive evidence for natural spread by vectors was found. The infected trees at these locations were eradicated. From 2000 until 2008 only two or three more infected trees were detected and eradicated. In 2010, however, some new serious cases of AP disease were found in the western parts of the Country. A survey program for orchards close to nurseries in the most important fruit districts was then started. During autumn 2011 orchards were inspected and symptomatic plants sampled, in orchards where no AP-like symptoms were found a random sampling was carried out. AP was found in both symptomatic and asymptomatic trees. Any infected tree was found in any of the surveyed nursery due to a strict use of healthy propagation material and good control of potential vector populations (Blystad *et al*., 2012).

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Fruit tree phytoplasma diseases and vectors in Belgium, Netherlands, and United Kingdom

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Abstract

In Belgium and Netherlands phytoplasma diseases mainly affect fruit trees, while in United Kingdom they are almost absent. Specific insect vectors were identified and strict measures to eliminate both pathogens and pests are actively implemented in Belgium and in Netherlands.

Key words: fruit trees, apple proliferation, pear decline, European stone fruit yellows, insect vectors.

Belgium

In 1981, apple proliferation (AP, '*Candidatus* Phytoplasma mali', EPPO A2 list) was discovered for the first time in Belgium. Until recently symptomatic trees had been reported sporadically and found infected, but they were subsequently destroyed. In September 2009, two apple trees close to each other were sampled by the Federal Agency for the Safety of the Food Chain in a nursery in the south-west of Belgium and tested positive by PCR (Olivier *et al*., 2010). Infected trees as well as adjacent trees have been uprooted and burned to prevent disease spread. Symptoms of pear decline were observed in the past in some orchards, but the presence of '*Candidatus* Phytoplasma pyri' (PD, EPPO A2 List) was never confirmed by molecular tests.

In 2010 and 2011 a limited survey was carried out in a few commercial and non-commercial orchards showed the presence of clear symptoms of witches' broom (AP) or early leaf discoloration (PD). Root and leaf samples were analysed by PCR and results revealed the presence of both quarantine organisms (Peusens *et al*., 2014). As consequence an extensive and systematic survey was carried out by the Federal Agency for the Safety of the Food Chain in 2011-2012 in 87 apple tree nurseries. Infected trees were destroyed and eradication measures were imposed in the nurseries concerned. The

NPPO of Belgium is currently focusing its official inspections on the production of propagation material and fruit trees. In 2004 an infection with European stone fruit yellows was detected in a single plum tree in a private garden.

Netherlands

Apple proliferation was detected for the first time in nursery trees in summer 1997. In 1998, it was found in a few orchards in the southern part of the Country. Strict measures were taken in these orchards to prevent any further spread (NPPO, The Netherlands). From 1995 pear trees with symptoms of PD were found, the number was increasing when grafted onto susceptible rootstocks, and since 2012 the NPPO declared this disease to be present in all parts of the pear growing areas in the Country.

United Kingdom

One single tree of British origin was found to be infected with AP in 1985, but no additional infections were recorded since. In 1992 Parry's disease was discovered to be present in pear orchards, but no phytoplasma identification was reported. European stone fruit yellows was detected in apricot at one location.

Table 1. Overview of the current status of fruit tree phytoplasmas in Netherlands (NL) and in Belgium (B).

Country	Phytoplasma	Disease	Incidence	Host	Symptoms	Detection method
NL	'*Ca.* P. mali'	Apple proliferation	Low	Apple	proliferation and early leaf-reddening	PCR
NL	'*Ca*. P. pyri'	Pear decline	Moderate	Pear	Early leaf-reddening, growth reduction	PCR
B	'*Ca*. P. mali'	Apple proliferation	Low	Apple	Proliferation, long and small peduncles, enlarged stipules, smaller fruit size lacking colour	PCR
B	'*Ca*. P. pyri'	Pear decline	Low/ moderate	Pear	Early leaf yellowing or reddening	PCR
B	'*Ca.* P. prunorum'	European stone fruit yellows	One report	Plum	Yellowing and decline	PCR

Phytoplasma vectors

In Belgium the population dynamic of psyllid phytoplasma vectors was monitored in several orchards, they were collected throughout the year, identified using a microscope and tested for '*Ca*. P. mali' presence. The following *Cacopsylla* sp. were present on apple trees: *C. mali, C. melanoneura* and *C. picta*. Some orchards were surrounded by hawthorn hedges and additional *Cacopsylla* species were found: *C. peregrina, C. affinis* and *C. crataegi*. In pear the infection by pear sucker is a major problem together with the production of honeydew by *C. pyri.* Another species, *C. pyricola,* was found but in a small number. In Netherlands three psyllid species were identified: *C. melanoneura, C. pyri* and *C. pyricola*.

Table 2. Current status of vectors of fruit tree phytoplasmas in Netherlands (NL) and Belgium (B).

Country	Vector	Population density	Phytoplasma	Vector host	Collection method	Identification method
NL	*C. melanoneura*	Low	AP	apple	beating tray	morphology
NL	*C. pyri*	High	PD	pear	beating tray	morphology
NL	*C. pyricola*	Low	PD	pear	beating tray	morphology
B	*C. picta* *C. melanoneura*	Very low Very low	AP	apple hawthorn	beating tray + sticky trap	morphology
B	*C. pyri*	High	PD	pear	beating tray	morphology
B	*C. pyricola*	Low	PD	pear	beating tray	morphology

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Phytoplasma diseases and their vectors in Czech Republic, Hungary and Poland

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Abstract

European stone fruit yellows is a long standing problem in apricot and peach orchards in Moravia and East Bohemia, Czech Republic (CR), and in stone fruits in several regions of Hungary and Poland. The study on '*Candidatus* Phytoplasma mali' infected apple trees revealed the presence of strains in all the three countries. Epidemic occurrence of "stolbur" was recorded in tomato, pepper and celery in South Moravia: *vmp*1 gene analyses revealed that four genetic variants are infecting annual crops and wild plants in CR. Most of Hungarian "stolbur" strains clustered in tuf-type b STAMP II. Transmission of "stolbur" *via* potato tubers and association of this phytoplasma with maize redness disease and its vector *Reptalus panzeri* were investigated in Hungary. New plant hosts and potential insect vectors of phytoplasmas belonging to different 16S ribosomal groups and subgroups were also identified in Czech Republic, Hungary and Poland.

Key words: '*Ca.* P. asteris', '*Ca.* P. mali', '*Ca.* P. pini', '*Ca.* P. prunorum', '*Ca.* P. solani'.

Introduction

Phytoplasmas are bacteria lacking a cell wall and inhabiting the phloem of plants and the hemolymph of insect vectors (Bertaccini *et al.*, 2012). More than a thousand phytoplasma-associated diseases causing high economic losses are known around the world. Phytoplasmas affect many plant species including vegetables, fruit trees, conifers, and ornamental plants. Severe devastation in crops, yield losses, decreasing of fruits quality and death of plants is often the final result of these infections. There is no suitable treatment against phytoplasma diseases and most of them are quarantine organisms included in EPPO A2 list of pests. Czech Republic, Hungary and Poland as countries

located in Central Europe, are exposed to invasion of variable biotic factors including phytoplasmas from both Western and Eastern Europe. We focused on detection and characterization of phytoplasmas especially causing economically important losses in these three countries. The aim of this study was also to summarize new plant hosts and phytoplasma vectors reported since 2009.

Czech Republic

'*Candidiatus* Phytoplasma prunorum' is a long-standing problem. A survey was done in apricot, peach and cherry orchards in Moravia and East Bohemia (Krška *et al*., 2012; Ludvíková *et al*., 2011). Different methods of DNA extraction and PCR detection were evaluated (Mašková *et al*., 2009), including comparison of symptom variability after graft-inoculation in different *Prunus* spp. (Nečas *et al*., 2012). Occurrence of '*Ca.* P. pyri' (PD), '*Ca*. P. mali' (AP) and '*Ca.* P. asteris' (16SrI-B and 16SrI-C subgroups) was confirmed in orchards as well as in wild growing fruit trees. Extensive RFLP study of AP revealed prevalence of P-I 16S-23S profile, rpX-A subgroup, subtypes AP-15 and AT-2 (Fránová *et al*., 2013a).

'*Ca.* P. solani' ("stolbur") was detected in many plant species and its epidemics have been reported in tomato, pepper and celery in South Moravia (Navrátil *et al*., 2009). Study of genetic variability of "stolbur" also by use of the polymorphic gene *vmp* 1 in annual crops and wild plants revealed the presence of four genetic variants (Fialová *et al*., 2009). Long term monitoring of "stolbur" vectors was done in intensive orchards and overwintering sites. The first mass occurrence of *Hyalesthes obsoletus* in South Moravia since 1950´ was observed in 2010 (Šafářová *et al*., 2011). Alarming information were obtained about the phytoplasma presence in overwintering *Cacopsylla pruni* and *C. picta* in which phytoplasmas were detected in up to 23% of the samples. A survey of potential phytoplasma vector occurrence was done also in vineyards and surrounding intensive vegetable plots: 136 hemipteran species were identified, 34 of them were phytoplasma vectors and/or insect hosts. Together with the main "stolbur" phytoplasma vector, *H. obsoletus*, the occurrence of the other vector *Anaceratagallia ribauti* with 50% of positive individuals was detected. *Trifolium pratense* (Fránová *et al*., 2009) and *Chenopodium album* (Šafářová *et al*., 2011a) were found to be new hosts species for "stolbur" phytoplasma that was also found to be associated with "bois noir" (BN) in grapevine (Starý *et al*., 2013).

Asparagus officinalis (Fránová and Petrzik, 2010) and *Plantago lanceolata* (Fránová and Šimková, 2009) were identified as hosts of '*Ca.* P. asteris' (AY, 16SrI-B). A phytoplasma belonging to 16SrI-C ribosomal subgroup was firstly reported in *Echinacea purpurea* (Fránová *et al*., 2009), *Rhododendron hybridum* (Přibylová *et al*., 2013), *Ribes rubrum* (Přibylová *et al*., 2011) and *Apium graveolens* (Fránová and Špak, 2013). Phytoplasmas belonging to subgroup 16SrIII-B were identified in *E. purpurea* (Fránová *et al*., 2013) and *Chenopodium album* and phytoplasmas belonging to 16SrVI-A subgroup were

reported in *Rhododendron hybridum* (Přibylová *et al*., 2009). The finding of *Ulmus minor* affected by elm yellows in Moravia represents the northernmost confirmed occurrence of phytoplasma on elm trees within Europe (Navrátil *et al*., 2009).
AP and PD were sporadically detected in *C. picta* (90 individuals tested/4 positive) and *C. pyri* (966/11), *C. pyrisuga* (47/1), *C. pyricola* (17/1) in apple and pear orchards in East Bohemia, respectively (Ludvíková *et al*., 2011a). Examination of hemipterans in vineyards revealed the presence of phytoplasmas belonging to subgroups 16SrI-B, 16SrI-C, 16SrI-F, 16SrIII-B, 16SrXII-A, and unexpectedly, in *Jassargus obtusivalvis* of phytoplasmas of the 16SrXIV group (Orságová *et al*., 2011).

Hungary

National survey on grapevine yellows revealed the distribution of BN in all wine regions of Hungary. Molecular characterisation of '*Ca.* P. solani' proved the presence of different genotypes all resulted in the tuf-type b. Most of the strains clustered in tuf-type b, STAMP cluster II, while one isolate from tomato resulted affiliated to STAMP cluster III. Grapevine strains from north-west Hungary clustered together in a monophyletic branch of the STAMP tuf-type a genotype (Fabre *et al.*, 2011).
Transmission of "stolbur" phytoplasma *via* potato tubers was studied in a 3-year experiment. Tubers and their daughter plants (4 varieties of high starch content) were tested with PCR-RFLP and real-time PCR analyses. In an average of three-years 83.8% and 0.5% of the tubers and daughter plants, respectively resulted infected with "stolbur" (Ember *et al*., 2011a). A 2010 survey carried out to verify maize redness presence and its known vector *Reptalus panzeri* in the maize production areas allow to detect the presence of '*Ca*. P. solani' in symptomatic maize plants and of *R. panzeri* at one locality (Acs *et al*., 2011).
The "flavescence dorée" (FD) vector *Scaphoideus titanus*, was found in the southern counties in 2006 and its spreading to north is continuous. In order to evaluate the risk represented by the wild reservoir as a source of FD outbreaks diverse wild perennial plants in vineyards were tested in 2008-2011. Phytoplasmas belonging to group 16SrV were detected by nested PCR/RFLP analyses in alders (86%) and in clematis (71%). Their characterisation on the *map* gene revealed that strains from both plants carried the same *map* gene sequence as the FD strains. This survey has also detected the presence of *Oncopsis alni* and *Dictyophara europaea* reported to be phytoplasma vectors (Ember *et al*., 2011). In 2013 FD phytoplasmas were firstly detected in grapevine and in one *S. titanus* sample collected in south-west Hungary (Kriston *et al*., 2013).
'*Ca*. P. pyri' the agent of pear decline was detected in *C. pyri* and *C. pyricola* and '*Ca*. P. prunorum' agent of European stone fruit yellows as well identified in central region of Hungary. Study on samples from 6 apple trees, originating from a small field destroyed by AP, near the Austrian border revealed the presence of '*Ca*. P. mali' with both P-I and P-II 16S-23S profiles, belonging to AT-1, AT-2, AP subtypes and only to rpX-A ribosomal subgroup (Paltrinieri *et al*., 2010).

Poland

Epidemiological study revealed the predominant presence of '*Ca*. P. prunorum' (16SrX-B) in stone fruit trees. Moreover, in single peach and sweet cherry trees '*Ca*. P. mali' (16SrX-A) and '*Ca*. P. pyri' (16SrX-C) were respectively identified (Cieślińska and Morgaś, 2011). Survey of apple orchards and several home gardens showed that apple trees were mainly infected by '*Ca*. P. mali'. Most of them were classified to subgroup rpX-A, subtype AP. RFLP analysis of 16S rDNA plus 16S-23S spacer region showed two restriction profiles for the '*Ca*. P. mali' isolates: P-I and P-II. '*Ca*. P. asteris' (AY, 16SrI-B) was also detected in several apple trees. PCR-RFLP analysis of 16S rRNA, *rpl22* and *rpS3* as well as *secY* and *tuf* genes has not showed sequence diversity among these '*Ca*. P. asteris' strains. '*Ca*. P. mali' was also identified in *C. melanoneura* and *C. picta*, while '*Ca*. P. asteris' was detected in a single batch of leafhoppers (Cieślińska and Kruczyńska, 2011; Cieślińska *et al*., 2012).

The presence of three phytoplasmas was demonstrated in 11 out of 13 tested coniferous plant species; 9 are new natural host plants. '*Ca*. P. pini' (16SrXXI), was detected in *Abies procera*, *Picea pungens*, *Pinus banksiana*, *P. mugo*, *P. nigra*, *P. sylvestris*, *P. tabuliformis* and *Tsuga canadensis* (Kamińska *et al*., 2011). '*Ca.* P. pruni' (16SrIII) was detected in *Picea abies* and *P. glauca*, while '*Ca*. P. asteris' in *Picea pungens* trees. Phytoplasmas were detected in about 27% of the witches' broom originated plants or showing pronounced shoot proliferation symptoms as well as in some asymptomatic hybrid trees (Kamińska and Śliwa, 2010; Kamińska and Berniak, 2011).

AY was reported for the first time in plants belonging to Brassicaceae family showing growth abnormalities: *Brassica* interspecific hybrids with green foliage and complete flower bud failure, Brussels sprout with stunted growth, severe leaf malformation and flower bud failure (Kamińska *et al*., 2012), Chinese cabbage with abnormal flowers, virescence and phyllody (Kamińska *et al*., 2012a). AY was also detected in oilseed rape (Zwolińska *et al*., 2011), European hazel with stunting and leaf yellows symptoms (Cieślińska and Kowalik, 2011) and *Fraxinus excelsior* showing ash yellows symptoms (Kamińska and Berniak, 2009). Oriental lily hybrids with leaf necrosis and flower malformation symptoms were found to be infected by AY (16SrI-B) and AP (Kamińska and Śliwa, 2011). A phytoplasma closely related members of 16SrI-C subgroup was detected in tomato plants in the western region of the country (Krawczyk *et al*., 2010). *Pisum sativum* with stunting, shoot proliferation and leaf chlorosis was found to be a new "stolbur" phytoplasma host (Zwolińska *et al*., 2012). The natural occurrence of phytoplasmas was firstly reported in *Rubus* spp. plants with stunting, short and thin shoots. Sequence analysis of 16S rRNA gene showed that most of the strains clustered together with the reference strain rubus stunt (RuS, '*Ca*. P. rubi', 16SrV-E), whereas the strains from loganberry and wild blackberry were classified as X-disease (16SrIII) and aster yellows groups, respectively (Cieślińska, 2011).

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Presence and distribution of phytoplasma diseases and vectors in Germany and Switzerland - current state of the art

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Abstract

Germany and Switzerland are neighboring countries located in the middle of Europe. Both countries have vineyards and orchards with different fruit tree species in which various incidences of phytoplasma diseases are present. Despite similar environmental conditions, regional climatic and biogeographic particularities may influence the epidemiology and ecology of a specific disease and of its vectors. During the COST action a survey based on questionnaires was carried out asking members to report the distribution of phytoplasma diseases and of their vectors in their countries. This chapter describes the current situation in Germany and Switzerland, based on compiled information from questionnaire data during these activities and on additional reports from national researchers.

Key words: grapevine yellows, fruit tree phytoplasmas, leafhopper, psyllids, spread.

Introduction

Phytoplasma diseases of fruit trees and grapevine are associated with several economic important diseases all over Europe; some of them are also quarantine organisms. The most widespread grapevine phytoplasmas are those of 16SrV-group causing "flavescence dorée" (FD) and '*Candidatus* Phytoplasma solani' (group 16SrXII-A) associated with "bois noir" (BN). Fruit tree phytoplasmas belong to the apple proliferation group (16SrX-A), which includes '*Ca.* P. mali', the agent of apple proliferation (AP), '*Ca.* P. prunorum', associated with European stone fruit yellows (ESFY), and '*Ca.* P. pyri', agent of pear decline (PD). While FD is transmitted by the leafhopper *Scaphoideus titanus* and BN by the cixiid planthopper *Hyalesthes obsoletus*, all European fruit tree phytoplasmas are transmitted by psyllids. Although cultivation practices and climatic conditions can be quite heterogeneous in different regions, transmission by insects and long-distance spread by infected planting material lead to a rapid diffusion of these phytoplasmas among all European countries. In case of mobile vectors or endemic plant reservoirs also isolated orchards and vineyards can be affected. For this reason, updated knowledge on disease distribution as well as on the presence of insect vectors is

fundamental for a comprehensive pest risk assessment and cross-national phytosanitary decisions.

Phytoplasma diseases and vectors in Germany

Phytoplasma detection in plants and insects has been carried out by standard molecular protocols using PCR-based assays. Psyllid identification was done using the key of Ossiannilson (1992) and the electronic key www.psyllidkey.eu. Vector capacity has been proven by transmission trials for *H. obsoletus*, *Oncopsis alni*, *Cacopsylla picta* and *C. pruni*.

The most spread grapevine-related phytoplasma disease is BN. It is present in 11 of the 13 viticultural regions of Germany. Two other locally distributed diseases on grapevine are: the Palatinate grapevine yellows (PGY) associated with phytoplasmas of the elm yellows group (16SrV-C) which is present in viticultural areas of Palatinate, Mosel and Franken and another grapevine yellows associated with a phytoplasma of the aster yellows group (16SrI) which was occasionally found in Palatinate and Mosel areas, respectively (Ipach *et al*., 2010). The cixiid planthopper *H. obsoletus*, the proven vector for '*Ca*. P. solani' to grapevine, has been found in all areas where the disease is present. It is feeding on two major host plants, *Convolvulus arvensis* and *Urtica dioica*, which serve also as natural reservoirs of the phytoplasma. Phytoplasmas associated with Palatinate grapevine yellows colonize *Alnus glutinosa* and are only erratically transmitted from this host to grapevine by the leafhopper *O. alni*. The vector(s) for the aster yellows-related infections in grapevine are still unknown. The quarantine disease "flavescence dorée" has not been found in Germany so far and also its vector, *S. titanus*, is not reported yet.

Phytoplasma diseases and vectors in Switzerland

Phytoplasma detection in plants and insects has been carried out by visual monitoring of symptoms as well as by molecular analyses. Psyllid identification was done using the key of Ossiannilson (1992) and the electronic key www.psyllidkey.eu. Vector capacity has not been proven by transmission trials in Switzerland and vector capacity for *S. titanus, H. obsoletus, C. mali, C. pruni* and *C. pyri* is therefore presumed from studies and trials carried out abroad.

Both important diseases on grapevine, BN and FD are present. While '*Ca*. P. solani' and its vector *H. obsoletus* are widespread all over the country (Kehrli *et al*., 2010; 2011), the agent of FD has so far only been found in the Ticino region. However its vector, the leafhopper *S. titanus*, has not only been reported in the canton Ticino, but also in the cantons Vaud, Geneva and Valais.

Pear decline is widespread in Switzerland (Bünter and Schaerer, 2012), whereas apple proliferation is restricted to Northwestern Switzerland, mainly to the cantons Aargau and Solothurn (Bünter and Schaerer, 2012). European stone fruit yellows is only reported

from some local stone fruit growing regions, mostly in the canton Valais, where its vector, *C. pruni*, is also present. In the canton Valais, two populations A and B, belonging to the *C. pruni* complex, coexist (Peccoud *et al.*, 2013) and both were shown to carry '*Ca*. P. prunorum', the ESFY agent. Mostly breeding and feeding on wild *Prunus* species, *C. pruni* quite likely also lives in cultivated *Prunus* species such as apricots. Moreover, current observations indicate that '*Ca*. P. prunorum' is more widespread than expected since symptoms on other *Prunus* spp. such as *P. persica* or *P. domestica* are difficult to identify. The phytoplasma presence was also confirmed in nurseries outside of the canton Valais. The acknowledged vector *C. picta* is present in the cantons Aargau and Solothurn, but has only found being infected with '*Ca*. P. mali' in the canton Aargau (Jarausch *et al*., 2011). No information is so far available concerning the presence or infection rate of putative vectors of '*Ca*. P. pyri'. The rubus stunt phytoplasma has occasionally been reported from local blackberry and raspberry cultivations. Potato "stolbur" is also found, but rarely, although its vector, *H. obsoletus*, is widespread.

Table 1. Summary of disease incidence, phytoplasma agents and vector status in Germany.

Disease	Incidence	Region/s	Phytoplasma /(sub)group	Vectors	Presence	Host/s
''Bois noir'' (BN)	wide	in 11 out of 13 viticultural regions	'*Ca.* P. solani' 16SrXII-A	*H. obsoletus*	widespread on herbaceous vegetation	*U. dioica, C. arvensis, Calystegia sepium* grapevine occasional feeding host
Palatinate grapevine yellows (PGY)	local	Palatinate, Mosel, Franken	elm yellows 16SrV-C	*O. alni*	widespread on *A. glutionsa*, transmitted to grapevine erratically	*A. glutinosa* grapevine erratic host
Grapevine yellows (GY)	local	occasional Palatinate, Mosel	'*Ca.* P. asteris' aster yellows 16SrI	no information	no information	no information
Apple proliferation (AP)	wide	all regions	'*Ca.* P. mali' 16SrX-A	*C. picta*	widespread (proven vector)	*Malus* spp.
European stone fruit yellows (ESFY)	wide	all regions	'*Ca.* P. prunorum' 16SrX-B	*C. pruni*	widespread	*Prunus* spp.
Pear decline (PD)	wide	all regions	'*Ca.* P. pyri' 16SrX-C	*C. pyri, C. pyricola*	no information	*Pyrus* spp.
Rubus stunt (RuS)	local	Bade-Wurrtemberg, Palatinate	'*Ca.* P. rubi' 16SrV-E	*Macropsis fuscula*	locally widespread	blackberry raspberry
Potato "stolbur"	local	Hesse, Palatinate	'Ca. P. solani' 16SrXII-A	*H. obsoletus*	widespread	grapevine, *U. dioica, C. arvensis*

Table 2. Summary of disease incidence, phytoplasma agents and vector status in Switzerland.

Disease	Incidence	Region/s	Phytoplasma/ (sub)group	Vectors	Presence	Host/s
''Bois noir'' (BN)	general	all viticultural regions	'*Ca*. P. solani' 16SrXII-A	*H. obsoletus*	widespread	grapevine, *U. dioica*, *C. arvensis*
				R. panzeri	localised and rare in canton Ticino	
				R. cuspidatus	localised and rare in canton Ticino	
"Flavescence dorée" (FD)	local	Canton Ticino	elm yellows 16SrV	*S. titanus*	localised in cantons Ticino, Vaud and Geneva	grapevine
				Dictyophara europea	localised, transmission erratic	grapevine, *Clematis vitalba*
				Orientus ishidae	localised, transmission unknown	grapevine?
Palatinate grapevine yellows (PGY)	unknown	Canton Ticino	elm yellows 16SrV-C	*O. alni*	localised, transmission erratic	grapevine, *A. glutinosa*
Apple proliferation (AP)	local	Cantons Aargau Solothurn	'*Ca*. P. mali' 16SrX-A	*C. picta* *C. melanoneura*	widespread	*Malus* spp., *Crataegus monogyna*
European stone fruit yellows (ESFY)	local	Canton Valais	'*Ca*. P. prunorum' 16SrX-B	*C. pruni*	widespread	*Prunus* spp.
Pear decline (PD)	wide	all fruit growing regions	'*Ca*. P. pyri' 16SrX-C	*C. pyri* *C. pyricola*	no information	*Pyrus* spp.
Rubus stunt (RuS)	local	local small fruit cultivations	'*Ca*. P. rubi' 16SrV-E	*Macropsis fuscula*	no information	blackberry, raspberry
Potato "stolbur"	rare	occasional	'*Ca*. P. solani' 16SrXII-A	*H. obsoletus*	widespread	grapevine, *U. dioica*, *C. arvensis*, *Solanum tuberosum*

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Recent insight on phytoplasma diseases and vectors in France

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Abstract

Phytoplasma diseases mainly affect the French viticulture, stone fruit and lavender production. Recent studies have pointed out the impact of wild plants as a source of infection or as reservoir for insect vectors. Two strains of "flavescence dorée" (FD) phytoplasmas are present in French FD outbreaks, surrounding wild *Vitis* regrowth and infected alders may constitute potential epidemic reservoirs. It was experimentally shown that cold winter temperature has an effect on egg-hatching dynamics in *Scaphoideus titanus* but also on operational sex-ratio and that *S. titanus* nymphs do not use vibrational communication to aggregate on plant. A draft sequence of the FD phytoplasma was produced and used to improve the taxonomy of the group 16SrV and to serve for the search of genetic determinants involved in the phytoplasma adaptation to insect vectors. "Bois noir" is endemic in French vineyards and '*Candidatus* Phytoplasma solani' strains detected belong to the stamp cluster IA and IV. '*Ca.* P. solani' is endangering lavender production as specific strains are rapidely propagated by *Hyalesthes obsoletus* populations living on lavender. Two cryptic species corresponding to *Cacopsylla pruni* are present in France as vector of European stone fruit yellows.

Key words: "flavescence dorée", "bois noir", lavender decline, *Scaphoideus titanus, Cacopsylla pruni.*

Strains of "flavescence dorée" phytoplasmas

Two strains of "flavescence dorée" (FD) phytoplasmas are present in French outbreaks where surrounding wild *Vitis* regrowth and infected alders may constitute potential epidemic reservoirs. FD is epidemically transmitted by the grapevine leafhopper *Scaphoideus titanus*, an insect of North American origin now widely distributed in the vineyards of Southern France and also present in Burgundy, Jura, Champagne and Loire valley. The *S. titanus* populations were shown to have been introduced from the USA and therefore low genetic diversity is encountered (Papura *et al*., 2009; 2012). Grapevine yellows are surveyed using a reference diagnosis method consisting of a Taqman triplex real-time PCR for FD and BN phytoplasmas as well as a *Vitis vinifera* endogenous control (Pelletier *et al*., 2009).

The sequencing or the restriction map of the gene *map* allows to differentiate genetic clusters: the one named mapFD2 is clonal and represent 85% of the disease cases, whereas the cluster mapFD1 only represents 15% of the FD cases and is mainly detected in South-Western France (Salar *et al*., 2009). These two strains were isolated and transmitted to broad bean in which there are experimentally maintained using *Euscelidius variegatus*. Experiments in controlled conditions showed that both strains multiply with the same kinetics in plant, so they should have the same epidemiological properties. The prevalence

of the strain mapFD2 in France may have resulted from an increased propagation from nurseries (Salar *et al*., 2013). Despite the control measures such as the pulling out of the infected grapes and the spread of insecticide, the disease is difficult to control due to the large viticulture areas involved. In order to use less insecticide, some growers organizations decided to improve the disease management by monitoring the insect vector populations and extensively surveying the vineyards for disease symptoms.
Alders were randomly sampled in France. So called alder yellows phytoplasmas (AldY) were detected in more than 85% of the alder trees. Most of the AldY *map* gene sequences showed some diversity, but formed a monophyletic cluster with other *map* gene sequences of FD, AldY and Palatinate grapevine yellows (PGY) strains. The *map* gene sequence of some AldY strains was found identical to the one of French mapFD1 type strains, while other AldY phytoplasmas clearly classified in the mapFD2 cluster. In South-western France, along rivers, uncontrolled rootstock regrowths have shown to constitute in many places a reservoir for FD phytoplasmas and *S. titanus* populations escaping the insecticide treatments.

Genomics, taxonomy and adaptation to insect vectors in FD phytoplasmas

The 670 kbp chromosome of "flavescence dorée" phytoplasma line FD92 was partially sequenced by pyrosequencing and SOLEXA. More than 94% of the chromosome could be assembled and the 22 largest contigs representing 85% of the chromosome were annotated. Out of 464 chromosomal coding sequences (CDS), 174 CDS (38%) were involved in information transfer (DNA replication, protein production, RNA modification and regulation), 88 CDS (19%) were encoding metabolic enzymes, 40 CDS (9%) corresponded to transporters, 8 CDS (1%) corresponded to cellular processes, whereas 145 CDS (31%) remained cryptic. At this stage of incomplete assembly, repeated sequences were underestimated and transposon and phage-related CDS (2%) could not yet be precisely evaluated. FD92 phytoplasma possesses a complete glycolytic pathway and has a prominent system for proteolysis, possibly resulting from the adaptation to its woody hosts.
It was shown upon sequencing of 5 chromosomal regions in a broad collection of phytoplasmas, that phytoplasmas belonging to the taxonomic subgroup 16SrV-C and –D corresponding to FD phytoplasmas, alder yellows phytoplasmas, Palatinate grapevine yellows detected in Germany and France and *Clematis* phytoplasmas detected in Italy may constitute a common genomic species. It was also the case for strains of rubus stunt that could also be differentiated at the 16SrDNA level, supporting therefore their description as '*Candidatus* Phytoplasma rubi' (Malembic-Maher *et al*., 2011).
Some alder yellows phytoplasmas detected on grapevine in France and in Germany as isolated cases could be transmitted to the broad bean *Vicia faba*. None of them could be experimentally transmitted from this host by *S. titanus*, on the contrary of mapFD1 and map-FD2 strains of FD. In order to identify genetic determinants responsible for

the adaptation of FD phytoplasmas to the *S. titanus* insect vector; the draft genome of the epidemic strain FD92 was searched for genes encoding surface proteins with high genetic diversity. *Vmp* genes which encode surface proteins VMP-A and VMP-B display low homology to the *Mycoplasma agalactiae* variable lipoprotein VPMA. VMP possess a putative signal peptide, large repeated domains of about 80 amino acids and a C-terminal transmembrane alpha helix. Such organization in repeated domains was reminiscent of surface proteins of Gram positive bacteria involved in the invasion of eukaryotic cells. *Vmp* genes were sequenced among a representative set of 16SrV phytoplasma strains. VmpA and vmpB sequences both formed 3 phylogenetic clusters, out of which the cluster II and III corresponded to strains epidemically propagated by *S. titanus*. As previously reported for the '*Ca.* P. solani' *vmp*1 (Cimerman *et al.,* 2009), *vmp*B genes were shown to be submitted to positive selection pressure. Phylogenetic analysis of the repeated domains indicated that VMP of cluster II and III quickly varies by duplication of the repeated domains. The hydrophilic central part of FD92 VMPs was cloned in pET28 expression vector in *E. coli* in fusion with a polyhistidine tag and purified. Anti-VMP polyclonal antisera detected the expression of VMPs in plant and insect. VMP-A was shown to interact in farwestern-blot assays with two proteins of 55 kDa and 120 kDa of the experimental vector *E. variegatus* and the natural vector *S. titanus*. VMPs represent valuable markers for studying the adaptation of FD phytoplasma to *S. titanus* insect vector and its sequence should be a useful tool for risk assessment of epidemics in the vineyard (Desqué *et al.,* 2013).

Biology and behavior of *S. titanus*

In agreement with the Northern American origin of the FD vector, it was experimentally demonstrated by submitting eggs from a natural population to temperature variations that the majority of hatchings occurred more quickly in cold rather than in mild winter simulated conditions (Chuche and Thiery, 2009). The authors have also examined how simulated fluctuating temperatures affect the operational sex ratio of hatching as well as nymph and adult fitness (body size, weight, and developmental rate) in this leafhopper. Female eggs were more sensitive to temperature variation. There was a weaker excess of males after incubation under warm conditions. Nymph instars that hatched from eggs exposed to warm temperature were larger and bigger that those exposed to cold temperature, but developmental rate of the two populations was not affected (Chuche and Thiery, 2012).
Nymphs of *S. titanus* have an aggregating behavior on grapevine that could result from communication among them. Although the use of substrate-borne vibrations to communicate is well known in adult leafhoppers, this behavior had not been so far investigated for nymphs. It was shown that nymphs of *S. titanus* do not use species-specific vibrations neither for intra- nor interspecific communication and also did not produce alarm vibrations when facing potential predators. It was therefore concluded that their aggregative behavior is independent from a vibrational communication (Chuche *et al.*, 2011).

"Bois noir" and lavender decline phytoplasma genetic diversity and epidemiology

The "bois noir" (BN) disease associated with the presence of '*Ca.* P. solani' is endemic in France. The phytoplasma is transmitted by *Hyalesthes obsoletus*, a planthopper residing in weeds such as bindweeds (*Convolvulus arvensis*) and stinging nettles (*Urtica dioica*) which also act as plant reservoirs. '*Ca*. P. solani' is affecting solanaceous crop such as tomato, tobacco, eggplant, pepper but also sugar beet and small fruit production such as strawberry. To follow phytoplasma strains distribution and spread genotyping tools are used, mainly sequencing *sec*Y and *stamp* genes as well as *vmp*1 sequencing or RFLP (Cimerman *et al.*, 2009; Pacifico *et al.,* 2009). In France, in all BN cases S1, S4 and S6 *sec*Y genotypes are present in almost equal proportion according to a survey conducted in 2004. Most of these cases corresponded to V1 and V4 *vmp*1-RFLP patterns (Pacifico *et al.*, 2009).

A gene encoding a variable surface protein of *'Ca.* P. solani' was recently isolated and used for strain genotyping (Fabre *et al*., 2011; 2011a). *Stamp* gene sequencing allowed to differentiate 56 different genotypes in the Euro-Mediterranean basin. Phylogenetic analysis showed the existence of four main stamp genetic clusters. French strains belong to genetic clusters tuf-type b-stamp IA corresponding to strains from bindweed in western Europe and tuf-type a-stamp IV corresponding to strains propagated from *U. dioica*.

The most important damages are caused to lavender fields where *H. obsoletus* are proliferating. Lavender decline is associated with several genetic variants of '*Ca*. P. solani' (Danet *et al*., 2010), its incidence was surveyed in twenty seven lavender fields in South East France from 2008 to 2010 and declining plants were collected and submitted to a '*Ca.* P. solani' specific Taqman real-time PCR assay at spring and autumn. The incidence of lavender decline increased over seasons in most of the fields surveyed. The presence of the phytoplasma was revealed in 19% to 56% of the plants according to seasons and cultivars and its prevalence was correlated with symptom severity. Better detection of the pathogen was obtained in autumn than in spring. In autumn '*Ca*. P. solani' was detected in 56% of the declining *Lavandula angustifolia* but only in 39% of the declining *L. angustifolia* x *L. latifolia* hybrids. Genotyping of the '*Ca.* P. solani' strains detected showed that fifteen different secY genotypes were associated with the disease. Fields were infected by 3 to 7 different '*Ca.* P. solani' strains. Three strains of genotypes S17, S16 and S14 were prevalently detected in most fields and prevalence of the S17 strain increased during the survey to reach 75% in 2010. The '*Ca.* P. solani' strains detected in wild plants surrounding lavender fields were mainly of S1 and S4 genotypes, indicating that the main reservoir of the disease were lavender themselves instead of weeds as it is usually the case for other diseases associated with '*Ca.* P. solani' presence. *H. obsoletus* could be commonly captured in lavender fields and was shown to mainly harbour the S17, S16 and S14 genotypes. The strain S17 could be insect-transmitted to *Catharanthus roseus*. Two independent cases of '*Ca.* P. trifolii' infection could also be evidenced on lavender and lavender hybrid (O. Sémétey *et al.,* unpublished data).

In the past years, susceptible and tolerant lavenders (*L. angustifolia)* or *Lavandula* hybrids were proposed as disease management strategy to growers. However, the bases

for tolerance are unknown. The titers of '*Ca.* P. solani' in the leaves and shoots of different lavenders and hybrids, either sensitive or tolerant was determined by quantitative PCR and symptom severity resulted to be not correlated to the tolerance status of all cultivars tested, nor to the phytoplasma titer (Gaudin *et al.*, 2011). It is suspected that tolerance to decline may be related to a lower susceptibility to the insect vector.

European stone fruit yellows and its psyllid vectors

'*Ca*. P. prunorum' induces economic damages essentially to apricot and Japanese plum production. It was shown that *Cacopsylla pruni* present in France as vector of European stone fruit yellows have overwintering and latency stage on conifers (Thebaud *et al*., 2009). Using microsatellite markers (Sauvion *et. al.,* 2009*)*, this psyllid was discovered to encompass two highly divergent genetic groups that are morphologically similar (Sauvion *et al.,* 2007). ITS2 sequences recently showed that the two genetic groups also are highly divergent in this sequence. A specific ITS2 PCR test was designed for the assignment of individuals to either genetic group based on amplicon size. Because none of the previously assigned individuals appeared heterozygous at the ITS2 locus it was inferred that the genetic groups of *C. pruni*, whose distribution is partly sympatric, constitute biological species that have not recently exchanged genes (Peccoud *et al*., 2013).
Eleven different genotypes of '*Ca*. P. prunorum' have been detected so far in France out of the 34 detected, based on the sequencing of four house-keeping genes namely *ace*F, *pnp*, *sec*Y and *imp* (Danet *et al*., 2011). '*Ca*. P. pyri' and '*Ca*. P. mali' are present but do not cause important losses to the pome fruit production.

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Overview of the phytoplasma and vector research in Austria, Croatia and Slovenia

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Abstract

"Bois noir" phytoplasma is present in the vineyards of all three countries, as well as its well known vector *Hyalestes obsoletus*. "Flavescence dorée" phytoplasma was detected in Slovenia first, and in Austria and Croatia within the timeframe of this COST Action. Molecular epidemiology and diversity investigations confirmed considerable diversity of both phytoplasmas. Besides the expected epidemiologic cycles, interesting and potentially new pathosystem niches have been investigated. The presence of the three most important fruit tree phytoplasmas, the agents of European stone fruit yellows, pear decline and apple proliferation, and of their vectors was detected in all three countries. Novel phytoplasma detection and research methods are being used, and innovative management strategies are developed.

Key words: "bois noir", '*Candidatus* Phytoplasma solani', "flavescence dorée", '*Ca.* P. pyri', '*Ca*. P. prunorum', '*Ca*. P. pini', epidemiology, vectors.

Introduction

The phytoplasma research in Austria, Croatia and Slovenia has some common features probably due to the geographic proximity, cultural links and overlaps in agricultural practices. The focus is on grapevine and fruit tree phytoplasma infection problems, and constant improvements in relevant diagnostic approaches. This overview summarizes the main achievements in phytoplasma and vector research from 2009 onwards entailing the reader's familiarity with the previous publications. The data were gathered from the published papers and conference contributions available to the authors for the years 2009-2013.

Austria

"Bois noir" (BN) associated with the presence of "stolbur" phytoplasmas ('*Candidatus* Phytoplasma solani') is presently widespread in all vine growing regions of Austria. Surprisingly current investigations show that BN epidemiology has changed dramatically within a few years. Investigations between 2003 and 2008 frequently ascertained the

presence of tuf-type b "stolbur" phytoplasmas in *Convolvulus arvensis* and grapevines, whereas infections of *Urtica dioica* were not common. *Hyalestes obsoletus* was rare or not detectable except in Styria. The search for alternative vectors proved that the Agalliinae leafhopper *Anaceratagallia ribauti* is able to transmit '*Ca*. P. solani' to *Vicia faba* and *Catharanthus roseus* under experimental conditions (Riedle-Bauer *et al*., 2008; Sára and Riedle-Bauer, 2009; Brader *et al.,* 2014). Since 2012, in contrast, mass occurrences of *H. obsoletus* on *U. dioica* were ascertained in all vine growing provinces of Austria. '*Ca*. P. solani' tuf-type b was frequently detected in *U. dioica* but rarely in *C. arvensis*. For a better analysis of epidemiological cycles *vmp1*, *stamp* and *secY* gene analyses were included in the study. The results allowed the conclusion that presently the main epidemiogical cycle of '*Ca*. P. solani' in Austria includes *H. obsoletus* as insect vector and *U. dioica* as weed phytoplasma source. The prevalent phytoplasma genotype shows a tuf-type b restriction pattern, a *tufB* gene sequence intermediate between tuf-type a and -b and *secY*, *stamp* and *vmp1* genes of nettle associated strains (Brader *et al.*, 2014). Systematic field investigations to manage BN demonstrated high recovery rates of diseased vines after pollarding. In addition statistical analysis linked recovery rates to grapevine cultivar and plant age (Riedle-Bauer *et al*., 2009).

Scaphoideus titanus, the vector of "flavescence dorée" (FD) was detected in southeast Styria in 2004. Since then, the insect has spread and is now present in parts of south and southeast Styria and Burgenland (Zeisner, 2008; AGES, 2013). First outbreaks of FD were recorded in 2009 in southeast Styria. Later a second focus in south Styria was identified. The phytoplasma was identified on local *Vitis vinifera* cultivars, on an American hybrid, as well as on *Clematis vitalba*. Mixed infections of '*Ca*. P. solani' and FD phytoplasmas were also recorded (Reisenzein and Steffek, 2011). As a consequence models aiming to simulate the current and the potential future distribution of FD and its vector *S. titanus* were developed. The models permitted the simulation of temporal and spatial dynamics with consideration of potential global warming and different management strategies. In addition, economic impact analyses allowed the evaluation of different intervention and abatement strategies and the estimation of the potential economic impact on Austrian viticulture (Steffek *et al*., 2011; Strauss *et al*., 2012).

During the last decade losses of apricot and pear trees due to European stone fruit yellows (ESFY) and pear decline (PD) have become a major concern. Surveys on the occurrence and distribution of psyllids in pome and stone fruit orchards revealed the presence of *Cacopsylla pruni, C. pyricola, C. pyri, C. pyrisuga, C. melanoneura* and *C. picta*. Phytoplasmas were occasionally ascertained in *C. pruni,* investigations in several pear orchards revealed infection rates of pear psyllids (*C. pyricola, C. pyri* and *C. pyrisuga)* between 3 and 8%. No phytoplasma presence was observed in *C. melanoneura* and *C. picta* (Lethmayer *et al*., 2011, C. Lethmayer, pers. comm.). Within the frame of an EFSA project the current distribution of '*Ca.* P. prunorum' and '*Ca.* P. pyri' was reviewed (Steffek *et al.*, 2011a; 2012). Based on this work different management options to review the phytosanitary status of these pests in the EU were elaborated.

Presence and temporal dynamics of *C. pruni* on *Prunus armeniaca, P. domestica, P. spinosa* and *P. cerasifera* 'Nigra', as well as phytoplasma infection rates of *C. pruni* and *Prunus* spp. were studied in detail from 2011 till 2013. *C. pruni* adults were observed on *Prunus* spp. from calendar week 10 to 12 till the end of June. Zero to 11.5% of the remigrant insects and 0-3.44% of the springtime generation insects, as well as 9.8% to 63.3% of the apricot samples, 20 to 40% of the plum samples and single blackthorn samples were infected (Maier *et al*., 2013). The transmission of '*Ca*. P. prunorum' during the propagation process was tracked by grafting infected scions on certified apricot seedling rootstocks. Whip grafting in winter resulted in significantly lower infection rates (0-15%) than budding in summer (46 to 88%) (Riedle-Bauer *et al*., 2012). Effects of kaolin, paraffin, orange and fennel oils on survival and feeding of *C. pruni* were tested under laboratory conditions (Riedle-Bauer *et al*., 2011). Molecular characterization of ESFY and PD strains was carried out by analysis of *imp*, *aceF* and *hflB* genes (Paleskić *et al*., 2013).

Movement of overwintered adults in a model apricot orchard was tracked by aid of a mark and recapture study. The experiments allowed monitoring of insect spread and estimation of insect numbers in an orchard. The method might be helpful for the development of insect vector management strategies (Riedle-Bauer *et al.,* 2014).

Croatia

The BN phytoplasma impact on the vine growing is still the largest among phytoplasma detected in Croatian vineyards. Correspondingly, BN molecular epidemiology and diversity are the most investigated topics. Multigene sequence analysis including housekeeping genes *tufB*, *secY* and BN-specific genes *vmp1* and *stamp* resulted in finding BN large molecular diversity. About 20 genotypes were found in grapevines, *C. arvensis* and *H. obsoletus* samples from various geographic locations. Closed *H. obsoletus*-bindweed-grapevine pathosystems have been found, but different pathosystems at some locations were also present (Šeruga Musić *et al*., 2011; 2012; 2013).

Parallel investigations of BN pathosystems in the vine growing regions on both sides of the Croatian-Hungarian border were performed and insect diversity was found in the eastern regions, but *H. obsoletus* was the most widespread BN vector along the whole border. Also, the prevalence of BN tuf-type b and *C. arvensis* pathosystems was shown with some interesting niches containing pathosystems potentially dominated by *U. dioica* and tuf-type a of BN (Škorić *et al*., 2011).

The first FD *foci* in Croatia were recorded in 2009-2010 (Šeruga Musić *et al*., 2011a) in both commercially well-known and indigenous grapevine varieties (Škorić *et al*., 2011a; Šeruga Musić *et al*., 2012). Besides initially recorded foci west and southwest of Zagreb, two new locations with diseased vines east and southeast of the capital were reported demonstrating the FD-zone widening (Škorić *et al*., 2011a). Subsequent FD studies resulted in FD presence confirmation in *C. vitalba* and *S. titanus,* and molecular

characterization demonstrated the circulation of FD1 and FD3 strains at different locations (Šeruga Musić *et al.,* 2012).
Even though symptoms of stone and pome fruit phytoplasmas were observed in the country long ago, the molecular characterization of pathogens and the vector survey are more recent (Križanac *et al*., 2010). '*Ca*. P. pyri' and '*Ca*. P. prunorum' are the most widespread and have the highest incidence in pears and several stone fruit species, respectively. *C. pruni* has been implicated in the ESFY transmission, whilst *C. pyri* and *C. pyrisuga* were found positive for PD. Molecular diversity of fruit tree phytoplasmas was richer than expected as was confirmed by multigene sequence analysis of some local strains (Danet *et al.,* 2011). Interestingly, BN was found in pears as often as PD. Moreover, some *C. pyrisuga* samples also harboured "stolbur" phytoplasmas. The epidemiological significance of these findings is still unresolved. The finding of *C. melanoneura* and *C. picta* preceded the molecular identification of AP.
'*Ca*. P. asteris' (aster yellows phytoplasma, AY) is well known for its global and species wide distribution. Besides an ongoing investigation of AY in Lombardy poplars, a phytoplasma closely related to 16SrI-B, whose characterization is in progress, was detected in oilseed rape (Škorić *et al*., 2012) in mixed infection with *Turnip mosaic virus*. AY was also sporadically detected in grapevines, *S. titanus*, fruit trees (pear, peach, Japanese plum) and *C. pyri* vectors (Križanac *et al*., 2010).
A phytoplasma, known to infect conifer species '*Ca*. P. pini', was detected in Croatia for the first time in 2011 (Ježić *et al.,* 2012). One *Pinus mugo* and several *P. halepensis* trees in north western continental and coastal habitats respectively, were found infected by this phytoplasma (Ježić *et al*., 2013).

Slovenia

The Slovenian phytoplasma research in the last five years has been marked by the efforts to understand the grapevine interactions with phytoplasmas via biochemistry, genomic and transcriptomic approaches, including the application of bioinformatic and statistical tools (Hren *et al*., 2009; Prezelj *et al*., 2013; Rotter *et al*., 2013). In addition, several new detection protocols like real-time PCR and loop-mediated isothermal amplification have been introduced in phytoplasma diagnostics (Nikolić *et al*., 2010; VITISENS FP7 project report).
The vineyard infections with BN are very common and besides the main BN vector *H. obsoletus*, *Euscelis incisus*, *S. titanus*, *Reptalus cuspidatus* and *R. panzeri* also harbour BN (Mehle *et al*., 2011), apparently without major epidemiologic significance. Although FD has been detected in grapevine and *C. vitalba* plants since 2005 (Mehle *et al*., 2011), in the last years its rapid spread from south west to north east was observed. Therefore, a great effort was made in development of new diagnostic protocols which would allow easier and early detection of this phytoplasma (Prezelj *et al*., 2013; VITISENS FP7 project report). To investigate genetic diversity of this phytoplasma in Slovenia a

wide analysis of strains from known and possible hosts and vectors was performed. The genotyping has revealed the occurrence of FD strains similar to FD70, and belonging to subgroups FD-D and FD-C. Among them, FD-D isolates prevailed in grapevine, and only FD-C strains were detected in symptomatic as well as asymptomatic *Clematis* plants (Mehle *et al.*, 2011a). Alders (*Alnus glutinosa* and *A. incana*) and the insect *Oncopsis alni* were found to host phytoplasmas with sequence similarities to three FD clusters, as well as those similar to other alder yellows strains (Mehle *et al*., 2011; 2011a). In some alder samples mixed phytoplasma infections with different combinations were also demonstrated. Besides *S. titanus*, harbouring FD (Mehle *et al*., 2011; 2011a), the mosaic leafhopper *Orientus ishidae* was found infected with FD, increasing the complexity of the FD pathosystems (Mehle *et al*., 2010).

With the exception of symptomatic *Echinacea purpurea* plants (Radišek *et al*., 2009), '*Ca*. P. asteris' has not been detected in the country. However, a new real-time PCR assay using TaqMan minor groove binder probes for its detection was developed (Hren *et al*., 2010).

The presence of AP, PD and ESFY is long time known, as well as the wide distribution of their common vectors, and does not differ from the usual scenarios. Besides in apple, AP was detected in cherry, apricot and European plum. ESFY was detected in *C. pruni*, PD in *C. pyri* and *C. pyricola* (Mehle *et al*., 2011).

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Overview of the phytoplasma and vector research in Bosnia and Herzegovina, Bulgaria, FYR Macedonia, Romania and Serbia

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Abstract

These countries showed increasing interest in phytoplasma research. Detection and identification of phytoplasmas and/or some of their insect vectors in fruit trees and grapevine was achieved. Deep study on multigene differentitation of aster yellows and "stolbur"-related phytoplasmas were also performed and diversity of some of the detected strains was demonstrated.

Key words: grapevine, fruit trees, aster yellows, "bois noir", "flavescence dorée", insect vectors.

Introduction

Phytoplasma associated diseases are long-time known and described in these countries, however only recently specific researches were founded and carried out in order to identify the phytoplasmas present in the majority of agricultural relevant crops. The availability of molecular tools together with the expertise gained also through the Action allow to start the management of these diseases in the best way, after discovering of phytoplasma presence and also vector related to several of the diverse phytoplasmas studied. In all five Countries the presence of "bois noir" associated mainly to "stolbur" tuf-type b phytoplasmas was reported, as well as the widespread presence of corn reddening associated with molecularly undistinguishable "stolbur" phytoplasmas. Wide presence of apple proliferation and European stone fruit yellows or pear decline is also a quite common feature in the majority of these Countries. A number of insect vector was also reported and studied mainly in Serbia and Bulgaria.

Bosnia and Herzegovina

The phytoplasma associated diseases detection in Bosnia and Herzegovina was greatly increased trough the action as well as the research on potential insect vectors. Main research was carried out on phytoplasmas infecting grapevine, and it was mainly related with "bois noir" strain characterization, however considering the presence in southern districts of *Scaphoideus titanus* (Delić *et al*., 2007) extensive monitoring were also performed. Presence and diffusion of grapevine yellows phytoplasmas was investigated in 2008 and 2010 in twelve vineyards located in two viticultural areas of Srpska where samples from different cultivars and weeds were collected for molecular analyses together

with some potential insect vectors. Phytoplasmas belonging to 16SrXII-A group were confirmed to be associated with grapevine yellows, while in a *Clematis vitalba* sample a phytoplasma belonging to 16SrV-C subgroup was identified. RFLP analysis of the tuf gene indicated the presence of the tuf-type b of "stolbur" phytoplasmas. *Dictyophara europaea* and *Reptalus cuspidatus* were identified but molecular analyses did not show phytoplasma presence in the tested insect samples (Delić *et al*., 2011).

Bulgaria

Rubus fruticosus in the region of Plovdiv showing severe stunting and bushy aspect resulted infected with a "stolbur" phytoplasma belonging to tuf-type b (Bobev *et al*., 2013). The same phytoplasma was reported in *Convolvulus arvensis* and *Prunus avium* (Avramov *et al*., 2011). Epidemiological studies on "bois noir" presence as well as on insect vectors indicate the presence of *Hyalesthes obsoletus* and *Reptalus* spp. infected with "stolbur". The presence of "flavescence dorée" vector *S. titanus* was also reported (Avramov *et al*., 2011a). In fruit trees all three main phytoplasmas belonging to the apple proliferation group were identified. The spread and the frequency of individuals from the psyllid genus *Cacopsylla* in four fruit tree orchards located in three different regions indicated that all psyllid species described as vectors of fruit tree phytoplasmas were present. *Cacopsylla pruni* specimens from two different regions were carrying '*Candidatus* Phytoplasma prunorum' (Etropolska *et al*., 2011).

FYR Macedonia

In this Country research was carried out on grapevine yellows presence and spreading in 13 locations of seven regions and "bois noir" tuf-type b was always identified (Mitrev *et al*., 2011).

Romania

"Stolbur" phytoplasmas were detected in several crops such as potato, tomato, pepper, eggplant and beet (*Beta vulgaris*); they were also detected in weeds, particularly *C. arvensis*, *Cuscuta* sp., and *Euphorbia falcata*. All infected samples had the same RFLP profile corresponding to the tuf-type b. "Stolbur"-affected potato plants produced a large number of spongy tubers that resulted in commercially unacceptable potato chips upon processing (Ember *et al*., 2011).
Apple proliferation and pear decline were also detected respectively in apple and pear.

Serbia

Phytoplasmas studied in Serbia enclose "stolbur" (Pavlović *et al*., 2011; 2012; Mitrović *et al*., 2013), European stone fruit yellows, pear decline, aster yellows, 16SrII-E,

16SrIII-B, bermudagrass white leaf, '*Ca.* P. ulmi', '*Ca.* P. rhamni'. The newly described '*Ca.* P. convolvuli' was also reported in bindweed together with "stolbur" phytoplasmas (Martini *et al*., 2012). Strains of phytoplasmas were also detected by multigene approach in "flavescence dorée" and apple proliferation samples from Serbia (Bertaccini *et al*., 2009; Paltrinieri *et al*., 2010; 2011; 2012). Epidemiological studies on "stolbur" and aster yellows insect vectors have been conducted (Jović *et al.*, 2009; Drobnjaković *et al.*, 2010). A new marker, groEL gene, has been developed and used for analyses of some of the examined phytoplasmas (Mitrović *et al*., 2011). Transmission trials carried out with a *H. obsoletus* population collected on nettle near a corn field allow the verification of the cixiid ability to transmit the corn reddening disease. Amplification of phytoplasma DNA was obtained after nested PCR assays from corn samples tested after 40 days from insect caging and from batches of *H. obsoletus* collected on nettle from the same population used for cage-transmission. RFLP analyses allow identification of the detected phytoplasmas as "stolbur" in both, corn experimentally infected and *H. obsoletus* specimens from the population used for transmission (Mori *et al*., 2013).

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Review of occurrence of phytoplasmas in Spain, Portugal and Malta

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Abstract

Phytoplasmas detected in Spain and Portugal are associated with relevant diseases in grapevine and fruit trees for which also main insect vectors were identified. Malta carried out small surveys only on grapevine phytoplasmas that resulted not present. Other phytoplasmas such as aster yellows, "stolbur", 16SrIII, 16SrVI and '*Candidatus* Phytoplasma pini' were detected in horticultural crops and/or in forest or urban trees.

Key words: grapevine, fruit trees, aster yellows, "bois noir", "flavescence dorée".

Introduction

Phytoplasma associated diseases are of relevant importance in southern European countries such as Spain and Portugal and during the Action extensive surveys allow to verify the presence of a number of them in diverse plant species of agricultural and environmental relevance. For the majority of phytoplasmas or phytoplasma groups detected insect vectors were also identified, facilitating their management after knowing their epidemiological spreading features.

Phytoplasmas and insect vectors involved in grapevine yellows diseases

Two diseases in cultivated grapevines, "flavescence dorée" (FD) and "bois noir" (BN), have been reported in Spain and Portugal. FD is the most aggressive disease but the symptoms of both are similar and mainly involve plant decline, desiccation of inflorescences, withering of clusters, irregular maturation of the wood, leaf rolling, vein banding, leaf yellowing on white variety and leaf reddening on red variety. The most spread disease in Spain is BN, it is present in different vine growing regions

such as "La Rioja", Alava, Navarre, Catalonia and Aragón. The first outbreak of FD in Spain was in 1996 in the French border of northeast of Catalonia and by now the disease is eradicated. FD is the most dangerous disease associated with phytoplasma presence in Portugal where it was detected since 2002 (Sousa *et al*., 2009) after the first identification of its vector, the ampelophagous leafhopper *Schapoideus titanus* Ball in 1998. The phytoplasma associated with FD in Spain and Portugal belongs to the ribosomal subgroup 16SrV-D. This strain shows also epidemic activity in other countries such as France and Italy. '*Candidatus* Phytoplasma solani' is the phytoplasma associated with BN. The study of the geographic distribution of "stolbur" isolates (tuf-type) in grapevine plants showed that tuf-type a was identified in La Rioja and Navarre and tuf-type b in Catalonia, Aragón and Navarre. In *Hyalesthes obsoletus* Signoret only tuf-type b was identified (Batlle *et al.,* 2009).

In Spain *S. titanus* is present in the north of the country: Catalonia (northeast) and Galicia (northwest). The limit in the distribution of this insect seems to be the south of Catalonia, any individual has been captured in the viticultural areas of the south of Catalonia (Ribera d'Ebre and Terra Alta). In Portugal, *S. titanus* is present in northeast and central regions as well in Madeira island.

The vector of BN, *H. obsoletus*, is present in all areas were also BN is present, but in low density populations. The highest number of individuals was recorded in Navarre and Aragón, whereas in Álava and Catalonia, the population of *H. obsoletus* was lower. The peaks of population take place between June 6th and July 14th.

In Malta small surveys were carried out in the main grapevine growing areas from 2009 and phytoplasma presence was not detected. Monitoring about insect vectors was not carried out.

Phytoplasmas and vectors involved in fruit tree diseases

European stone fruit yellows is widespread in Spain. '*Ca.* P. prunorum' has been identified in plum, apricot, nectarine, peach and almond crops. The vector *Cacopsylla pruni* was identified for the first time in Spain in fruit areas near Barcelona in 2003. The population of *C. pruni* is low in Spain, showing higher population in Extremadura and Catalonia (Barcelona and Tarragona) and lower in Aragón and Valencia.

Pear decline disease is widespread in several pear fruit areas of Spain. The main affected varieties are Llimonera (Jules Guyot), Abate Fettel and Bartlett. Blanquilla variety shows the lowest incidence of the disease. Apple proliferation is spread only in the north of Spain, Asturias and Basque country. *'Ca*. P. pyri' and '*Ca*. P. mali' are present in pear and apple orchards in Spain; '*Ca.* P. pyri' was recently identified also in the central region of Portugal (Table 1).

Cacopsylla pyri is, as in other Mediterranean areas, the pear decline vector in Spain and Portugal. It is also a pest in pear orchards of both countries and mainly the only *Cacopsylla* detected in the orchards.

Sampling carried out in apple plots of different geographic areas of Spain indicated the presence of the two species of *Cacopsylla* reported as vectors of the apple proliferation disease. The evolution of *C. picta* and *C. melanoneura* populations showed two peaks, one for adults re-immigrants in early April and the other for new generations between June and July (Table 2). The populations of *C. picta* are higher than those of *C. melanoneura* (Laviña *et al.,* 2011).

Table 1. Presence of phytoplasma diseases in fruit trees and grapevine in Spain.

Region	Phytoplasma	Strain	Phytoplasma disease	Incidence	Host/s
Spain	'*Ca*. P. pyri'		PD	medium	pear
Spain	'*Ca*. P. prunorum'		ESFY	medium	plum, apricot
Spain	'*Ca*. P. prunorum'		ESFY	low	peach, nectarine
Basque country/ Asturias	'*Ca*. P. mali'		AP	low	cider
Spain	'*Ca*. P. solani'	tuf-types a and b	"bois noir", "stolbur"	low	grapevine, parsley, strawberry, carrot ……
North Catalonia	"flavescence dorée"	16SrV-D	FD	eradicated	grapevine

Table 2. Phytoplasma vectors identified in Spain and Portugal.

Vectors	Population density		Phytoplasma		Infection rate		Vector/ plant hosts
	Spain	Portugal	Spain	Portugal	Spain	Portugal	
C. pyri	high	high	'*Ca.* P. pyri'	'Ca. P. pyri'	6%	nd	pear
C. pruni	low	-	'*Ca*. P. prunorum'	-	10-30%	-	*Prunus mahleb; P. spinos*a; *Prunus* sp.
C. picta	medium	-	'*Ca*. P. mali'	-	30%	-	*Malus* sp.
H. obsoletus	low	?	'*Ca*. P. solani'	'*Ca*. P. solani'	30-80%	nd	*Convolvulus* sp.; *Lavandula* sp.; *Solanum nigrum* Portugal: grapevine
S. titanus	medium	high	FD	FD	n.d	high	grapevine

Other phytoplasmas detected

Phytoplasmas belonging to the "stolbur" group (16SrXII) were detected in willow (*Salix babylonica* Linn) showing yellows ball-like structures and small leaves symptoms collected in the east of Spain (Alfaro *et al*., 2011), in *Dianthus caryophyllus,* in solanaceous and in other horticultural crops.

Phytoplasmas clustering in the 16SrIII group, '*Ca.* P. pruni', were detected in faba bean (*Vicia faba* L.) plants showing symptoms of shoe stringed leaves, phyllody and flower abortion in the south of Spain (Castro *et al*., 2004). *'Ca*. P. pini' has been detected in *Pinus halepensis* in the Mediterranean coast of Spain. Phytoplasmas related to group 16SrVI, '*Ca.* P. trifolii', where identified in *Capsicum annuum* plants showing short internodes and green flowers buds. '*Ca*. P. asteris' has been detected in ornamental and horticultural plants.

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Phytoplasmas and phytoplasma vectors in Greece, Israel, Italy and Turkey

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Abstract

A number of phytoplasma diseases were reported for the first time in both Greece and Turkey, while in Italy beside some new finding the work was mainly carried out to verify epidemiological features of grapevine and fruit tree phytoplasmas towards their sustainable management. In Israel there was confirmation of phytoplasma presence mainly in horticultural crops and identification of possible insect vector species.

Key words: grapevine, fruit trees, '*Candidatus*' Phytoplasma, molecular characterization.

Introduction

The improvements on phytoplasma and vector knowledge in Italy, Greece, Turkey and Israel obtained in the frame of the COST FA0807 are of great impact for the Mediterranean areas of these Countries and for the EU as well. The information concerns new plant hosts of phytoplasmas, new vectors, development of new techniques for pathogen detection, and production of novel tools and strategies for disease containment. Also, the characterization of phytoplasma infecting already established hosts allowed to give more detailed information concerning taxonomy, etiology and epidemiology of several crops including grapevine, fruit tree species, cereals and ornamental plants. In particular a new phytoplasma infecting bindweed has been found and described by Martini and colleagues (2012).

Grapevine

Grapevine appears to be the most investigated crop in terms of phytoplasma characterization and of phytoplasma group and subgroup involved in the grapevine yellows (GY) etiology. For "flavescence dorée" (FD) interesting achievements allowed Filippin and colleagues (2009) to identify different plant species as new hosts of the phytoplasma agent such as *Clematis vitalba* and *Ailanthus altissima*. Also, the same research group showed the capacity of *Dictyophara europaea* to transmit the FD phytoplasma from *C. vitalba* to grapevine. FD phytoplasma characterization has been also conducted in different areas of Italy by other groups using ribosomal and non ribosomal genes in single and multilocus sequence analysis. The use of these techniques made it possible to draw a more precise picture of the presence and the distribution of

FD phytoplasma strains in Italy (Bertaccini *et al*., 2009). Recently in Turkey has been also detected for the first time the presence of 16SrV and 16SrIX phytoplasmas (Ertunc *et al*., 2013), the latter phytoplasma group is the same of phytoplasmas associated with a tremendous diseases of almond and other stone fruit trees in EU geographic contiguous Countries such as Lebanon.
Also for "bois noir" (BN) several works underlined the molecular diversity among the BN stains based on analysis of different phytoplasma genes (Pacifico *et al*., 2009), helping to investigate the presence of BN in wild plants, mainly weeds, in vineyard and their role as reservoir for the phytoplasma and its vector *Hyalesthes obsoletus*. The BN ecology in fact is critical for the disease management in particular for the production of healthy plant material to be provided to nursery companies and for surveys and tests to be carried out by the phytosanitary services. The BN phytoplasma was detected and studied in Israel, Italy and Turkey.

Fruit trees

Among the plant quarantine phytoplasma apple proliferation (AP) took an important part in the research activities in Italy, Turkey and Greece often related to outbreaks of the disease such as in Italy and Greece (Rumbou *et al*., 2011). The phytoplasma characterization have been accompanied to the investigation on vector/s ecology allowing to draw a more precise map of the disease in Italy based on phytoplasma strain information on 16S rDNA and multilocus sequence analysis (Casati *et al*., 2011). In Turkey also European stone fruit yellows and pear decline were studied together with other phytoplasmas infecting fruit trees.

Israel

Symptoms related to phytoplasma presence were observed in several ornamental and horticultural crops located in a number of production areas of Israel. Molecular analyses focused on carrot revealed that a high percentage of symptomatic plants tested positive for *Spiroplasma citri*. Some of the plants were infected by *S. citri* and a phytoplasma. Leafhopper species known to vector phytoplasmas and/or spiroplasmas, have also been trapped in several locations (Gera *et al*., 2011).

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Chapter 2

The phytoplasmas and phytoplasma vectors in COST FA0807 international Countries


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Relevant phytoplasma diseases in COST FA0807 International Countries

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Abstract

A short report about main phytoplasma-related activity carried out in these Countries is presented in relationship with COST activity and collaborations in the frame of the action.

Key words: Epidemic, management, phytoplasma disease, insect vector, prevention.

Introduction

The International Countries Institutions approved during the life of this action are 20 (Figure 1) with about 50 participants in total (14% of the Action participants). These countries interacted actively sharing relevant information about disease management, hosting some STSMs and producing joint publications with COST members of the Action. A dedicated scientific journal "Phytopathogenic Mollicutes" printed in India, available also on line, and edited between Italy and India was also developed and started to be printed during the Action. It published full papers presented at the meeting on dissemination of management strategies held in Barcelona and will continue to publish research related to phytoplasmology. Members of the majority of International Countries interacted actively attending some of the meetings held during the Action and presented papers (Thailand, India, Saudi Arabia, Brazil, Chile, Canada, Colombia and Iran). The four Institutions from four countries with reciprocal agreement contributed to the network by adding important data and information about detection, vector identification and management strategies for crops and species that are present also in COST Countries or that could be imported or traded into, since they represent relevant agricultural environments having commercial trading with EU (South Africa, Argentina, New Zealand and Australia). Selected activity carried out by some Countries is presented in this chapter. Specific significant achievements related to the Action in some of the other International Countries are summarized below.

South America

Chile. The research was focused on grapevine and fruit trees phytoplasmas in which "stolbur", X disease, ash yellows, and aster yellows were mainly detected (Gonzales *et al*., 2011; Longone *et al*., 2011). Some potential insect vectors were identified and tested to verify phytoplasma presence mainly in vineyards. In *Amplicephalus curtulus* Linnavuori & De Long, phytoplasmas belonging to subgroup16SrI-B and 16SrXII-A were identified, while in *Paratanus exitiosus* (Beamer) phytoplasmas of the subgroups

16SrI-B, 16SrVII-A and 16SrXII-A were detected. Phytoplasmas belonging to subgroup 16SrI-B and 16SrVII-A were identified in *Convolvulus arvensis* L. and *Polygonum aviculare* L., to subgroup 16SrXII-A in *C. arvensis*, and to subgroup16SrVII-A in *Galega officinalis* L. (Fiore *et al*., 2012). The epidemic situation appears to be mainly linked to environmental factors and there are not severe outbreaks, probably also related to the strict quarantine control and the reduced importation of propagation materials.

Brazil. A number of plant species are affected by phytoplasma diseases, comprising wild and cultivated crops. Amongst the recently botanic species reported as natural phytoplasma host is sweet orange (*Citrus sinensis*) with huanglongbing symptoms in which '*Candidatus* Liberibacter' species are described as major agents (Gasparoto *et al*., 2012). The majority of phytoplasmas identified in Brazil belongs to groups 16SrI and 16SrIII (Montano *et al*., 2011). Relevant studies are also carried out to find a sustainable management of the corn stunt disease that is associated with phytoplasma and spiroplasma presence (de Oliveira *et al*., 2013).

Africa

Mauritius. Severe disease outbreaks were reported in tomato, onion, watercress and okra cultivations and associated phytoplasmas were aster yellows and "stolbur" (Gungoosingh-Bunwaree *et al*., 2010; 2011; 2013). One interesting feature is the genetic variability found in the detected strains. Research are in progress focused on transmission ways of the detected phytoplasmas in that restricted environment (small island).

South Africa. Grapevine yellows is a widespread phytoplasma disease and symptoms were observed for the first time in South Africa in 2006. Since then, the agent associated with the disease has been identified as aster yellows (AY) phytoplasma (Engelbrecht *et al*., 2010; Carsterns *et al*., 2011). Symptoms of the disease initially occurred on grapevines in two production regions, Olifants River and Wabooms River, but recently it spread also near Robertson and Trawal in the Western Cape Province. *Mgenia fuscovaria* (Stal) was identified as a vector for the disease (Douglas-Smith *et al*., 2010). Monitoring and eliminating infected plants represented the main management way to contain the epidemic. More joint studies are in progress to better characterize the epidemic aster yellows strain.

Asia

Iran. Witches' broom disease of lime (WBDL) associated with '*Candidatus* Phytoplasma aurantifolia' presence, is responsible for major losses of Mexican lime trees (*Citrus aurantifolia* L.) and it is among the major phytoplasma problems in the country. A comprehensive program to contribute in controlling this destructive agent was carried out enclosing genomics, transcriptomics, epigenomics, proteomics and metabolomics analyses that were able to provide some insight into plant stress tolerance mechanisms (Mardi *et al*., 2011). The recent production of a specific antiserum with new technologies is also helping in massive detection of infected citrus plants and therefore improved the disease management in this country (Shahryari *et al*., 2011; 2013).

Azerbaijan. During a survey on temperate fruit tree orchards in the Guba region North of Azerbaijan, a yellows on peach was associated with a '*Ca.* P. brasiliense' strain that was molecularly characterized. It represents the first strain of this phytoplasma studied in a plant other than hibiscus and elsewhere than Brazil (Balakishiyeva *et al*., 2010).
Thailand. Sugarcane white leaf (SCWL) is the most destructive known sugarcane disease in Asia, especially in Thailand and is transmitted by the leafhoppers *Matsumuratettix hiroglyphic*us (Mutsumura) and *Yamatotettix flavovittatus*. So far there is no sugarcane resistant variety or effective method to control this disease. Bacterial symbionts in *M. hiroglyphicus* were characterized to exploit possibility of their use for insect population control method. Dominant bacterial types found were an unknown Betaproproteobacterium (BAMH) and '*Candidatus* Sulcia muelleri'. In addition culturable bacteria isolated and cultured include Gram-positive *Bacillus* spp. and Gram-negative *Pseudoacidovorax* spp. (Wangkeeree *et al*., 2012).

Oceania

New Zealand. '*Ca.* P. australiense' occurs in New Zealand and Australia where it is associated with plant diseases in native, weed and crop plants. Between January 2009 and July 2010, four new diverse hosts of '*Ca*. P. australiense' have been identified in New Zealand: potato, Jerusalem cherry, swan plant and celery, as well as in a new disease in boysenberry. Partial *tuf* gene sequence analysis of 29 strains from the new plant hosts revealed that they belong to two separate subgroups, tuf variant VII and tuf variant IX. Two of the strains, one from potato and the other from celery, contained a mixed infection of both phytoplasma subgroups (Liefting *et al.*, 2011).

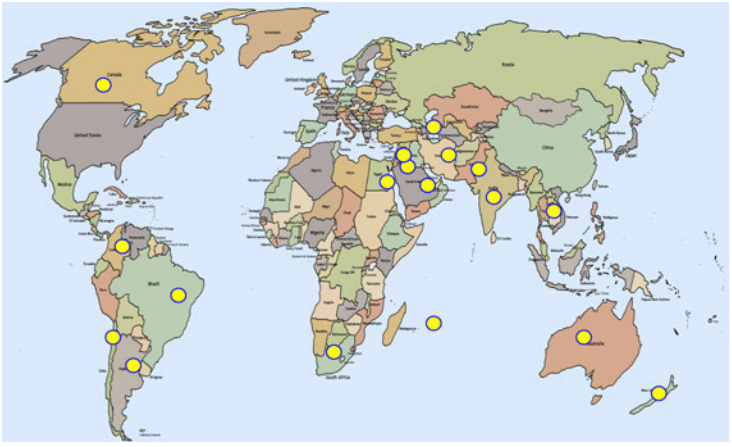

Figure 1. International countries participating in the FA0807 Action: Canada, Colombia, Chile, Brazil, Argentina, South Africa, Mauritius, Egypt, Saudi Arabia, Lebanon, Jordan, Iran, Azerbaijan, Pakistan, India, Thailand, Australia and New Zealand.

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Almond witches' broom phytoplasma: disease monitoring and preliminary control measures in Lebanon

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Abstract

A national survey was conducted in Lebanon in 2009/2010 and updated in 2012 in order to determine the extent of spread of almond witches' broom disease associated with '*Candidatus* Phytoplasma phoenicium' in the country, as well as to conduct preliminary research about the disease epidemiology and further molecular characterization of the phytoplasma. Over four years of continuous work, Lebanese and Italian efforts were joined to map the endemic areas as opposed to focus areas, to discover the phytoplasma vector(s) and to elaborate preliminary management plans.

Key words: phytoplasma, stone fruits, nursery, control measures.

Introduction

'Candidatus Phytoplasma phoenicium', a member of the 16S rDNA phytoplasma group IX, is considered the aetiological agent of almond witches' broom (AlmWB) disease, which caused in Lebanon the death of more than 100,000 almond trees in the last decade (Abou-Jawdah *et al.,* 2002). In the last few years, severe infections, frequently associated with a noticeable yield reduction, have also been observed on peach and nectarine trees (Abou-Jawdah *et al*., 2009). Almond witches' broom determines on almonds the death of the trees in a few years, causing impressive economical losses for the farmers, drastically reducing the production. Moreover, since the disease is spreading

also in nectarine and peach orchards, it represents a very dangerous threat not only for the Lebanese, but also for all the Mediterranean cultivations of these stone fruits.
A national survey was conducted in Lebanon in 2009/2010 and updated in 2012 in order to determine the extent of spread of almond witches' broom disease (AlmWB) associated with '*Candidatus* Phytoplasma phoenicium' presence in the country, divided in 26 districts, where local offices of the "Ministry of Agriculture extension services" are located. Training sessions for sixty technicians have been conducted in order to train the extension service personnel about the disease symptoms and the sample collection methods.

The national survey of orchards and nurseries

During 2012, according to the national census, all 561 stone fruit cultivated regions have been visited, in order to collect leaf samples for the detection of phytoplasma presence. During the survey, 1,419 orchards have been monitored; each orchard was located by GPS, in order to record its position and to draw a regional map on the spread of the disease in the area using the GIS (Geographic Information System) software. During the visits all the trees present in each orchard were monitored for almond witches' broom symptom presence. In order to confirm the infection of symptomatic plants or to verify the presence of the pathogen in trees showing doubtful symptoms, an average of 15 leaf samples were collected in each district and processed for '*Ca.* P. phoenicium' identification. A common protocol of detection has been established and shared among three laboratory units at AUB, LARI and USEK departments, by using the semi-specific primer pair AlWF2/AlWR2 (Abou-Jawdah *et al*., 2003). A total of 231 samples were collected and processed.
A complete survey of 279 mother plants at the LARI-Tal Amara station (used for the Lebanese certified seedling production), as well as of 136 registered and non-registered nurseries has been implemented, in order to check the phytosanitary status of the nursery sector in Lebanon. A scientific committee has been created to discuss and share, every 6 months, the partial achieved results and the strategies to be implemented among all the involved partners and subjects.
The research about the phytoplasma vector(s) is still on going and in order to reduce the *foci* of infection in the country, an eradication plan has been first implemented in a pilot area (Zahle district) in 2012 and then extended to seven districts in 2013.

Results

Almond, peach and nectarine orchards were severely affected and the number of infected trees was increasing. Out of 231 samples, 158 tested positive to the analyses (Table 1). The presence of the disease, detected in 16 out of 26 districts in 2010, was also confirmed in two new districts in 2012 (Bent Jbeil and Bcharre); positive samples have been detected in stone fruit orchards located in 221 out of 561 monitored villages (Figure 1).

Figure 1. Map of the almond witches' broom spread in the Lebanese regions.

Table 1. Results of the PCR analyses conducted in 2012 on stone fruits.

	Location	Number of collected samples	Positive samples (16SrIX specific PCR)
North Lebanon	Akkar	12	9
	Donniyé	13	7
	Koura	9	8
	Tripoli	1	1
	Batroun	20	17
	Bcharré	3	2
	Zgharta	9	9
	Jbeil	9	5
	Kesrouane	5	1
	Metn	3	0
	Baabda	2	0
South Lebanon	Aley	4	2
	Chouf	12	8
	Jezzine	18	13
	Saida	3	1
	Sour	5	2
	Marjeyoun	20	15
	Hasbaya	17	11
	Bent Jbeil	6	3
	Nabatiyé	4	2
Bekaa Valley	Hermel	6	5
	Baalback	15	7
	Zahlé	7	3
	West Bekaa	13	13
	Rachaya	15	14
Total		231	158

Two additional 16SrIX subgroups were detected (-F and -G) in addition to the prevalent subgroup -D (Molino Lova *et al.*, 2011).

Concerning the nursery sector, 282 samples were collected and analyzed from registered and non-registered nurseries as well as 279 samples from mother trees in Tal Amara station. All the mother plants were negative for AlmWB phytoplasma presence. However, five out of 136 visited nurseries were found selling seedlings that tested positive for AlmWB; a prompt decision from the Lebanese Ministry of Agriculture was adopted to destroy all the seedlings.

Extension workshops have been organized for 593 farmers and 47 nurserymen. In 2012, pilot areas were selected, elimination of 182 AlmWB-infected trees and their replacement have been implemented in 14 villages. Again during 2013, a plan for tree

elimination and crop replacement has been implemented by the Ministry of Agriculture to reduce the impact of the disease and to support the rural affected areas in Baalback, West Bekaa, Rachaya, Hasbaya, Marjayoun, Bent Jbeil and Jezzine districts. A total of 6,206 infected trees have been eliminated until the end of October, 2013 in 91 villages. The 674 involved farmers will receive from the Ministry of Agriculture new fruit tree seedlings, adapted to the different regions, in compensation for their economic losses.

Conclusions

The complex epidemiology of phytoplasma diseases, mainly of '*Ca*. P. phenicium' necessitates cooperation of researchers in different disciplines in order to understand the disease epidemiology and to develop appropriate disease containment and management strategies. Legislation and control measures are urgently necessary to limit the diffusion of almond witches' broom in Lebanon but also to avoid its spread to other Middle East countries and to Europe.

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'*Candidatus* Phytoplasma solani' associated with grapevine "bois noir" disease in Jordan

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Abstract

During a survey carried out in Jordanian vineyard plants showing typical grapevine yellows disease symptoms were observed. Total DNA was extracted from leaf veins for PCR analysis. PCR products sequenced shared 99.5% sequence identity with '*Candidatus* Phytoplasma solani'. Phylogenetic analyses confirmed the affiliation of phytoplasma strains identified in grapevine to '*Ca*. P. solani' (subgroup 16SrXII-A), opening an avenue to future studies on the dissemination and impact of "bois noir" disease in Jordan vineyards.

Key words: grapevine yellows, disease, Jordan, PCR/RFLP, sequencing.

Introduction

"Bois noir" (BN) is a disease of the grapevine yellows (GY) complex associated with '*Candidatus* Phytoplasma solani' (subgroup 16SrXII-A) presence, and mainly transmitted by the polyphagous cixiid *Hyalesthes obsoletus* Signoret (Alma *et al*., 1987; Quaglino *et al*., 2009; 2013). BN is largely spread in Europe and in countries of the Mediterranean basin, but in the last years its presence was reported also in South America, South Africa, Iran and China (Botti and Bertaccini, 2006; Gajardo *et al*., 2009; Karimi *et al*., 2009; Duduk *et al*., 2010). In the present work BN presence in Jordan is reported.

Materials and methods

During a survey carried out in vineyards in August and October 2012, grapevine plants showing typical grapevine yellows disease symptoms were observed. In the same vineyards, bindweed plants showing stunting and leaf chromatic alteration were found, suggesting the involvement of phytoplasmas in the disease etiology. Total DNA was extracted from leaf veins of 25 symptomatic and two asymptomatic grapevines, and

from five symptomatic and two asymptomatic bindweeds. Phytoplasma detection by nested PCR assays was carried out using universal primer pairs P1/P7 followed by R16F2n/R16R2 (Lee *et al*., 1998). DNAs from periwinkle plants infected by '*Ca*. P. asteris' strain SAY (group 16SrI), '*Ca*. P. solani' strain STOL (group 16SrXII), and '*Ca*. P. ulmi' strain EY1 (group 16SrV), were used as positive controls. DNAs from healthy periwinkle and reactions without template DNA were employed as negative controls. R16F2n/R16R2 PCR products amplified from grapevine and bindweed samples were sequenced and analysed through the software BlastN (sequence identity), BioEdit (alignment), iPhyClassifier (group/subgroup affiliation), and MEGA 5 (phylogeny).

Results and discussion

Amplification of a band of the expected size (1,250 nt) in three grapevines and in five bindweeds, and in the positive controls through 16S rDNA nested PCRs was observed. No amplification was obtained with DNA from 22 symptomatic grapevines, probably because samples were collected late in the growing season and phytoplasma distribution in plants was non-uniform (Skoric *et al*., 1998; Constable *et al*., 2003), nor from asymptomatic plants and negative controls. PCR products were sequenced by commercial services in Italy (Primm, Milan) and Korea (Macrogen Inc., Soul). Representative 16S rDNA nucleotide sequences were deposited in NCBI GenBank with accession number KC835139 (from grapevine) and KC835140 (from bindweed). The 16S rDNA nucleotide sequences shared >99.5% sequence identity with '*Ca*. P. solani' reference strain STOL (AF248959), and carried identical STOL-unique signature sequences and distinguishing sequence blocks (Quaglino *et al*., 2013; Salem *et al*., 2013). Phylogenetic and in *silico* RFLP analyses confirmed the affiliation of phytoplasma strains identified in grapevine and bindweed in Jordan to '*Ca*. P. solani' (subgroup 16SrXII-A), opening an avenue to future studies on the dissemination and impact of "bois noir" disease in Jordan.

These studies may add new information about BN, previously reported in neighboring countries (Davis, *et al*., 1997; Choueiri *et al*., 2002; Contaldo *et al*., 2011). Further studies will investigate the role of *Hyalesthes obsoletus* Signoret, a polyphagous cixiid responsible for the BN phytoplasma transmission in Europe, and other possible insect vector(s) in the BN spread in Jordan.

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Potential vectors of '*Candidatus* Phytoplasma phoenicium' in Lebanon

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Abstract

Almond witches' broom disease spread rapidly over the Lebanese territory in the last years causing severe economic damages. Previous investigations on insect vectors were focused on leafhoppers. A survey carried out through the years 2010-2012 was aimed at the cixiid-fauna present in Lebanese almond and nectarine orchards and its natural infection by phytoplasmas. The list of the collected genera and, among them, the carriers of '*Candidatus* Phytoplasma phoenicium' is presented.

Key words: Almond witches' broom , cixiids, stone fruits, vectors, field samplings.

Introduction

The presence and rapid spread of almond witches' broom (AlmWB) in Lebanon, causing serious economic losses, entails the activity of one or more insect vectors. Previous investigations focused on the family Cicadellidae showed *Asymmetrasca decedens* Paoli to be the most abundant species in stone fruit orchards and able to carry '*Candidatus* Phytoplasma phoenicium'. Although other leafhopper species, usually present in very low density in the orchards and belonging to the subfamilies Typhlocybinae and Deltocephalinae were found positive to the phytoplasma as well, their vector ability has never been proved by transmission trials (Abou-Jawdah *et al*.; 2003, Dakhil *et al*., 2011).
Beside the leafhoppers some cixiid (planthopper) species are known as vectors of phytoplasmas to various crops (Maixner, 1994; Jović *et al.*, 2007; Pinzauti *et al.*, 2008). For this reason this survey was focused on the cixiid-fauna present in almond and nectarine orchards of Lebanon with particular attention on their natural infection by phytoplasmas.

Materials and methods

The project was realized through the years 2010-2012 based on intensive samplings carried out by means of Malaise and yellow sticky traps. The survey took place in two AlmWB infected orchards, one in the north and one in the south of Lebanon, planted with almond and nectarine trees, respectively. Additional direct samplings with a hand-held D-vac were performed in the same key-orchards and their surroundings (Figure 1). All cixiids captured with the different methods were identified by morphological features and subsequently analysed for phytoplasma detection.

Total DNA was subjected to direct and nested PCR assays, using the primer pair AlWF2/AlWR2 (Abou-Jawdah *et al.*, 2003) and the primer pair P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996), followed by the primers R16F2n/R16R2 (Gundersen and Lee, 1996) universal for phytoplasmas. Nested PCR products were then sequenced and analysed by *in silico* RFLP to identify the 16Sr group/subgroup.

Figure 1. a) Malaise trap in Kfarkela orchad; b) yellow sticky trap in Kfarkela orchard; c) and d) direct sampling by means of D-Vac.

Results

The following cixiid genera were collected during the field samplings: *Cixius*, *Tachycixius*, *Eumecurus*, *Oliarus*, *Pentastira*, *Pentastiridius* and *Hyalesthes*. Several species were distinguished within each genus the taxa of which are in many cases still unclear.
The expected fragment of approximately 390 bp was obtained with the primer pair AlWF2/AlWR2 in the four genera *Cixius*, *Tachycixius*, *Eumecurus* and *Hyalesthes*. Nucleotide sequence analyses confirmed the presence of '*Ca.* P. phoenicium'-related phytoplasmas while *in silico* RFLP analyses on R16F2n/R16R2 amplicons evidenced that the strains infecting insect specimens in Lebanon belong to the subgroup 16SrIX-B.

Discussion

These results highlight the role of planthoppers in the epidemiology of AlmWB disease. Nevertheless transmission trials are needed to assess their vector activity and further studies about their identification at the species level are required. Concerning the latter, a deep systematic revision is desirable to solve the taxonomic critical situation of cixiid family. Moreover, since almost no information is known about their biology, further investigations to verify their host plants and their population dynamics are recommended to better understand the disease spread and epidemiology to define suitable control strategies.

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Advances in knowledge about phytoplasma diseases in Argentina

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Abstract

Up to now phytoplasmas enclosed in four ribosomal groups (16SrI, 16SrIII, 16SrVII and 16SrXIII) have been detected in Argentina infecting more than 20 plant species. The X-disease (16SrIII) is one of the most spread phytoplasma group; not only it is represented by a high number of subgroups, but it has also a wide host range. Among the 16SrIII subgroups described, -B, -J and two new subgroups -W and –X have been reported. Also new phytoplasmas have been identified belonging to 16SrI-B and –S (new subgroup), 16SrVII-B and -C and 16SrXIII-C subgroups. Epidemiological studies show that phytoplasmas in important crops such as garlic and alfalfa are present with high prevalence and low incidence. The transmission of the agent associated with alfalfa witches' broom (ArAWB) is being studied under controlled conditions. Genes encoding immunodominant membrane proteins were identified in this local phytoplasma genome and specific antisera against proteins potentially involved in phytoplasma-host interactions are being produced.

Key words: phylogenetic relationship, 16S rDNA, characterization, interaction, vectors.

Introduction

Phytoplasmas enclosed into four different groups have been detected so far in Argentina: 16SrI (aster yellows); 16SrIII (X-disease); 16SrVII (ash yellows), and 16SrXIII (Mexican periwinkle virescence), in over 20 plant species, some economically important such as garlic (*Allium sativum* L.), China-tree (*Melia azedarach* L.), strawberry (*Fragaria x anannassa* D.) and alfalfa (*Medicago sativa* L.), causing variable damages and losses. Phytoplasmas were also detected in ornamental, native and weed plant species (Table 1).

The first phytoplasma detected and studied in Argentina was the China-tree decline phytoplasma. China-tree was introduced in Argentina from Asia for ornamental

and forestry purposes. In the late 1970s, the first symptomatic trees were seen; they showed leaf size reduction and yellowing, shoot proliferation, internode shortening, severe stunting and gradual necrosis of the plant. Vázquez *et al*. (1983) described the China-tree decline as a mycoplasma-like disease. The association of the China-tree decline disease with a phytoplasma was later confirmed by polymerase chain reaction assays (Gómez *et al*., 1996). A polyclonal antiserum, produced from infected China-tree plants, offered a rapid and simple tool to detect affected plants and to associate this pathogen with symptoms in other important crops. The antiserum, when used at a higher concentration, detected other phytoplasmas present in garlic (*Allium sativum* L.), peach (*Prunus persica* L.) and tona (*Toona ciliata*) (Gómez *et al*., 1996). Later studies have been initiated on the distribution, host range and classification of newly detected phytoplasmas in Argentina.

Data from pathogens found in other regions cannot be used directly in these areas because different pathogens influenced by the role that insect vectors play in each case may be present (Lee *et al*., 2000). For this reason, the work is focused on detection and molecular characterization of new phytoplasmas, on identification of different species of Hemiptera vectors, and on the components involved in the phytoplasma transmission. Epidemiological studies of phytoplasmas diseases in different crops and study of proteins potentially involved in host-pathogen interaction are also in progress.

Identification and molecular characterization

The phytoplasmas detected in Argentina have been recognized by symptom observation and detected by PCR amplification using universal primers (Lee *et al*., 1993; 1998). In most cases, amplicons produced by PCR assays have been analyzed with RFLP procedures, cloned, sequenced and compared with database sequences. Phylogenetic studies have been made and specific primers were designed when necessary, in order to increase the efficiency in detection. When possible, the pathogen has been transmitted to periwinkle (*Catharanthus roseus* G Don) by grafting or by *Cuscuta subinclusa* transmission.

Up to now four 16Sr phytoplasma groups in more than 20 plant species have been identified (Table 1). The highest number and widest distribution of strains was found in 16SrIII (X-disease) group affecting vegetables, woody, ornamental and native plant species, showing typical leaf size reduction, witches' broom and internode shortening. According to the classification scheme based on PCR/RFLP of 16S rDNA, the strains belong to subgroups -B, -J (Galdeano *et al*., 2004; Fernández *et al*., 2013a; Guzmán *et al*., 2014) and to two new subgroups -W and –X (Galdeano *et al*., 2013). As regards 16SrI (aster yellows) group, subgroups -B and -S were found in periwinkle, carrot and chamomile (Torres *et al*., 2004; 2011). Phytoplasmas belonging to 16SrVII (ash yellows) group were detected in herbaceous plants, with representatives of subgroups

-B and -C. The predominant symptoms were witches' broom, phyllody and leaf size reduction (Conci *et al*., 2005; Meneguzzi *et al*., 2008; Fernández *et al*., 2013). The 16SrXIII (Mexican periwinkle virescence) group was detected in China-tree and in strawberry (Arneodo *et al*., 2007; Fernández *et al*., 2008) associated with decline, redness and leaf size reduction.
As a result, in Argentina at least in three phytoplasmas have been identified as belonging to unique subgroups. Several of these pathogens were identified exclusively in South America, suggesting mechanisms of evolutionary divergence mediated by geographical isolation (Lee *et al*., 2000).

Epidemiological studies

Epidemiological studies were carried out in production areas of crops systematically affected by phytoplasma diseases. When possible, apart from disease distribution, the associated Hemiptera fauna has been studied as well as yield losses.
Alfalfa (*Medicago sativa* L.) is one of the most widespread cultivated forage species. Almost 19 years ago, an outbreak of a disease causing severe witches' broom was observed in alfalfa fields for seed production in the 'Cuyo' region (Andean Midwest) (Hijano and Pérez Fernández, 1995). A phytoplasma was detected, named Argentinean alfalfa witches' broom (ArAWB), and characterized as a new subgroup (16SrVII-C) of the ash yellows group (Conci *et al*., 2005). More than 30 leafhopper species have been identified within alfalfa crops and surrounding weeds. In three of them the ArAWB phytoplasma was consistently detected by PCR and potential insect vectors were identified. A high prevalence of the disease was registered in the studied region, and the disease incidence increased (reaching 12%) with crop age leading to premature plant death (Meneguzzi, 2009).
In garlic (*Allium sativum* L.) production fields, the main Argentina's export vegetable, a phytoplasma has been identified in the disease known as "garlic decline" (Conci *et al*., 1998). According to 16S ribosomal gene sequence analysis and RFLP pattern, the garlic decline phytoplasma (GDIII) was related to 16SrIII (X-disease) subgroup -J (Galdeano *et al*., 2004). In entomofauna studies, many Cicadellidae species were found and this phytoplasma was detected in at least one of them (Catalano *et al*., 2008). Further studies are focused on the detection of other potential insect vectors. The disease occurs in all producing areas of the country with high prevalence (23-100% fields had at least one diseased plant) and low incidence (0.03-0.78%) (Galdeano *et al*., 2009). Cultivars Morado and Blanco are more susceptible to GDIII since incidence and prevalence are significantly higher than in cultivars Colorado. Blanco and Colorado are traditionally planted in Argentina, however the Morado cultivar has been introduced from Asia within the last 25 years, and represents a high percentage of the garlic cultivated area at this time. The severity of the disease is extremely high in garlic and leads to death of the plant in early infections. However, the apparent absence

of an efficient vector reduces the disease incidence, and symptom remission during sprouting has been also observed (Galdeano *et al*., 2009).
The strawberry leaf phytoplasma (16SrXIII) has been reported exclusively associated with strawberry (*Fragaria x ananassa* D. cv camarosa) crops in Argentina. Epidemiological studies showed that the strawberry lethal redness is present with a 62% of prevalence and low incidence (0.45%) in strawberry production areas (F.D. Fernández, pers. comm.).
The increasing spread of the China-tree decline led to a drastic reduction of its use for forestation. The phytoplasma (ChTDIII) was classified within subgroup 16SrIII-B (Gómez *et al*., 1996; Galdeano *et al*., 2004). Distribution studies established that throughout the central and northwestern provinces of Argentina there are trees affected by this phytoplasma, while in the northeast provinces and Tucumán (two regions sharing similar agro-ecological characteristics) another phytoplasma (China-tree yellows; ChTYXIII) belonging to 16SrXIII (Mexican periwinkle virescence) group of the new subgroup –C is associated with the disease. In addition, several trees sampled in the northeastern provinces and Tucumán were found to have double infections with 16SrIII-B and 16SrXIII-C phytoplasmas. In the northeastern provinces, both phytoplasmas (ChTDIII and ChTYXIII) were found at similar frequency (Arneodo *et al*., 2007).

Vectors

Hemiptera insects (1 Membracidae and 37 Cicadellidae species) collected from alfalfa fields and surrounding weeds have been identified, the relative abundance of each species was established and the presence of the phytoplasma within the insect was determined (Meneguzzi, 2009). The population curves of infective insects were established in three species that were detected as potential vectors of the ArAWB phytoplasma. Based on such results, the transmission of the agent associated with alfalfa witches' broom (ArAWB) is being studied in controlled conditions. The insects are reared in order to study each species transmission parameters. The analysis of preliminary data obtained from transmission tests led to the hypothesis that *Ceresa nigripectus* (Hemiptera, Membracidae) would act as a ArAWB terminal host (Meneguzzi, 2009). *C. nigripectus* was reared under experimental conditions and it was possible to obtain a stable population throughout the year. The number of nymph stages, duration of each stage as well as the total duration of its biological cycle has been determined. Also the area of the plant where the females lay their eggs, the shape, size and color thereof and duration of egg stage were determined. Other plant species that could act as secondary hosts of the species have been found too (Pérez Grosso *et al*., 2013). Currently the characteristics of this species immature stages are being studied. Furthermore, transmission tests using ArAWB infected plants as a source of inoculum are performed in order to establish the acquisition, latency and inoculation times required for the disease establishment.

Table 1. Argentinean 16Sr phytoplasma groups/subgroups, plant host and geographic location.

16Sr group	Subgroup	Phytoplasma (acronym)	Plant host	Geographical location (Province)
I	-B	maize bushy stunt (MBS)	Zea mays	Buenos Aires,Tucuman, Salta, Santa Fe, Chaco, Misiones
	-S	Argentinean *Catharanthus* little leaf (ACLL)	*Catharanthus roseus* G Don (periwinkle), *Daucus carota* L. sub-sp. *sativus* (carrot) and *Matricaria chamomilla* L. (chamomile)	Córdoba, Corrientes
III	-B	China-tree decline (ChTDIII)	*Melia azedarach* L.	Córdoba, Corrientes, Chaco, Tucumán, Mendoza, Santa Fe, Misiones, San Juan
		Caesalpinia little leaf (CaesLL)	*Caesalpinia gilliesii*	Córdoba
		Gaura yellows phytoplasma (GYP)	*Gaura parviflora*	Córdoba
		Argentinean peach yellows (ArPY)	*Prunus persica* L. (peach)	Jujuy
		tomato little leaf (TomLL)	*Solanum lycopersicum*	Tucumán
		Catharanthus little leaf (CatLL)	*C. roseus*	Corrientes
	-J	*Bellis* virescence (BellVir)	*Bellis perennis* (daisy)	Córdoba
		Conium witches' broom (CWB)	*Conium maculatum* L.	Córdoba
		sunflower phytoplasma (SunPhy)	*Helianthus annuus* L.	Buenos Aires
		garlic decline (GDIII)	*Allium sativum* L. (garlic)	Mendoza, Córdoba, San Juan, Buenos Aires
		tomato reddening (TomRed)	*Solanum lycopersicum* (tomato)	San Juan
		sugar beet decline (SBd)	*Beta vulgaris* L. var. *saccharata* (sugar beet)	Río Negro
		Cucurbita virescence (CucVir)	*Cucurbita maxima* var. *zapallito* (zucchini)	Córdoba
	-W	*Heterothalamus* little leaf (HetLL)	*Heterothalamus alienus*	Córdoba
	-X	*Conyza* witches' broom (ConWB)	*Conyza bonariensis* (L.) Cronquist	Córdoba
	*	*Chrysanthemum* virescence (CVir)	*Chrysanthemum* sp.	Córdoba
		Bidens phyllody (BiPP)	*Bidens subalternans* var *simulans* Sherff	Córdoba

16Sr group	Subgroup	Phytoplasma (acronym)	Plant host	Geographical location (Province)
VII	-B	*Artemisia* witches´ broom (ArtWB)	*Artemisa annua* L. and *C. bonariensis*	Córdoba, San Juan
	-C	Argentinean alfalfa witches' broom (ArAWB)	*Medicago sativa* L. (lucerne)	San Juan, Mendoza
		Argentinean strawberry phyllody (ASP)	*Fragaria* x *ananassa* D. (strawberry)	Corrientes
	*	*Bacharis* witches' broom (BaWB)	*Baccharis flabellata* (Hook. et Arn.) var. *flabellata*	Córdoba
XIII	-C	China-tree yellows (ChTYXIII)	*M. azedarach*	Chaco, Formosa, Corrientes, Misiones, ucumán
	*	strawberry red leaf (StrawRL)	*Fragaria* x *ananassa*	Tucumán, Corrientes

*, new undefined subgroup

Phytoplasma-host interactions

The great molecular diversity of diseases associated to phytoplasma presence seems to require studying each particular pathosystem in each region. The development of new strategies like the analysis of phytoplasma genes encoding immunodominant membrane proteins, as well as secretion and effector proteins, supports not only the study of each phytoplasma, but also the knowledge of colonization strategies, adaptation and pathogenicity mechanisms. Consequently, the genes of interest are being identified in local phytoplasma genome, and specific antisera against proteins potentially involved in phytoplasma-host interactions are being produced in order to characterize and localize pathogen proteins in infected tissues.

SecA is a protein with a highly conserved structure among many bacterial species, unique to bacteria, and differs remarkably from those present in the chloroplasts which make it an ideal candidate for the generation of a specific antiserum against phytoplasmas. For this reason, an internal fragment of the SecA coding gene (840 bp) has been amplified from purified China-tree DNA infected with ChTYXIII (16SrXIII-C), cloned and expressed in bacterial culture. The purified SecA protein (~38 kDa) has been used in antiserum production (Fernández *et al.*, 2011).

Phytoplasma effector proteins are involved in the interaction with their hosts and may interfere with different host cell processes, such as intracellular trafficking, gene expression and defense responses. The export of these proteins directly into the host cytoplasm represents a critical role in the host-phytoplasma interaction with marked impact on pathogenicity mechanisms. Therefore, SAP11ar coding gene (381 bp) has been amplified from purified *C. roseus* DNA infected with ACLL (16SrI-S), cloned and expressed in bacterial culture. The purified SAP11ar protein (~10.9 kDa) was confirmed by MALDI-TOF analysis and is being used in antiserum production.

Phytoplasma membrane proteins are in direct contact with their hosts and are supposed to play a crucial role in the phytoplasma spread within the plant and in the insect vector. Three types of highly abundant and immunodominant membrane proteins (IDP) have been identified within the phytoplasmas: Amp, IdpA, and Imp. The study of genes encoding immunodominant membrane proteins of ChTYXIII (16SrXIII-C) has been recently initiated with the objective of characterize them and compare the nucleotide sequence with phytoplasmas from other geographic locations.

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Phytoplasma diseases in trees of Bogotá, Colombia: a serious risk for urban trees and crops

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Abstract

Phytoplasmosis are emergent diseases that affect many urban tree species in Bogotá, Colombia. Molecular techniques indicate that 16SrI and 16SrVII were the two major groups of phytoplasmas affecting *Acacia melanoxylon*, *Croton* spp., *Eugenia myrtifolia, Fraxinus uhdei, Liquidambar styrciflua, Magnolia grandiflora, Pittosporum undulatum, Populus nigra* and *Quercus humboldtii* from 2008 to 2013. Groups 16SrV and 16rXII were also detected in and *L. styraciflua.* Symptoms such as witches' brooms, epicormic shoots, tufted foliage, atypical elongation of apical shoots, small leaves change dramatically the architecture of the tree crowns. In December of 2013, a preliminary prevalence study showed that the proportion of affected trees varied between 36% in *Croton* spp. to 100% in *F. uhdei*. Symptoms suggesting phytoplasma involvement have also been observed in other tree species of the area. Phytoplasmosis has spread to trees growing in the surrounding rural areas of Bogotá, where they may act as source of inoculum for crops and other plants. For example, phytoplasmas of groups 16SrI and 16SrVII have been detected in strawberry crops of the surrounding Sabana de Bogotá. Since there are no effective treatments, knowledge of the ecology and epidemiology of these diseases seems the only possibility to propose management strategies.

Key words: 16SrI, 16SrV, 16SrVII, 16SrXII, symptoms, prevalence.

The importance of urban trees

According to the Department of Economics and Social Affairs of the United Nations, in 2011 about 50% of the human population lived in urban areas. It is expected that by 2025 58% and by 2050 67% of the population will live in cities (UN, 2014). Therefore, the conservation of the urban biodiversity, including the trees, should be considered an important issue for the cities administrators. Trees provide ecological services that include: reduction of air pollution, control of rain water, carbon storage, improvement of water quality and reduction of energy consumption and, sheltering for bird, insect populations and other organisms. In addition, trees generate social benefits such as human well-being and have an aesthetic value that for example can increase the value of properties.

In Bogotá in 2012, the estimated number of trees per 100,000 inhabitants was of 15,757.9. This is equivalent to 0.2 trees per inhabitant, although the OMS recommends one tree per 0.33 inhabitants (Observatorio Ambiental de Bogotá, 2013). The low number of trees in Bogotá, the economic cost of growing and maintaining them, in

addition to the importance for the biodiversity and quality of life, justify the study of the diseases that affect the health status of the urban trees.

Phytoplasmas in *Fraxinus uhdei* in Colombia

The first disease associated with phytoplasmas in Bogotá was described in 2001 in *Fraxinus uhdei* (Oleaceae) (Griffiths *et al*., 2001; Filgueira *et al.*, 2004). *F. uhdei* is native to Central America and was introduced to Bogotá in the decade of 1950, from United States. It adapted easily to the tropical conditions of Bogotá and soon dispersed to other cities and rural areas of the Andean zone, located between 1,100 and 2,700 m of altitude. In the decade of 1990 *F. uhdei* was the predominant tree species of Bogotá in streets and parks of the city. At that time, the health status of the trees began to decay and symptoms attributed to two independent causes were described. One type of damage was caused by the feeding habits of *Tropidosteptes chapingoensis* (Hemiptera: Miridae). Other symptoms including yellowing, dead branches, dieback, decline, small leaves, deliquescent branching, tufted foliage and epicormic shooting suggested the presence of phytoplasmas (Griffiths *et al*., 2001; Franco-Lara and Filgueira, 2005). These symptoms resembled those of ash yellows, a disease that affected several species of *Fraxinus* and *Syringa* in North America and is associated with *'Candidatus* Phytoplasma fraxini*'* presence (group 16SrVII) (Sinclair and Griffiths, 1996; Griffiths *et al*., 1999). The prevalence of the phytoplasma disease of *F. uhdei* has been estimated in Bogotá during these years. Observations made in 2000 on 600 trees showed that 100% of the trees had symptoms, and that 92% of them were moderately or severely affected. Since the disease was recorded, a large number of *F. uhdei* were removed, the trees that remain barely survive (Figure 1E). Between May 2004 and June 2005, a survey was carried out to estimate the infection degree of *F. uhdei* in the Andean zone of Colombia. Bogotá, Medellín, Pereira, Manizales, Armenia Tunja and Sabana de Bogotá (semi-rural area surrounding Bogotá) were visited and 106 trees resulted symptomatic. Symptoms associated with phytoplasmas were observed in all the surveyed areas. In average, 96% of the trees had tufted foliage, 69% deliquescent branching and 50% epicormic shoots, suggesting the presence of the disease in other areas of the Andean region (Figure 1A) (Franco-Lara and Filgueira, 2005).

The presence of *'Ca.* P. fraxini*'* has been demonstrated in the *F. uhdei* of Bogotá. Initially, the molecular detection of phytoplasmas in these trees was extremely difficult due probably to the low concentration and heterogeneous distribution of phytoplasmas, unknown patterns of seasonal distribution, occurrence of inhibitors for PCR assays. To confirm the presence of phytoplasmas in *F. uhdei* DAPI tests on stems and petioles of symptomatic trees were carried out (Sinclair *et al*., 1992). The sections of symptomatic trees showed non-defined, cloudy spots present exclusively in the phloem (Figure 1B). Those spots were distinguishable from nuclei, rarely detected in the phloem and were not observed in the negative controls leading to the conclusion that they corresponded to phytoplasmas. Transmission assays to periwinkle (*Catharanthus roseus*) using dodder

(*Cuscuta* sp.) were also performed. In the cases in which dodder connected effectively trees and periwinkle plants, the latters showed symptoms such as virescence, small leaves and excessive production of shoots in the basal part, whereas the non-infected plants were symptomless (Figure 1C). The phytoplasma presence was confirmed in the periwinkle plants by nested PCR of the 16SrRNA gene and by DAPI test (Perilla *et al*., 2010).
Phytoplasmas of group 16SrVII have been detected in *F. uhdei*. Nested PCR tests with primers for the 16SrRNA and EF-Tu genes of phytoplasmas were performed on tree DNA extracts from Bogotá and Manizales. Amplicons obtained with P1/P7 or P1/Tint (Smart *et al*., 1996) followed by R16F2n/R16R2 (Gundersen and Lee, 1996) or PhytufL22 (AAGCGCCTGAAAGAAAAA)/ PhytufR24 (TGATGCCACGTAATTAAAA) were sequenced. Sequences of both genes showed more than 99% of identity with ash yellows phytoplasmas from North America (Table 1). Additional confirmation for the presence of phytoplasmas of group 16SrVII in samples from Bogotá and Manizales was obtained by cloning R16F2n/R16R2 amplicons and analysing them by RFLP(Figure 1D).
At the beginning of the decade of 2000, it was considered that *'Ca*. P. fraxini*'* was limited to the genus *Fraxinus* and *Syringa*, and its geographical distribution was restricted to North America (Sinclair and Griffiths, 1996; Lee *et al*., 2000). When in 2001 it was detected in Bogotá, it was considered an exotic disease affecting only this tree species.

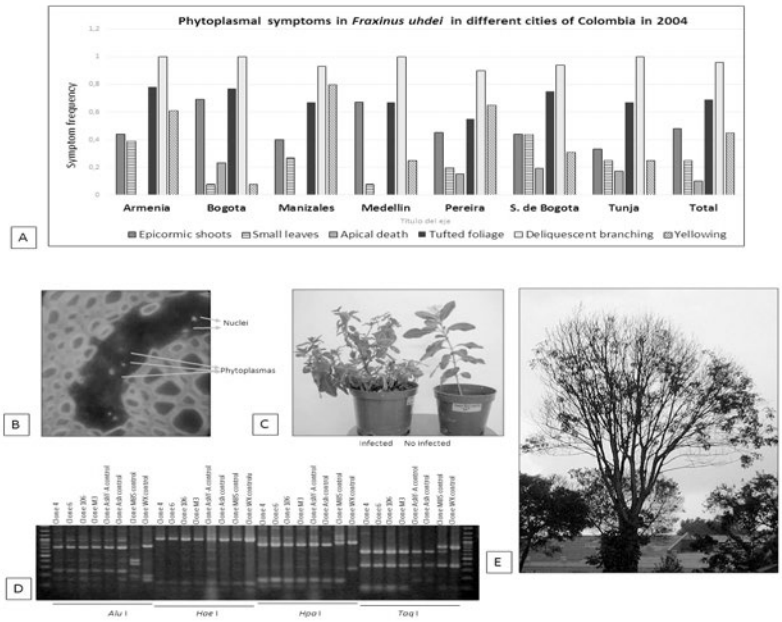

Figure 1. Phytoplasmas of group 16SrVII in *F. uhdei* in Bogotá (E). A. Frequency of symptoms associated with phytoplasmas in different zones of central Colombia. B. Detection of phytoplasmas in the phloem of a leaf petiole of *F. uhdei* by DAPI test (400 X). C. *C. roseus* infected with phytoplasmas using dodder from *F. uhdei* as source of inoculum. Left, infected symptomatic plant and right, non-infected, symptomless plant. D. RFLP on ash yellows phytoplasmas from Bogotá (4, 6 and 106) and Manizales (M3) compared with AshY, MBS and WX phytoplasmas on agarose gel electrophoresis at 3% in TBE buffer and stained with ethidium bromide.

Table 1. Identity of sequences of phytoplasmas of *F. uhdei* from Bogotá compared to ´*Ca*. P. fraxini´ from North America.

Gene	GenBank n. of Colombian sequences	Max score	Total score	Query cover	Identity	GenBank n. of North American sequences
16SrRNA	AY687786	1698	1698	100%	100%	JQ868445
	AY661425	1539	1539	100%	99%	
	AY661423	1498	1498	100%	99%	
Factor EF-Tu	AY661424	723	723	55%	100%	JQ824219
	AY685053	723	723	55%	100%	

Phytoplasmas in other tree species in Bogotá

Between 2006 and 2007, alterations of the morphology of the crown of many tree species began to be observed in Bogotá. A very notorious example was *Liquidambar styraciflua* (Hamamelidaceae), a North American species introduced to replace in part the *F. uhdei* affected by phytoplasmas. Later, symptoms suggesting phytoplasmas were also observed in other introduced species such as *Acacia melanoxylon* (Fabaceae), *Eugenia myrtifolia* (Myrtaceae), *Magnolia grandiflora* (Magnoliaceae), *Pittosporum undulatum* (*Pittosporaceae)*, *Populus nigra* (Salicacea) and native species such as *Quercus humboldtii* (Fagaceae) and *Croton* spp. (Euphorbiaceae). In all these trees, there is evidence of changes in the growth patterns of branches or the formation of atypical vegetative structures that produces tree morphologies divergent from the normal.

The most important symptoms have been described for all the tree species (Sinclair and Griffiths, 1994; Boa, 2003; Phytoplasma Resource Center, 2011, Forestry Images, 2011). In general, the most common symptoms are: tufted foliage (branches with slow twig growth and short internodes that cause foliage to appear bunched), deliquescent habit of ramification (several slowly growing twigs near the branch end with no clear leader), witches' brooms, epicormic shoots (shoots or leaves that develop from buds in the trunk or branches that should be inactive), atypical elongation of apical shoots, small leaves. In *Croton* spp. the main symptom is the production vegetative structures similar to leaves that develop from buds and cover large areas of trunks and branches. These leaflets are brownish-green and densely covered with trichomes (Figure 2). All these symptoms suggest disturbances of the growth regulators of the trees, characteristic of phytoplasma diseases (Lee *et al*., 2000; Christensen *et al*., 2005, Bertaccini and Duduk, 2009). So far, symptoms such as virescence and phyllody have not been observed in trees of Bogotá. In several species yellowing of the foliage was present and although it could be attributable to other causes, it was included in the description since it is very often associated with phytoplasma presence. For *A. melanoxylon, E. myrtifolia, F. uhdei* and *M. grandiflora* it has been shown that the symptoms remain through the year but gradually become more severe making the trees almost unrecognisable.

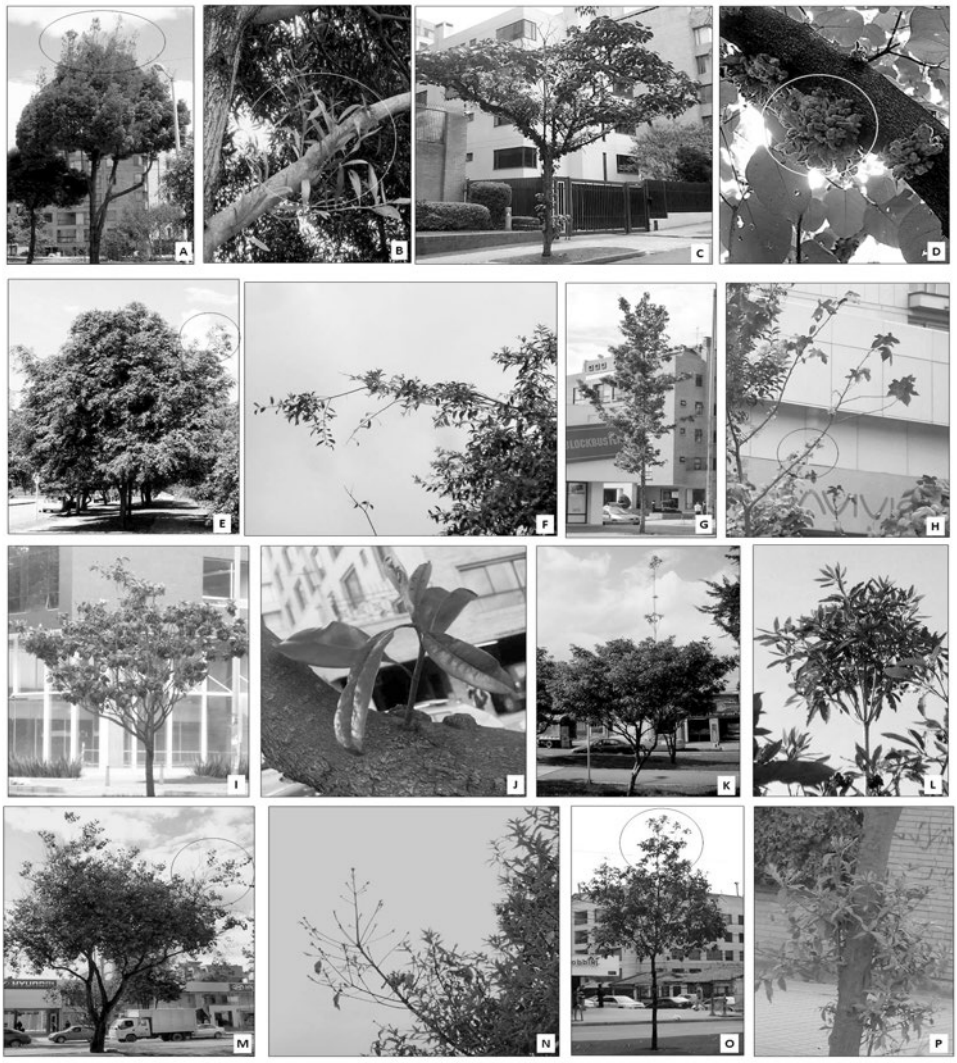

Figure 2. Pictures of symptomatic trees in Bogotá and details of symtoms. (A) *A. melanoxylon* showing elongations of apical shoots and (B) epicormic leaves. (C) and (D) *Croton* sp., with leaflets. (E) and (F) *E. myrtifolia* with tufted foliage apical shoots. (G) *L. styraciflua* with general deformation of the crown and (H) small leaves. (I) *M. grandiflora* showing tufted foliage and elogation of apical shoots*I* and (J) epicormic shoots. (K) *P. undulatum* with an elongated branch showing atypical branching patterns. (L) Witches´ broom in *P. undulatum*, (M) *P. nigra* with general deformation of the crown and (N) deliquescent branching pattern. (O) *Q. humboldti* with tufted foliage and defoliation and (P) epicormic shoots.

A preliminary survey to estimate the presence of phytoplasmas was carried out in 2013 and showed that the prevalence varied from 36% in *Croton* spp. to 100% in *Fraxinus,* but this may be an underestimation. To confirm the phytoplasma presence

molecular tests including nested PCR, followed by RFLP and sequence analysis have been carried out. The presence of phytoplasmas was tested with primers that anneal to the 16S rRNA-23SrRNA gene. Direct reactions were performed with P1A/P7A (Lee *et al.,* 2004) and amplicons used as templates in subsequent nested reactions using internal primers R16mF2/R16mR1 or R16F2n/R16R2 (Gundersen and Lee, 1996). Second nested reactions were performed in many cases using fU5/rU3 (Seemüller *et al.,* 1994) and PhyRNAF3.3 (AATAGTGGAAAAACTATC)/PhyRNAR2.6 (TGACGGGCGGTGTGTACAAACCCCG) primers. So far the evidence obtained by RFLP and sequence analysis suggests that 16SrI and 16SrVII are the two major groups of phytoplasmas affecting the trees in Bogotá (Figure 3A, Table 2) (Perilla and Franco-Lara, 2012; L. Franco-Lara, unpublished). Groups 16SrV and 16SrXII have also been detected in *L. styraciflua* (Franco-Lara *et al*., 2010; Franco-Lara *et al*., unpublished). Moreover, the presence of mixed infections in the same tree has been observed (Figure 3B). So far, sequences corresponding to groups 16SrI and 16SrVII have been obtained for all the tree species with two exceptions. Sequences of 16SrI have not been obtained for *A. melanoxylon* although it has been detected by RFLP, and 16SrVII sequences have not been obtained for *F. uhdei* in the tests performed in the last few years, while its presence has been confirmed by RFLP analyses.

These results indicate that phytoplasmas of groups 16SrI and 16SrVII are able to infect many different families of plants and that frequently they share the same plant host. Interestingly, the sequences of 16SrVII of all the trees tend to show similarities of 99.9 to 100% with previous sequences of *Fraxinus* from Bogotá and North America. This observation suggests that the original source of inoculum may be the *F. uhdei* plantlets brought from North America to Bogotá many years ago, from where these phytoplasmas may have spread to new host species. On the other hand, phytoplasmas of the group 16SrI found in Bogotá tend to be more variable than 16SrVII reflecting the fact that this is a group in which large polymorphisms were reported in 16S rDNA (Lee *et al*., 2004). However, the sequences 16SrI can be separated in two main groups. One, in which sequences are more similar to reference strains of subgroups 16SrI-B and 16SrI-D and a second of sequences that tend to cluster together but not with any known subgroup (L. Perilla and L. Franco-Lara, unpublished). This may indicate at least two sources of inoculum for 16SrI.

Perspectives

The evidence obtained so far indicates that phytoplasmas represent a serious risk for the trees of Bogotá. In addition to the species that we have studied, symptoms suggesting phytoplasma presence are also visible in other tree species of the area such as *Podocarpus oleifolius*, *Pinus patula*, *Salix umboldtiana*, *Prunus serotina*, *Carica pubescens*. There are also some protected forests near Bogotá, composed primarily of native trees, that may also be are at risk, but the occurrence of phytoplasmas in them was not tested.

Furthermore, there is evidence of phytoplasmas affecting crops growing near Bogotá. This is the case of strawberry in which the groups 16SrI and 16SrVII have been associated with symptoms of virescence and phyllody (L. Franco-Lara, unpublished). The situation of the trees of Bogotá is extremely worrying . As far as we know, there are no other cases reported in which so many plant species of different families are affected by phytoplasmas in the same geographic region.

Table 2. Principal symptoms, prevalence and groups of phytoplasmas detected in trees of Bogotá.

Tree species	Symptoms[1]	Threshold[2]	Prevalence[3]	16Sr groups[4]	GenBank accession numbers[5]
Acacia melanoxylon	Epicormic shoots, tufted foliage, atypical elongation of apical shoots	2/3	26/55 (47%)	-VII	KJ093443
Croton spp.	Leaflets, epicormic shoots	1/2	12/33 (36%)	-I, -VII, -I + -VII	JQ730858 HG764348* HG764349* HG764350 HG764351
Eugenia myrtifolia	Tufted foliage, atypical elongation of apical shoots	1/2	35/43 (81%)	-VII, -I	KJ093442 KJ200361
Fraxinus uhdei	Tufted foliage, epicormic shoots, small leaves, deformation of the crown, atypical elongation of apical shoots	3/5	50/50 (100%)	-I, -VII, -I + -VII	JQ730859 HG764354
Liquidambar styraciflua	Small leaves, tufted foliage, deformation of the crown, atypical elongation of apical shoots, epicormic shoots	3/5	21/22 (95%)	-I, -V, -VII, -XII, -I + -VII	KJ200363 KJ200364 KJ200365
Magnolia grandiflora	Tufted foliage, atypical elongation of apical shoots, deformation of the crown, yellowing	2/4	46/70 (66%)	-I, -VII	KJ093444 KJ200362
Pittosporum undulatum	Atypical elongation of apical shoots, tufted foliage, deformation of the crown, yellowing	2/4	17/20 (85%)	-I, -VII	JQ730861
Populus nigra	Deformation of the crown, yellowing, atypical elongation of apical shoots, tufted foliage	3/4	14/15 (93%)	-I, -VII, -I + -VII	JQ730860* HG764355*
Quercus humboldtii	Yellowing, tufted foliage, general deformation of the crown, witches´ brooms, atypical elongation of apical shoots, deliquescent branching	3/6	17/23 (74%)	-I, -VII, -I + -VII	

[1] Principal symptoms associated with phytoplasma in each species, in order of importance
[2] A tree was considered symptomatic when it showed at least the threshold number of symptoms associated with phytoplasma
[3] Prevalence: number of diseased trees / total number of trees. The percentage of diseased plants is presented in parenthesis
[4] Groups of phytoplasmas detected in different species by RFLP and/or sequence analysis. (+) cases in which mixed infections have been detected in the same tree
* Cases in which sequences obtained from the same tree corresponded to different groups

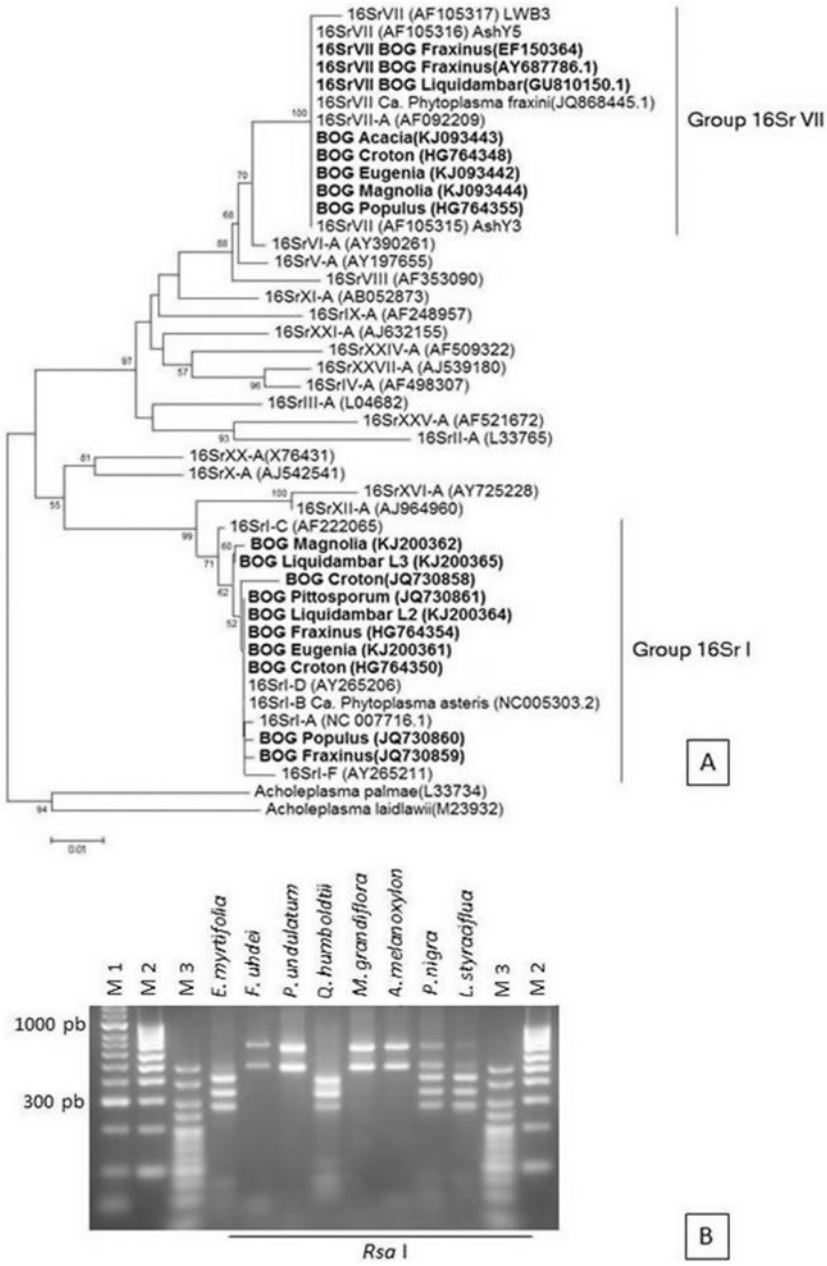

Figure 3. (A) Dendogram built with sequences from a region of 511 nucleotides of the16SrRNA gene. The tree was constructed by Neighbour Joining with a bootstrap of 1,000, using Mega 5.2 software. (B) RFLP of R16F2n/R16R2 amplicons in agarose gel as described in figure 1D. M1, M2, M3, Hyper ladder (Bioline) 500, 100, and 25 bp respectively. *E. myrtifolia*, 16SrI; *F. uhdei*, 16SVII; *P. undulatum*, 16SVII, *Q. humboldtii*, 16SrI; *M. grandiflora*, 16SrVII; *A. melanoxylon*, 16SrVII and *P. nigra* and *L. styraciflua*, mixed infections 16SrI and 16SrVII.

There are no information about the insect vectors of phytoplasmas in Colombia. However, in the last three years attempts to determine the insect vectors that transmit phytoplasmas in Bogotá have been made. From May 2011 to April 2012, leafhoppers were captured in grass areas nearby symptomatic trees. Taking into account the external morphology of the insects they were classified in nine morphotypes (MF01-MF09). All of them belong to the Typhlocybinae, Deltocephalinae or Xestocephalinae subfamilies in the Cicadellidae family (M. Wilson, personal communication). The identification of the species is underway. Phytoplasmas of groups 16SrVII were detected in three morphotypes and 16SrI plus 16SrVII in six morphotypes (Franco-Lara and Perilla, 2012). Transmission tests were performed with MF04 and MF06 using insects field-collected. Interestingly the two morphotypes were able to transmit both groups of phytoplasmas 16SrI and 16rVII to common bean, under experimental conditions (L. Franco-Lara, unpublished).

Bogotá is located in the Sabana de Bogotá, a plateau of 425,000 ha in the Cordillera de los Andes at 2,600 m of altitude. It is a continuous plateau delimited by mountains, many of which are covered with forests comprising many native species. Apart from Bogotá, there are other 21 small cities and towns surrounded by agricultural lands and grasslands where the vegetation is relatively constant. Since Colombia is located in the tropical zone, day temperatures (13 to 25°C) and night temperatures (-5 to 10°C) in the Sabana remain more or less constant during all the year. There are no seasons in the sense of the temperate zones, but in particular there is no winter. Therefore the Sabana provides a continuum of landscape structure and climatic conditions that may facilitate the dispersion of vectors and phytoplasmas. If this is so, an unanswered question is why the symptoms associated with phytoplasmas appeared only recently. The observation of phytoplasma-related symptoms in trees began in *Fraxinus* in about 2000 but it is unclear when did these symptoms actually appeared. It is important to take into account that phytoplasma symptoms could not be recognized even by trained phytopathologists. Thus, some tree species may have had undetected symptoms before 2000. However, observations in *A. melanoxylon, Croton spp. L. styraciflua* and *M. grandiflora* among others, indicate that at least in these species the symptoms began to be observable around 5 or 6 years ago.

Although the weather in the tropical zone is pretty constant, in Colombia it is affected by the ENSO (El Niño Southern Oscillation), a meteorological phenomenon that strongly influences the climate of the Pacific basin. Every 3 to 8 years approximately, the movement patterns of the marine currents change, affecting the climate of the intertropical zone. In the Andean region of Colombia ENSO produces rain deficit and draughts. For example, a very severe ESNO took place in 1997-1998 and during the first decade of the 21st century four ENSO seasons have occurred. It is conceivably that in those very dry periods when the plants hosts are under hydric stress, insect vectors may change hosts temporarily looking for better conditions. If this is the case, phytoplasmas may spread to new, different plant species, expanding their host range in the years in which droughts are more severe.

By studying the ecology and epidemiology of this pathosystem we may be able to provide management strategies that might reduce phytoplasma associated diseases impacts in the biodiversity and economy of urban trees. Therefore, more information about possible herbaceous hosts, plant resistance, species, habits and ecology of insect vectors, interactions of phytoplasmas of different groups in the same individual plant is needed.

Acknowledgement

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Coffee crispiness and nogal cafetero witches' broom associated with '*Candidatus* Phytoplasma pruni'-related strains in Colombia: multilocus gene characterization

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Abstract

Coffee is among the most important cultivated crops in Colombia, where it is frequently shaded with nogal cafetero, a tree species also used for timber and reforestation programs. Since 1998 severe cases of phytoplasma-related symptoms were observed in coffee plantations, and starting on 2002 also in nogal cafetero. Both diseases were associated to 16SrIII phytoplasma presence and different strains from coffee and nogal cafetero were identified having 99% of homology between them and to several 16SrIII strains in the 16S ribosomal gene. RFLP profiles of ribosomal protein gene and elongation factor indicate the presence of differentiable strains in a coffee sample, one of which is the prevalent in nogal cafetero. These results indicate a complex disease aetiology involving more phytoplasma strains in the two studied plant species.

Key words: Coffee, nogal, witches' broom, phytoplasma, 16SrIII.

Introduction

Coffee crispiness, a disease of uncertain etiology, has been endemic in coffee (*Coffea arabica* L.) plantations in Colombia for at least 60 years. Symptoms typically consist of bud proliferation, abundant short and narrow leaves, phyllody, floral abortion, monospermic fruit, and dwarfing of plants (Figure 1). In severe cases, coffee crispiness disease (CCD) can significantly affect production; it was observed for the first time in the western Department of Antioquia and, by 1950, it was present in the central region of the country, subsequently it was detected in the southern Department of Huila. The disease affect all the *C. arabica* cultivars planted in Colombia, which historically include Typica, Bourbon, Caturra, and Colombia. Since 1997, farmers reported an increase in the symptom severity of CCD in places located over 1,500 m above sea level. This has been observed both in new plantings and in plants regenerated from stumps, a common practice in Colombian plantations (Galvis *et al*., 2007). By 2002 the disease was first reported in the shade tree 'nogal cafetero' [*Cordia alliodora* (Ruíz & Pavón) Oken] in18 months old plants, in the southwest Department of Caldas and after it was also observed in Caldas, Risaralda, Cauca, Valle del Cauca and in the germplasm banks in Quindio (Hernandez *et al*., 2004).

Figure 1. Symptomatic nogal cafetero trees in the germplasm banks in Quindio, showing severe symptoms with witches' broom, branch malformation, abnormal shoot sprouting, dried buds and early defoliation.

Coffee is a major crop in Colombia and plants are often shaded by nogal cafetero, which is also used for timber and reforestation in Central and South America and Africa. It is a frequent component of agroforestry systems, especially since it provides dark coffee wood, and it is also useful in the production systems of certified coffees. It is a species that is well suited to be intercropped with agricultural plantations, being an important component of agroforestry systems. The wood is highly sought after by woodworkers for its quality, basic density, physical and mechanical properties and resistance to rot and moths (Rincon, 2009).

Although epidemics are confined to limited areas dispersed throughout the Colombian "coffee belt", incidence and severity are as high as 90%, resulting in total yield losses. Coffee crispiness disease has been associated with 16SrIII phytoplasma infection (Galvis *et al*., 2007, Mejia *et al*., 2010). Phytoplasmas of the same group were identified through PCR analyses in nogal cafetero plants showing witches' broom, branch malformation, and abnormal shoot sprouting (Galvis and Ospina, 2006, Rincon, 2009, Mejia *et al*., 2010). Further molecular characterization was therefore performed, as a contribution towards epidemiological studies aimed to prevent the disease from spreading.

Plant samples

Symptomatic branches and roots, from three *C. arabica* plants from Gigante region (Department of Huila) and five plants belonging to different genotypes from Paraguaicito (Department of Quindio) showing severe symptoms of crispiness and withches' broom were collected. Shoots, leaves and stems for a total of 23 samples were tested (Table 1).

RFLP and sequence analysis

After nucleic acid extraction following the procedure by Prince *et al*. (1993) ribosomal DNA was amplified by nested PCR reaction using primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by R16F2n/R2 (Gundersen and Lee, 1996) for RFLP analyses and by R16mF2/mR1 for sequencing. Ribosomal protein genes including 3' end of *rps19*, *rplV* (*rpl22*), *rpsC* (*rps3*) genes were amplified using primers rpL2F3/

rp(I)R1A and group specific primers rp(III)F1/rp(III)R1 (Martini *et al*., 2007). Two pairs of primer cocktails were used for *tuf* gene amplification, Tuf340/Tuf890 and Tuf400/Tuf835 in direct and nested PCR respectively (Makarova *et al.*, 2012).
Publicly available 16S rDNA, ribosomal protein and elongation factor sequences from different subgroups of the 16SrIII group (Zhao *et al*., 2009; Mello *et al*., 2011; Makarova *et al*., 2012; Davis *et al*., 2013; Galdeano *et al*., 2013), were retrieved from GenBank and aligned with the 16S ribosomal DNA *rp* and *tuf* sequences from nogal cafetero and coffee using CLUSTALX and BioEdit. *In silico* restriction analysis and virtual RFLP plotting was performed using pDRAW32 software (http://www.acaclone.com). Each sequence was digested with several restriction enzymes and a virtual 4.0% agarose gel electrophoresis image was plotted for virtual RFLP pattern comparisons. A similarity coefficient (F) was calculated for each pair of phytoplasma strains according to described methods (Lee *et al*., 1998; Nei and Li, 1979). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.2 (Tamura *et al*., 2011). Phylograms were generated to estimate the relationships based on the neighbour-joining method, with the reliability provided by 1,000 replications bootstrap test.

Table 1. Description of sample tested.

Strain	Samples	Source	Province	Genotype	Tissues
NcWB01	M1-H	nogal cafetero	Paraguacito-Quindio	NS-II-1-1	leaves
NcWB02	M1-B	nogal cafetero	Paraguacito-Quindio	NS-II-1-1	shoots
NcWB03	M1-T	nogal cafetero	Paraguacito-Quindio	NS-II-1-1	steam
NcWB04	M1-R	nogal cafetero	Paraguacito-Quindio	NS-II-1-1	roots
NcWB05	M2-R	nogal cafetero	Paraguacito-Quindio	S-IX-1-2	roots
NcWB06	M2-B	nogal cafetero	Paraguacito-Quindio	S-IX-1-2	shoots
NcWB07	M2-H	nogal cafetero	Paraguacito-Quindio	S-IX-1-2	leaves
NcWB08	M2-T	nogal cafetero	Paraguacito-Quindio	S-IX-1-2	steam
NcWB09	M3-H	nogal cafetero	Paraguacito-Quindio	S-IX-1-2	leaves
NcWB10	M3-B	nogal cafetero	Paraguacito-Quindio	S-IX-1-2	shoots
NcWB11*	M3-T	nogal cafetero	Paraguacito-Quindio	S-IX-1-2	steam
NcWB12	M4-H	nogal cafetero	Paraguacito-Quindio	NS-II-1-1	leaves
NcWB13	M4-B	nogal cafetero	Paraguacito-Quindio	NS-II-1-1	shoots
NcWB14	M4-R	nogal cafetero	Paraguacito-Quindio	NS-II-1-1	roots
NcWB15*	M5-H	nogal cafetero	Paraguacito-Quindio	S-IX- Paclo	leaves
NcWB16	M5-B	nogal cafetero	Paraguacito-Quindio	S-IX- Paclo	shoots
NcWB17	M5-R	nogal cafetero	Paraguacito-Quindio	S-IX- Paclo	roots
NcWB18	M5-T	nogal cafetero	Paraguacito-Quindio	S-IX- Paclo	steam
CCD01**	MC-B	coffee	Gigante-Huila	*C. arabica*	shoots
CCD02*	MC-B2	coffee	Gigante-Huila	*C. arabica*	shoots
CCD03	MC-H1	coffee	Gigante-Huila	*C. arabica*	leaves
CCD04	MC-H2	coffee	Gigante-Huila	*C. arabica*	leaves
CCD05	MC-T	coffee	Gigante-Huila	*C. arabica*	stem

Results and discussion

All symptomatic samples resulted positive to phytoplasma presence. Two phytoplasma strains from coffee (CCD01 and CCD02) and nogal cafetero (NcWB11 and NcWB15) respectively, were amplified in nested PCR on *16S rDNA*, *rp* and *tuf* genes using P1/P7, R16mF2/mR1 and rpIIIF1/rpIIIR1, Tuf400/Tuf835 primers.

According to the similarity coefficient comparison (Zhao *et al*., 2009) the virtual RFLP patterns derived from the CCD02 and NcWB15, R16F2n/R2 fragment were identical to each others (similarity coefficient 1.00) and were also undistinguishable from patterns of phytoplasmas belonging to 16Sr group III-J (GenBank accession: AF147706). The virtual RFLP patterns derived from the same amplicons of CCD01 and NcWB11 were different from the reference patterns of all previously reported 16Sr groups/subgroups. The most similar was the profile of 16SrIII- U (GenBank accession: HM589213), with a similarity coefficient of 0.97 (Figure 4). This strain may represent a new subgroup within the 16SrIII group. Comparative virtual RFLP patterns identify the key enzyme *Hpy*CH4V that distinguish the new subgroup pattern from those of previously recognized ribosomal groups/subgroups. Actual laboratory restriction digestions with enzyme *Hpy*CH4V confirm the new subgroup pattern (data not show).

In phytoplasmas from both studied species, actual RFLP analysis conducted with *Hha*I, *Hpa*II (Figures 2 and 3) and *Bst*UI (data no show) on the strains NcWB11 and NcWB15 revealed patterns that differed from those described above. They apparently correspond to profiles of two distinct phytoplasmas, but sequencing of the 16S rRNA revealed the occurrence of sequence heterogeneity in each strain. This condition was reported previously for other members of group 16SrIII (Jomantiene *et al*., 2002; Mello *et al*., 2006). The presence of sequence heterogeneity may explain the discrepancy observed between actual and virtual RFLP profiles (Figures 2, 3 and 4). These results suggest that, in some cases, such as those studied in the present work, key enzymes defined by virtual RFLP analysis do not necessarily behave as such when used to perform actual RFLP. Thus, in these cases, the results of virtual RFLP are not confirmed by actual RFLP analysis (Mello *et al*., 2011).

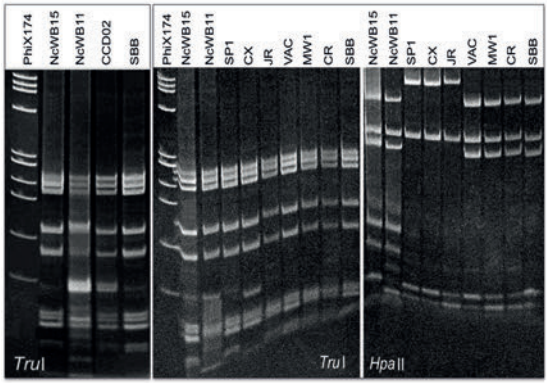


Figure 2. Restriction profiles of R16R2n/F2 amplicons of coffee crispiness and nogal cafetero witches' broom phytoplasmas compared with those of other reference strains in group 16SrIII. NcWB15 and NcWB11: nogal cafetero; CCD02: coffee; SBB, *Solanum marginatum* big bud from Equador, 16SrIII-(F/J*), *variant of the designed subgroup or pattern type; SP1, spirea stunt (16SrIII-E); CX, peach X disease (16SrIII-A); JR, poinsettia branch inducing (16SrIII-H); VAC, vaccinium witches' broom (16SrIII-F); MW1, milkweed yellows (16SrIII-F); CR, *Crepis biennis* yellows (16SrIII-B). Marker: phiX174, phiX174 *Hae*III digested.

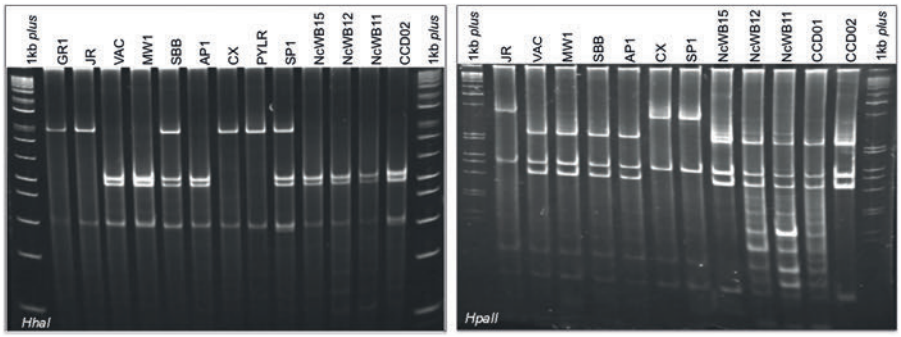
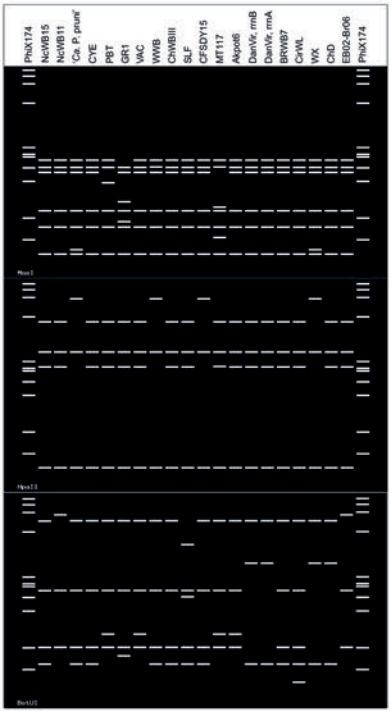


Figure 3. Restriction profiles of R16F2n/R2 amplicons of strains from coffee crispiness and nogal cafetero witches' broom phytoplasmas compared with those of reference strains in group 16SrIII with the enzyme *Hha*I and *Hpa*II. NcWB11, NcWB12 and NcWB15: nogal cafetero; CCD01 and CCD02: coffee; GR1, goldenrod yellows (16SrIII-D); JR, poinsettia branch inducing (16SrIII-H); VAC, vaccinium witches' broom (16SrIII-F); MW1, milkweed yellows (16SrIII-F); SBB, *Solanum marginatum* big bud from Equador, 16SrIII-(F/J*), *variant of the designed subgroup or pattern type; PYLR, peach yellows leaf roll (16SrIII-A); SP1, spirea stunt (16SrIII-E); CX, peach X disease (16SrIII-A); CR, *Crepis biennis* yellows (16SrIII-B). Marker: 1Kb *plus* (Invitrogen).



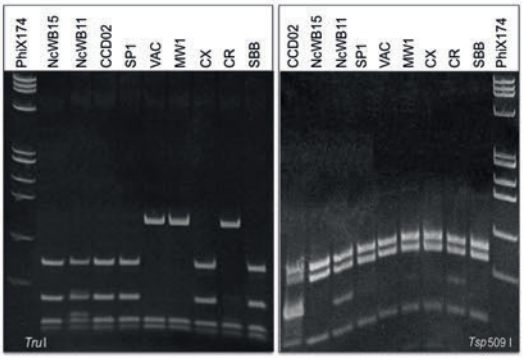
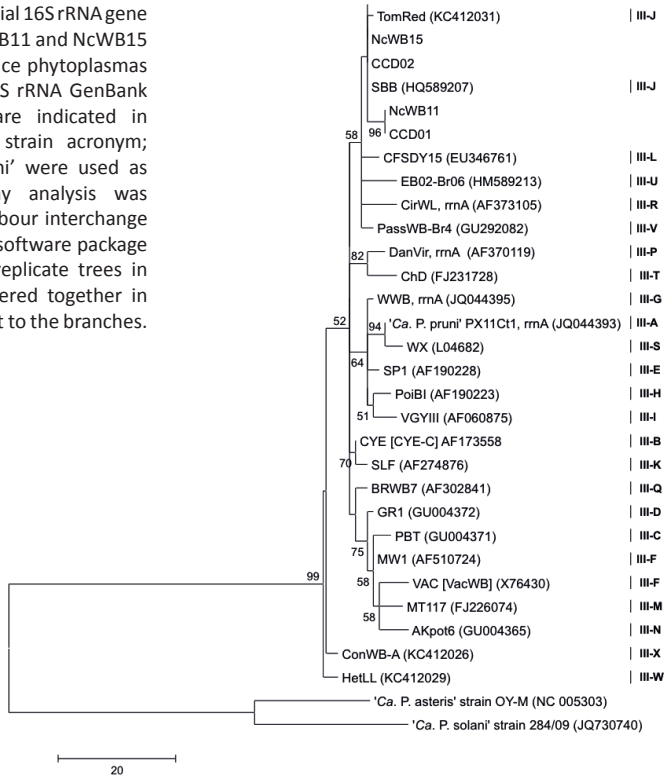


Figure 5. RFLP analyses of amplicons (300 bp) obtained after nested-PCR with the primer pair rp(III)F1/rp(III)R1 from two nogal cafetero witches' broom disease (NcWB11- NcWB15) and one of coffee crispiness disease (CCD02) phytoplasma strains. SP1, spirea stunt; VAC, vaccinium witches' broom; MW1, milkweed yellows; CX, peach X disease; CR, Crepis biennis yellows; SBB, Solanum marginatum big bud from Equador. Marker: phiX174, phiX174 *Hae*III digested.

Figure 4. Virtual RFLP patterns from *in silico* digestions of 16S rRNA gene R16F2n/R16R2 fragments constructed using pDRAW32 software (www.acaclone.com) and the iPhyClassifier on line program (Zhao *et al.*, 2009) from phytoplasma strains NcWB15 and NcWB11. From top: recognition sites for restriction enzymes *Mse*I (*Tru1*I), *Hpa*II and *BstU*I. Marker: phiX174, phiX174 *Hae*III digested.

Figure 6. Phylogenetic tree of partial 16S rRNA gene from strains CCD01, CCD02, NcWB11 and NcWB15 from Colombia and from reference phytoplasmas within the 16SrIII subgroups. 16S rRNA GenBank sequence accession number are indicated in parentheses preceded by the strain acronym; *'Ca.* P. asteris' and '*Ca*. P. solani' were used as outgroups. Maximum-parsimony analysis was conducted using the close neighbour interchange (CNI) algorithm implemented in software package MEGA5.2. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Bar indicated 20 nt substitutions.



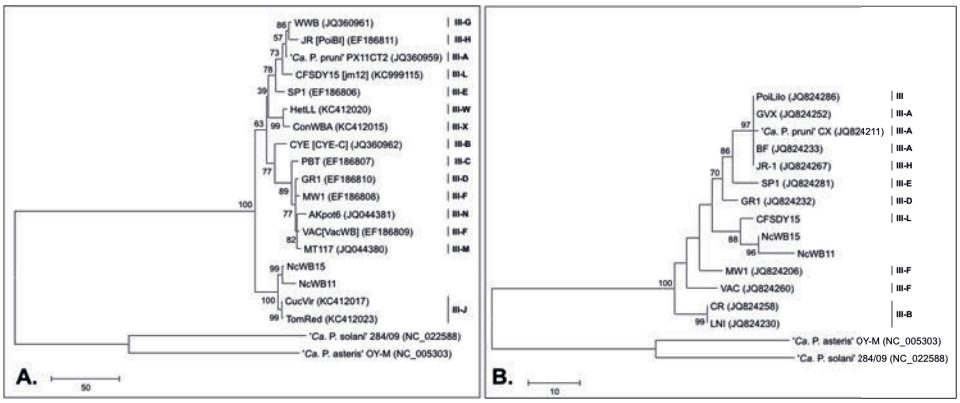


Figure 7. Phylogenetic trees from ribosomal protein and elongation factor sequences from strains NcWB11 and NcWB15 from Colombia and from reference phytoplasmas within the 16SrIII subgroups. A. Ribosomal protein L22 (rplV) and S3 (rpsC) complete and partial CDs. B. Conserved region within the *tuf* gene, amplified with primer cocktails Tuf400/Tuf835. Accession numbers are indicated in parentheses preceded by the strain acronym; *'Ca.* P. asteris' and '*Ca*. P. solani' were used as outgroups. Maximum-parsimony analysis was conducted as described in Figure 6.

Differences between the strains were also detected in tuf sequences since the two strains show 7 SNPs including a specific restriction with *Dde*I. Further characterisation by amplification of a 300-bp fragment on the 16SrIII group ribosomal protein operon followed by RFLP analyses with *Tru1*I and *Tsp509*I produced a unique profile for strains CCD02 and NcWB15, while strain NcWB11 was undistinguishable from some of the described rp subgroups. This indicates the presence of two rp differentiable strains in the coffee sample, one of which is the prevalent in nogal cafetero (Figure 5), however the identification in nogal cafetero of two phytoplasma strains that are differentiable indicates that disease epidemiology is complex and involve probably other species in the wild environment. Search for insect vectors is in progress.

The recent years outbreaks of the phytoplasma diseases in the two species may have resulted from a combination of the phytoplasma's presence and the use of chemical for weed control that could have modified the vector population composition. The close phylogenetic relationship between the phytoplasmas identified in coffee and in nogal cafetero suggests also that coffee could be an alternative host for the phytoplasmas infecting nogal cafetero (Figures 6 and 7).

Coffee was introduced in the Country only 200 years ago and 16SrIII phytoplasmas were shown to be quite widespread in South America. Our results indicate a disease etiology involving more phytoplasma strains in agreement with evolution of the species under non epidemic conditions as it is for phytoplasma diseases of these two species in Colombia.

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Chapter 3

Diseases and insect vectors


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Diseases and insect vectors

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Abstract

The most important phytoplasma diseases in the Action Countries are those affecting fruit trees and grapevine. A short review of their presence and impact on agricultural systems is reported together with the update information about their recognized insect vectors.

Key words: apple proliferation, pear decline, European stone fruit yellows, grapevine yellows, "flavescence dorée", "bois noir".

Introduction

Phytoplasmas are microorganisms phloem-limited therefore only phloem-feeding insects can potentially acquire and transmit them. All known phytoplasmas are transmitted by insects in the order Hemiptera. However, vector species are restricted to only a few families of the suborder Auchenorrhyncha: namely, Cercopidae, Cixiidae, Derbidae, Delphacidae, Cicadellidae and in the Sternorrhyncha: Psyllidae (Weintraub and Beanland, 2006). Within a family, some species are known to be phytoplasma vectors, while others are not. But even within the same species different populations, mostly geographically separated, may occur, which are competent pathogen vectors in one area, but not in another. Updated knowledge on the distribution of a disease as well as the presence and vector status is very important for pest risk assessment and phytosanitary decisions. Therefore, the main goal of WG 2 "Epidemiology and vector ecology" of the COST action was to establish a vector monitoring system throughout Europe to identify phytoplasma vector species, monitor their spread throughout the COST countries, and to coordinate research into these and other means in which phytoplasmas are spread. Thus, WG2 activities included morphological identification and molecular characterization of vector species and populations, a questionnaire survey about distribution of phytoplasma diseases and their putative vectors throughout

different European regions, research of different means of disease spread and the modelling of particular epidemiological cycles and their consequences for direct control strategies. As a result, a comprehensive database was established procuring a current overview on the dispersal of the most important phytoplasma diseases and vectors in most Action member countries and models for particular pathosystems were generated.

Fruit tree phytoplasmas and their vectors

The genetically closely related '*Candidatus* Phytoplasma mali', '*Ca.* P. prunorum' and '*Ca.* P. pyri' (Seemüller and Schneider, 2004) are associated with economically important diseases in European fruit tree areas, respectively apple proliferation (AP), European stone fruit yellows (ESFY) and pear decline (PD). Whilst most phytoplasmas are transmitted by insect vectors of the hemipteran suborder Auchenorrhnycha (leafhoppers, planthoppers, treehoppers), all these fruit tree agents are spread by Sternorrhyncha of the superfamily Psylloidea (Weintraub and Beanland, 2006). Interestingly, the psyllid vector species all belong to the genus *Cacopsylla* and both, phytoplasmas and psyllid vectors, were geographically limited to Europe and the Palearctic ecozone. Only PD has probably been introduced from Eurasia to North America along with their vectors (Jarausch and Jarausch, 2010). Although the relationship between these phytoplasmas, their hosts and the vectoring psyllids is almost highly specific, there are some interconnections and biological factors which influence disease epidemiology, and vector ecology and which should be taken into consideration for risk assessment and vector control. For instance, a psyllid species can be a proven vector in one region or country, but not in one neighbouring, because populations are genetically different; a *Cacopsylla* species can even split into two different subspecies with potentially opposed characters regarding phytoplasma transmission capacities. Furthermore, a vector may transmit only a particular phytoplasma strain or subtype but not another.

Apple proliferation is one of the most economically important phytoplasma disease on apple in Europe (Seemüller *et al.,* 2011). Its agent, '*Ca.* P. mali', has been listed A2 quarantine pest by EPPO since 1992. A systematic review showed that AP has been detected in most EU countries where apple is grown commercially (MacLeod *et al.*, 2012). The disease has been reported from most western, southern and central European countries. Outside the EU, AP was found in the Balkan states (Albania, Bosnia and Herzegovina, Croatia, Serbia), Turkey, Moldova, Switzerland, and Ukraine. The northern limit of its distribution is Norway (for review MacLeod *et al.*, 2012).

Two psyllids, *Cacopsylla picta* (Foerster, 1848) (syn. *C. costalis*) and *Cacopsylla melanoneura* (Foerster, 1848) are recognized vectors of '*Ca*. P. mali' (Frisinghelli *et al*., 2000; Jarausch *et al*., 2003; Tedeschi *et al*., 2002). *C. picta* is distributed only in Europe (Ouvrard, 2014) and is monophagous on *Malus* spp. The insect completes one generation per year and aestivates, then hibernates, as adult on conifers (Mayer and Gross, 2007; Mattedi *et al.*, 2008). At the end of winter (March/April), *C. picta*

remigrates from the overwintering sites to apple trees for oviposition. *C. melanoneura* has a similar life cycle as *C. picta* but the overwintered adults reappear earlier in the year, and the new generation leaves its host plant earlier than *C. picta* to its aestivation and overwintering habitats (Mattedi *et al*., 2008; Tedeschi *et al.*, 2002; 2009). A significant difference is that the principal host plant of *C. melanoneura* is not apple but *Crataegus monogyna* (hawthorn), a common shrub. *C. melanoneura* has a Palaearctic distribution (Ouvrard, 2014) and is oligophagous on *Rosaceae*. In most of the studied areas both species are present (Jarausch *et al*., 2003; Delic *et al*., 2005; Carraro *et al*., 2008; Mattedi *et al*., 2008), in others so far only *C. melanoneura* has been found on apple (Tedeschi *et al*., 2002).

Jarausch *et al.* (2011) found that '*Ca*. P. mali' multiplies very rapidly in its vector *C. picta* so that newly hatched individuals can become infective within two weeks, and are able to transmit the disease inside the orchard in the same season indicating a polycyclic pathosystem with two main transmission periods.

Comprehensive studies on the vector capacity of *C. picta* and *C. melanoneura* and on the role of hawthorn as source of '*Ca*. P. mali' in different European regions led to contradictory results. Thus, *C. picta* has been proven main vector of '*Ca.* P. mali' in Germany (Jarausch *et al.*, 2003; 2011) and northern Italy (Frisinghelli *et al*., 2000; Carraro *et al*., 2008). In contrast, *C. melanoneura* was identified as main vector in Aosta Valley (Tedeschi *et al*., 2002) whereas in Germany *C. melanoneura* has been proven to be non-vector for '*Ca*. P. mali' (Mayer *et al.*, 2009). Furthermore, the German population preferred hawthorn as host plant which, however, was not found infected with the phytoplasma, whereas the north western Italian population seems to be able to move between apple and hawthorn. Accordingly, hawthorn has been found infected with '*Ca*. P. mali' and, thus, may play a role in the epidemiology of AP in this region (Tedeschi *et al*., 2009). On the contrary, recent ecological and genetic results indicate a differentiation between *C. melanoneura* populations associated with apple and hawthorn in Trentino, showing no significant exchange between these host plants (Malagnini *et al.*, 2013). The role of hawthorn as *inoculum* source of AP is still unclear.

European stone fruit yellows is an economically important disease on several cultivated *Prunus* spp. (e.g. apricot, peach, Japanese plums) in Europe (Marcone *et al*., 2010; 2011). Infected trees may completely die off from one year to the other. Its agent, '*Ca*. P. prunorum', is native to Europe and is not qualified as a quarantine pest by EPPO or EFSA (MacLeod *et al.,* 2012). The phytoplasma disease can be found in 14 of the 27 EU countries. ESFY has been reported from most southern and central European countries, with its northern limit in south-east England (Kent) and northern Germany (Schleswig Holstein). Outside Europe, ESFY has been found in the Balkan States, Turkey, Ukraine and most recently in Azerbaijan (MacLeod *et al.*, 2012; Steffek *et al.,* 2012).

The only described vector of '*Ca.* P. prunorum' in various European countries was until recently the psyllid *Cacospylla pruni* (Scopoli, 1763), an oligophagous species on *Prunus* (Carraro *et al.*, 1998). *C. pruni* was considered as an European and Central

Asian species that was known from almost all of Europe (Ouvrard, 2014). Sauvion *et al.* (2007; 2009) found indications for the existence of two genetically well differentiated groups of *C. pruni* by microsatellite genotyping. Recently, they demonstrated that *C. pruni* is in fact a complex of two cryptic species (Peccoud *et al.,* 2013), one (A) with a climatic preference for the regions with sweet winters in west of Europe, and the other (B) widespread in Europe. The two species are morphologically very similar but specific PCR primers allowed rapid assignment of *C. pruni* individuals (Peccoud *et al.,* 2013).
Both species are able to transmit '*Ca.* P. prunorum'. Indeed, the insects used by Thébaud *et al.* (2009) were a mixture of individuals A and B. The natural infection rate and the transmission capacity of *C. pruni* seemed vary stunningly. For instance, low natural infection rates of 1-3% were reported from Germany (Jarausch *et al.*, 2007; 2008) or France (Thébaud *et al.*, 2009), while ten times higher natural infection and important transmission rates were described by Carraro *et al.* (2004) in North eastern Italy. Analyses of a collection of 500 European populations of *C. pruni* A or B gave first indications for different transmission capabilities of the two *C. pruni* groups (N. Sauvion, unpublished). The biological cycles of the two species are identical. The psyllids migrate between conifers in mountainous regions where they aestivate and overwinter, and *Prunus* spp. (especially wild *Prunus* like blackthorn) at lower altitude where they breed (Jarausch and Jarausch, 2010). Thébaud *et al.* (2009) showed that *C. pruni* needs a long effective latency period of 8 months for multiplication and transmission of '*Ca*. P. prunorum'. From this transmission cycle, they infer that local secondary spread of ESFY in apricot orchards is marginal (contrary to the polycyclic spread of AP by *C. picta)*, and suggested disease management strategies at a regional scale, respecting the role of wild *Prunus* spp. in ESFY epidemics.
Pear decline (PD) is one of the most important diseases of pear and induces a more or less quick decline of the tree (Seemüller *et al*., 2011a). A decline-like disorder called 'moria del pero' has already been reported in Italy around 1950. In North America, PD was first described in British Columbia (1948) and spread then along the Pacific Coast. Devastating epidemics of PD were observed in the 1950s and 1960s along the Pacific coast of North America and in Italy (Jarausch and Jarausch, 2010). Its agent '*Ca*. P. pyri' is listed A2 quarantine pest by EPPO since 1978, and is also of quarantine significance for COSAVE (South America).
The phytoplasma disease can be found in 15 of the 27 EU countries (MacLeod *et al*., 2012). During the WG2 questionnaire survey, PD has been reported from most southern and central European countries, with its northern limit in south-east England and northern Germany. Outside of the EU and USA, PD has been found in Albania, Bosnia-Herzegovina, Croatia, Serbia, Kosovo, Turkey and Switzerland as well as in Canada, Iran, Lebanon and Libya (for review MacLeod *et al.*, 2012).
Seven distinct west Palaeartic *Pyrus*-feeding psyllid species have been described by Burckhardt and Hodinkinson (1986). They considered this group as problematic: "(it) is a complex of related but distinct species with overlapping geographical distributions.

Entomologists have usually applied names *pyri* or *pyricola* to all members of the complex and have failed to recognize morphological and biological differences between the species. Therefore, the literature concerning these species should be considered with a particular attention. A pictorial key supplemented with a dichotomous key is now available on web for the identification of the central European psyllids of the genus *Cacopsylla* which develop on Rosaceae, in particular *Pyrus*-feeding psyllid species, that should minimize the risk of misidentification" (http://www.psyllidkey.com).
Cacopsylla pyri (Linné, 1758) and *Cacopsylla pyricola* (Foerster, 1848) have been described as vectors of '*Ca.* P. pyri'. *C. pyri* is reported from Europe, the Caucasus, Central Asia, the Russian far east and China, while *C. pyricola* naturally occurs in the western palaearctics and has been introduced into the USA and Canada in the early 19th century. The two species are oligophagous on *Pyrus* species such as *P. communis*, *P. eleagrifolia*, *P. pyraster*, *P. amygdaliformis* and *P. salicifolia* where they produce several generations per year, while *C. pyrisuga* is univoltine; the adults overwinter on conifers and remigrate to *Pyrus* by middle March to April. *C. pyricola* has been described as vector of '*Ca*. P. pyri' only for Great Britain (Davies *et al.*, 1992) and North America (Jensen *et al.*,1964), while *C. pyri* was described as the vector in France (Lemoine, 1984), Italy (Carraro *et al*., 1998a) and Spain (Garcia-Chapa *et al*., 2005). Presentations during the COST meetings showed that individuals of *C. pyricola* and *Cacopsylla pyrisuga* (Foerster, 1848) have been found naturally infected with '*Ca*. P. pyri' (e.g. Austria, Italy, Czech Republic, Slovenia, Croatia, Hungary, Bosnia and Herzegovina, Poland, Romania Slovenia, and the Netherlands), but their vector capability has not yet been proven by transmission trials.

Grapevine yellows and their vectors

Two grapevine yellows due to phytoplasma infection are mainly affecting the European vineyards. Both agents at the origin of these diseases are listed as A2/EPPO quarantine pests. They are spread by sap feeding hemipteran insect vectors and to some extent by exchanges of infected planting material from grapevine nurseries. Present knowledge on the etiology and epidemiology of these grapevine yellows has been recently reviewed (Constable, 2010; Belli *et al.*, 2010).
Three strains of "flavescence dorée" (FD) phytoplasma are mainly spread from grapevine to grapevine, but surrounding wild *Vitis* regrowth, infected alders and *Clematis* may constitute potential epidemic reservoir. The genetic diversity of FD phytoplasmas has extensively been studied. It appeared that three main genetic clusters of FD phytoplasmas are present in Europe (Martini *et al*., 1999; 2002; Arnaud *et al*., 2007). In France, the genetic cluster mapFD2 is clonal and represents 85% of the disease cases, whereas the cluster mapFD1 only represents 15% of the FD cases and is mainly detected in south-western France (Salar *et al*., 2009). In Italy, mapFD3 strains (also called FD-C strains) are detected in addition to mapFD2 (also called FD-D strains) and mapFD1 strains (mostly present in north-western Italy and called FD-C/Piedmont strains). In north-eastern

regions of Italy and in Slovenia mapFD3 strains are usually more abundant than mapFD2 strains. In Serbia, only mapFD3 strains have up to now been detected. In northern Spain, Portugal and Switzerland only the mapFD2 strains have been detected so far.

The leafhopper, *Scaphoideus titanus* (Ball, 1932), an insect of North American origin, is an efficient vector of FD phytoplasma (Schvester *et al.*, 1961; Mori *et al.*, 2002). Since its introduction in Europe certainly in south-western France (Papura *et al.*, 2012), it has expanded its geographic distribution and represents an important risk factor for FD spreading. Its current distribution ranges from south Italy to Hungary, and from Portugal to Romania. If the vector is of North American origin, the FD phytoplasma is widespread in alders (*Alnus glutinosa*) in south-western France (Malembic-Maher *et al.,* 2007), but also all over Europe. Sixty to eighty percent of the alders are healthy carriers of the phytoplasma. More than 140 map genotypes have been detected in *A. glutinosa* in France, Italy, Germany, Serbia and Hungary, including the three mapFD1, mapFD2 and mapFD3 clusters. Transmission from alder to alder is achieved by *Oncopsis alni* (Maixner and Reinert, 1999), which occasionally transmits the phytoplasma to grapevine (Maixner *et al.*, 2000). In Italy and Serbia, mapFD3 strains are present in wild clematis (*Clematis vitalba*) from which they can be transmitted to grapevine by *Dictyophara europea* (Filippin *et al.*, 2009). The importance of phytoplasma transmission from alders and clematis to grapevine remains to be determined, but it cannot provoke a FD outbreak in the absence of the leafhopper *S. titanus*. In south-western France, along rivers, uncontrolled rootstock regrowth has shown to constitute in many places a reservoir for FD phytoplasmas and *S. titanus* populations escaping the insecticide treatments.

"Bois noir" (BN) is endemic in the Euro-Mediterranean area, and is associated with the accidental transmission of different "stolbur" phytoplasma strains that are maintained by epidemiological systems based on different endemic weed species as reservoir plants. BN is associated with '*Candidatus* Phytoplasma solani' presence (Quaglino *et al.*, 2013). The phytoplasmas are present in the natural vegetation and transmitted from and to herbaceous plants mainly by planthoppers of the family Cixiidae (Cvrkovic *et al.*, 2011). Since recently, *Hyalesthes obsoletus* (Signoret, 1865) was the only species known to transmit BN by occasional feeding on grapevine. However, it does not acquire phytoplasmas from infected grapevines. Instead, the root feeding nymphs acquire the pathogen from herbaceous host plants (nettle and bindweed). Where other vector species are probably involved in BN transmission, e.g. in south-eastern Europe (Cvrkovic *et al.*, 2011), their vector status and their life history need to be investigated carefully as a prerequisite for appropriate risk analysis as well as effective control of BN. Recent progress was made with the demonstration that *Reptalus panzeri* (Löw, 1883) is a BN vector in Serbia (Cvrković *et al.,* 2013).

BN is considered the result of an occasional branching of the natural transmission cycles to grapevine as a dead end host for the pathogen. Spread of BN is less epidemic than the one of FD and not affected by infected grapevine presence in the vineyard. Typical for

BN are long term fluctuations of disease incidence, with short epidemic outbreaks and decreasing disease levels during endemic stretches.
The genetic variability of BN strains is high and exhibits geographic patterns (Pacifico *et al.,* 2009; Quaglino *et al.*, 2009; Fabre *et al.*, 2011; 2011a; Johannesen *et al.*, 2012). Most important for the epidemiology of BN is that genetic diversity could be linked to plant host specificity. The variability of the *tuf* gene is diagnostic for the host plant association of BN strains (Langer and Maixner, 2004), since tuf-type a strains are specific to nettle, while tuf-type b strains are typical for bindweed, though associated with other weeds, too. In addition, populations of *H. obsoletus* from nettle and bindweed exhibit signs of adaptation to their respective host plants, e.g. differences in phenology or survival (Cargnus *et al.*, 2012; Johannesen *et al.*, 2011; Maixner, 2007). Genetically distinct host races of *H. obsoletus* have been identified in central Europe (Imo *et al*., 2013). The host affiliation of "stolbur" strains and vector populations results in distinct epidemiologic cycles based on the different plant host species. This implies the risk that new plant/vector or plant/"stolbur"-strain combinations could result in altered disease cycles and changing infection pressure to grapevine. Possible reasons for such changes include altering environmental conditions or cultural practice, host plant shift of phytoplasmas or insect vectors, and their range extension or dissemination. The phenomenon of the recent severe outbreaks of the nettle type (tuf-type a) of BN in central Europe was likely the result of the host shift of local populations of *H. obsoletus* from bindweed to nettle in combination with the range extension of Italian populations and associated tuf-type a strains to the north (Johannesen *et al.*, 2012).
The nature of BN epidemiology with grapevine being just an accidental host prevents epidemic outbreaks on the one hand, but impedes effective disease control on the other hand, since reservoir plants and vectors are common in the natural vegetation and not restricted to vineyards. Detailed information about the elements involved in local disease spread (predominant host plants, vector species, "stolbur" strains) is necessary to set up selective control measures.

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Molecular characterization of '*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma pyri' strains from Romania

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Abstract

Apple proliferation and pear decline diseases associated with '*Candidatus* Phytoplasma mali' and '*Ca*. P. pyri', respectively have an increasing importance for fruit growing production in Romania. During 2012, samples from different apple and pear varieties and from insects were collected from four fruit production regions in the southern and northern parts of Romania to test for phytoplasma presence. Shoots proliferation and enlarged stipules were observed on some of the apple trees whereas some pear trees showed premature foliage reddening. Universal primer pair P1/P7 followed by F1/B6 primers as well as primers fAT/rAS specific for apple proliferation phytoplasma group were used for amplification of 16S rRNA gene. Phytoplasmas were detected in 8 out of 35 apple and four out of 11 pear samples. Two restriction patterns were obtained on PCR products amplified with F1/B6 primers and digested with *Hpa*II, *Mse*I and *Ssp*I enzymes. No phytoplasmas were detected in insect samples. This is the first report on molecular characterization of '*Ca.* P. mali' and '*Ca*. P. pyri' strais found in Romania.

Key words: apple proliferation, pear disease, phytoplasma, PCR-RFLP identification.

Introduction

'*Candidatus* Phytoplasma mali' ('*Ca.* P. mali') and '*Candidatus* Phytoplasma pyri' ('*Ca.* P. pyri') (Seemüller and Schneider, 2004) are agents associated with the apple proliferation (AP) and pear decline (PD), respectively, serious diseases of fruit trees. Both phytoplasmas are closely related each other and together with '*Ca*. P. prunorum' belong to the apple proliferation group, 16SrX (Lee *et al*., 1998; Seemüller *et al*., 1998). '*Ca.* P. mali' is classified in subgroup-A and '*Ca.* P. pyri' in subgroup-C of 16SrX group. AP and PD are widespread in many European countries. Moreover, the presence of PD was also confirmed in north Africa and Asia (Ben Khalifa *et al.*, 2007; Liu *et al.,* 2007). *'Ca.* P. mali' is transmitted mainly by *Cacopsylla picta* Förster (Frisinghelli *et al.,* 2000; Jarausch *et al.,* 2003) and *C. melanoneura* Förster (Tedeschi and Alma, 2004). It was reported that *Fieberiella florii* Stal (Krczal *et al.,* 1988; Tedeschi and Alma, 2004) play also role in the spreading of this agent. *'Ca*. P. pyri' is transmitted by *C. pyri* L. (Carraro *et al.,* 1998; Çağlayan *et al*., 2010) in continental Europe and Turkey and by *C. pyricola* (Davies *et al.,* 1992) in the UK and in North America (Hibino *et al.,* 1971).

The affected trees display different symptoms which can be observed earlier in the season and are more pronounced during summer and autumn. Witches' broom symptom resulting through the abnormal development of axillary buds, small leaves with enlarged stipules, delayed flowering, flowers with numerous petals, small fruits with incomplete coloration and lacking in flavor and premature foliage reddening are generally associated with AP in apple trees (EPPO, 2006). Symptoms associated with pear decline disease develop along slow or quick decline of trees. Quick decline symptoms include a rapid wilting of leaves and fruits that culminate shortly with death of trees, while the slow decline is a progressive weakening of trees, in which the curl leaves, premature reddening and falling of the leaves are the most common symptoms (EPPO, 2006).

The apple proliferation disease has been occurring in Romania for a long time. Symptoms characteristic for AP were observed on wild apple trees and in apple orchards in central part of Romania in fifties and sixties of the last century. The first data on AP phytoplasma detection in the phloem elements of apple trees, symptomatology as well as its distribution in different regions of the country and economical importance and control were reported by Gheorghiu (1975). The disease incidence was assessed on 37% in intensive and 22% in traditional orchards.

Pear decline symptoms in central part of Romania were firstly described by Bălăşcuţă *et al*. (1979) and Minoiu and Ionică (1991). Over the last decades, Ploaie *et al*. (2006; 2008) brought new contributions to research on fruit trees phytoplasmas. Apple and pear production is economically important in Romania, therefore the phytoplasmas are subjected to the official control of Phytosanitary Regulations that complies with the European standards. Due to the diversity of AP and PD symptoms in different fruit growing regions and difficulties with detection of their agents, the molecular tools becomes obligatory to control both of these phytoplasmas.

The aim of this work was the detection and molecular characterization of the phytoplasmas infecting apple and pear trees in Romania, based on the 16S rDNA and the 16S-23S rDNA spacer region analysis.

Material and methods

Field survey and samples collection

During autumn 2012, shoot and leaf samples of symptomatic and asymptomatic apple and pear trees growing in seven commercial and experimental orchards located in four geographic regions (Dâmbovița, Argeş and Bistrița-Năsăud Counties and Bucharest) of southern and northern parts of Romania were collected and tested for the presence of phytoplasmas. Samples from single apple and pear trees growing in abandoned orchard in Bucharest were also included. Apple trees showed shoots proliferation (witches' broom), small leaves with enlarged stipules and rosettes, whereas curled leaves and their premature reddening were observed on pear trees. Psyllids in spring time and

leafhoppers in autumn were caught by beating on apple trees in Bucharest. A total of 35 apple samples from 12 cultivars, 11 pear sample from six pear cultivars and 10 insect samples of three species were tested for phytoplasma presence (Table 1).

Table 1. Results of phytoplasma detection in apple and pear samples collected in 2012 in different regions of Romania.

Location	Sample	Plant species/varieties/insects*	Symptoms	PCR results
Dâmboviţa	GD1.DB	apple/Golden Delicious, CO	enlarged stipules	+
	J1.DB	apple/Jonathan, CO	enlarged stipules	-
	I1.DB	apple/Idared, CO	leaf reddening	-
	GD2.DB	apple/Golden Delicious, CO	leaf reddening	-
	GD3.DB	apple/Golden Delicious, CO	leaf reddening	-
	I2.DB	apple/Idared, CO	leaf reddening	-
	Root.DB	rootstock MM 106	witches' broom	+
	GD4.DB	apple/Golden Delicious, CO	witches' broom	+
Argeş	Id1.AG	apple/Idared, CO	enlarged stipules	-
	W1.AG	apple/Wagner, CO	enlarged stipules	-
	F1.AG	apple/Florina, CO	leaf rosetting	-
	F2.AG	apple/Florina, CO	leaf rosetting	-
	GD1.AG	apple/Golden Delicious, CO	enlarged stipules	-
	Is1.AG	apple/Iris, CO	enlarged stipules	+
	Id2.AG	apple/Idared, CO	enlarged stipules	-
Bucharest	Un1.Bu	apple/unknown, AO	leaf reddening	-
	GD1.Bu	apple/Golden Delicious, AO	enlarged stipules	+
	Un2.Bu	apple/unknown, AO	leaf reddening	-
	GD2.Bu	apple/Golden Delicious, AO	witches' broom	+
	A1.Bu	apple/Aura, EO	leaf reddening	-
	G1.Bu	apple/Generos, EO	leaf reddening	-
	C1.Bu	apple/Ciprian, EO	enlarged stipules	-
	GD3.Bu	apple/Golden Delicious, EO	enlarged stipules	-
	Jp1.Bu	apple/Jonaprim, EO	leaf reddening	-
	Id1.Bu	apple/Idared, EO	leaf reddening	-
	R1.Bu	apple/Redix, EO	leaf reddening	-
	E1.Bu	apple/Everest, EO	leaf reddening	-
	GD4.Bu	apple/Golden Delicious, EO	enlarged stipules	-
	F1.Bu	apple/Florina, EO	leaf reddening	-
	Id2.Bu	apple/Idared, EO	enlarged stipules	-
	F2.Bu	apple/Florina, EO	leaf reddening	-
	GD5.Bu	apple/Golden Delicious, EO	enlarged stipules	-
	GD6.Bu	apple/Golden Delicious, EO	no symptoms	-
B. Năsăud	G1.BN	apple/Generos, EO	witches' broom	+
	G2.BN	apple/Generos, EO	witches' broom	+
Dâmboviţa	Un1.DB	pear/unknown, CO	leaf reddening	-
	L1.DB	pear/L12172P/BN, EO	leaf reddening	-
	Un2.DB	pear/unknown, CO	leaf reddening	-

Location	Sample	Plant species/varieties/insects*	Symptoms	PCR results
Argeş	N1.AG	pear/Napoca, CO	no symptoms	-
	N2.AG	pear/Napoca, CO	leaf reddening	-
	E1.AG	pear/Euras, CO	leaf reddening	-
Bucharest	W1.Bu	pear/Williams, AO	leaf reddening	+
	C1.Bu	pear/Conference, AO	leaf reddening	+
	BH.Bu	pear/Beaure Hady, AO	leaf reddening	-
	W2.Bu	pear/Williams, AO	leaf reddening	+
	Un1.Bu	pear/unknown, AO	leaf reddening	+
	Cm1.Bu-Cm6.Bu	*Cacopsylla melanoneura*, CO	-	-
	Ff1.Bu-Ff2.Bu	*Fieberiella florii*, CO	-	-
	Mp1.Bu-Mp2.Bu	*Metcalfa pruinosa*, CO	-	-

*CO, commercial orchard; EO, experimental orchard; AO, abandoned orchard

DNA extraction

Total DNAs were extracted from 1 g of fresh leaf midribs using a modified CTAB method (Maixner *et al.,* 1995) and dissolved in 100 µl TE 1X buffer. The DNA from batch of 1-5 insects were extracted by InnuPrep Plant DNA commercial kit (AnalytikJena, Germany) following the manufacturer's instructions.
DNA extracts from phloem tissue of apple and leaf midribs of *Catharanthus roseus* infected with AP-15 subtype, kindly provided by M. Cieślińska as well as DNA from healthy plant were used as positive and negative controls, respectively.

Identification of phytoplasmas by PCR-RFLP analysis

Total DNA templates were subjected to PCR using two sets of universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.,* 1995) followed by F1/B6 (Davis and Lee, 1993; Padovan *et al.,* 1995) or R16F2n/R16R2 (Gundersen and Lee, 1996) to amplify the 16S rDNA fragment. Additionally, direct PCR assays were conducted with universal primers fU5/rU3 (Lorenz *et al.,* 1995) as well as primer pair fAT/rAS (Smart *et al.,* 1993) specific for AP ribosomal group. The PCR reactions were carried out in a Thermocycler PTC-200 (MJ Research, USA) and 10 µl of the amplification products were analyzed by electrophoresis in 1.2% agarose gel followed by staining in ethidium bromide; DNA bands were visualized in an UVi-Tec transilluminator (Syngen, USA). The molecular weight of the products was estimated by comparison with a 100 bp DNA ladder (Fermentas, Vilnius, Lithuania).
To distinguish the apple proliferation subgroups in the Romanian phytoplasma strains on the basis of restriction fragment length polymorphism (RFLP) patterns, the nested-PCR products amplified using primers F1/B6 were digested with the enzymes *Mse*I, *Hpa*II and *Ssp*I (Fermentas, Vilnius, Lithuania) following manufacturer's instructions. The DNA fragments were separated on 2.5% agarose gel in 0.5% TBE buffer, stained

with ethidium bromide and visualized under UV transillumination. The RFLP profiles were compared to the patterns of reference strains and to the previously published ones (Lee *et al.,* 1998; Paltrinieri *et al.,* 2010).

Results

The results of PCRs performed with specific primers fAT/rAS showed that 8 out of 35 apple and 4 out of 11 pear samples were infected with phytoplasmas belonging to apple proliferation group (Table 1). The presence of AP phytoplasma was identified in DNAs from symptomatic plants and from positive control. No phytoplasmas were detected in insect samples, samples from asymptomatic trees and healthy control.

RFLP analyses of nested PCR products amplified with F1/B6 primers and digested with *Mse*I and *Ssp*I, showed that all infected apple samples gave the restriction patterns characteristic for '*Ca*. P. mali' classified to the subgroup 16SrX-A (apple proliferation ribosomal group). 16S rDNA fragments from pear samples after digestion with the same enzymes showed profiles referable to those of '*Ca*. P. pyri' (16SrX-C) (Figure 1). In turn, the RFLP analyses on F1/B6 amplicons digested with *Hpa*II showed two restriction profiles: P-I and P-II (Figure 1, Table 2). The P-I profile was detected in majority of '*Ca*. P. mali' strains (Figure 1, Table 2).

'*Ca*. P. mali' was present in 23.5% of tested apple samples whereas about 40% of pear samples were infected with '*Ca*. P. pyri'. The presence of phytoplasmas was not confirmed in the insect samples from apple, but further study is required, especially considering that most of the insect species involved in spreading of fruit trees phytoplasmas were already identified in Romanian orchards. The apple proliferation phytoplasma was detected in cultivars Golden Delicious, Iris and Generos in all surveyed locations: Dambovița, Argeş, Bucharest and B. Năsăud (Table 1). The most affected cultivar was Golden Delicious which is the second most important in apple production in Romania covering about 20% of apple growing area (NIS, 2013). Pear of the cultivars Williams, Conference and unknown cultivars found in abandoned orchard in Bucharest were also infected by phytoplasmas.

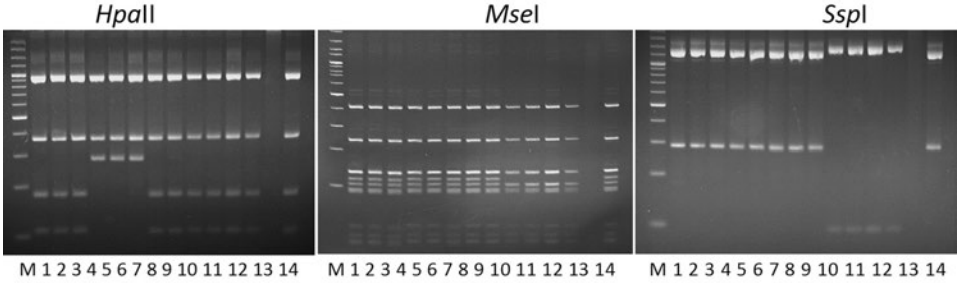

Figure 1. RFLP patterns of F1/B6 amplicons digested with *Hpa*II, *Mse*I and *Ssp*I enzymes. M: molecular marker 100 bp. Samples: apple 1-8, pear 9-12, healthy plant 13, positive control '*Ca*. P. mali' 14.

Table 2. Results of RFLP analyses of 16S rDNA and the 16S-23S rDNA spacer region of phytoplasmas infecting apple and pear trees in Romania.

No.	Species/Cultivar	Sample	Restriction pattern			Profile*
			*Mse*I	*Hpa*II	*Ssp*I	
1	apple/Golden Delicious	GD1.DB	A	A	A	PI
2	apple/Rootstock	Root1.DB	A	A	A	PI
3	apple/Golden Delicious	GD2.DB	A	A	A	PI
4	apple/Golden Delicious	GD1.Bu	A	B	A	PII
5	apple/Golden Delicious	GD2.Bu	A	B	A	PII
6	apple/Generos	G1.BN	A	B	A	PII
7	apple/Generos	G2.BN	A	A	A	PI
8	apple/Iris	Is1.AG	A	A	A	PI
9	pear/Williams	W1.Bu	B	A	B	PI
10	pear/Conference	C1.Bu	B	A	B	PI
11	pear/Williams	W2.Bu	B	A	B	PI
12	pear/Unknoun	Un1.Bu	B	A	B	PI

* profile distinction only based on RFLP profiles obtained after *Hpa*II restriction digest

Discussion

In Romania, typical symptoms of phytoplasma diseases were observed on apple trees in the 1960s (Gheorghiu, 1975) and on pear trees in the 1970s (Bălăşcuţă *et al*., 1979) respectivelly, but until now their associated phytoplasmas were not identified. The RFLP analysis revealed the presence of two distinct phytoplasmas: '*Ca*. P. mali' and '*Ca*. P. pyri' in apple and pear samples, respectively. Two different profiles, P-I and P-II were generated after *Hpa*II-digestion of PCR products amplified with F1/B6 primers. Both of these profiles were also obtained for '*Ca*. P. mali' strains from Italy, Hungary and Serbia (Casati *et al.,* 2010; Paltrinieri *et al.,* 2010), Poland (Cieślińska *et al.,* 2012) and the Czech Republic (Fránová *et al.,* 2013). The profile P-I was detected in the majority of apple from Romania whereas the P-II profile was detected only in three apple isolates from Bucharest and B. Năsăud.

This is the first report on identification of '*Ca*. P. mali' and '*Ca*. P. pyri' in Romania and characterization the strains of the both phytoplasmas based on molecular methods.

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Fruit tree phytoplasmas and their possible insect vectors in Turkey

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Abstract

The most studied phytoplasma diseases in Turkey are European stone fruit yellows (ESFY) and pear decline (PD). Results of a survey carried out by PCR/RFLP analysis on samples from more than 500 cultivated and wild *Prunus*, 116 pear and some symptomatic apple, pomegranate, persimmon (kaki) and olive samples are reported. ESFY and PD were the most common phytoplasmas in both germplasm and commercial orchards. However, also '*Candidatus* Phytoplasma mali' was detected in different regions of Turkey, its incidence was much lower compared to other 16SrX group phytoplasmas. Many overwintered individuals of *Cacopsylla pruni* were collected from *Abies* sp. and *Pinus* spp., and were molecularly typed as B. DNAs of individual psyllid species of *C. pruni* and *C. pyri* were analysed and the presence of '*Ca.* P. prunorum' and '*Ca.* P. pyri' was confirmed, respectively. As potential vectors of '*Ca.* P. mali' *C. picta, C. affinis*, and *C. melanoneura* were reported. Healthy pear and plum plants were inoculated by '*Ca.* P. pyri' and '*Ca.* P. prunorum' by using *C. pruni* and *C. pyri*, respectively. Experimental transmission trials of '*Ca.* P. mali' are in progress.

Key words: stone and pome fruits, PCR/RFLP, phytoplasmas, *Cacopsylla* spp.

Introduction

With the exception of some tropical fruits, almost all the fruit species can be grown in Turkey. In the world there are about 138 fruit species, and out of these 75 can be grown in Turkey that is the leader of production in apricot, hazelnut and sweet cherry in the world (Anıl, 2001; Asma, 2007). Although fruit tree phytoplasmas have been studied since 1999, most reports were on symptomatologic, microscopic and molecular characterization of European stone fruit yellows (ESFY), pear decline (PD) and apple proliferation (AP) diseases and on their epidemiology (Çağlayan *et al.*, 2011). First symptomatologic observations on ESFY was reported by Çağlayan and Gazel (1999) and then molecular detection of '*Candidatus* Phytoplasma prunorum' (ESFY) on Japanese plum (*Prunus salicina*) in Izmir (Jaraush *et al.*, 2000) and on apricot in Mersin province (Çağlayan *et al.*, 2004) were reported. In the following years ESFY was detected on almond, myrabolan, plum and peach trees (Sertkaya *et al.*, 2005), and then it was first time reported on germplasm orchards located in different part of Turkey

with a high infection rate as 54.8% (Ulubaş Serçe *et al.*, 2006). Responses of the Turkish apricot cultivar Tokaloğlu, Alkayısı, Şekerpare, Karacabey and Hacıhaliloğlu to '*Ca*. P. prunorum' presence under greenhouse conditions were studied and all tested plants resulted susceptible to ESFY (Ulubaş Serçe *et al.*, 2007).
'*Ca*. P. prunorum' causes economic losses on apricot and plums in Turkey. The pathogens negatively affects the cultivar Precoce de Tyrinthe apricot for both yield (decreased to 77%) and pomologic characteristics in different ratios (Gazel *et al.*, 2009). '*Ca.* P. prunorum' has recently been detected on almonds in Çanakkale province (Çağlayan *et al.*, 2011) and new cooperations with other Mediterranean countries were established for the large scale surveying for almond witches' broom disease not yet detected in Turkey. Recently phytoplasma-like symptoms were observed on cherries, pomegranate, persimmon (kaki) and also olives. The main symptoms on cherries were proliferation of branches, off-season flowering and declining, whereas yellowing and reddening were the main symptoms on pomegranate, kaki and olives.
Survey results for some fruit tree phytoplasmas and their potential vectors are summarized here.

Phytoplasma diseases in stone fruit trees

ESFY was detected in apricot, plum and almond trees. Main symptoms were yellowing, longitudinal leaf rolling, early bud break and off season flowering, on apricots; quick dieback on plums; late bud-break on some branches, leaf drops and proliferation on almonds (Figure 1).
The results of ESFY survey studies by PCR/RFLP analyses between 2002-2009 are summarized in Table 1.

Figure 1. Symptoms of European stone fruit yellows on apricot (A), plum (B) and almond (C) plants.

Table 1. Infection rate of '*Ca.* P. prunorum' in different stone fruit species obtained by PCR/RFLP analyses from surveys carried out between 2002 and 2009.

	Plum	Apricot	Almond	Peach	N. of positives/ Total	Infection rate (%)
Germplasm	14/32	11/29	2/6	1/11	28/78	35.89
Commercial orchards	12/192	9/129	3/81	0/30	24/432	5.55
Total	26/224	20/158	5/87	1/41	52/510	10.19

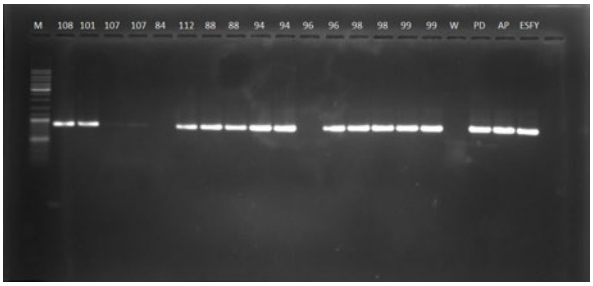

Figure 2. Nested PCR amplification of phytoplasma ribosomal DNA from symptomatic apricot (84, 88, 101, 107, 108, 112); plum (94, 96); almond (98); peach (99) trees and water control (W), using the universal primers P1/P7 followed by primers fU5/rU3. M: Marker, #SMO331 (MBI Fermentas), positive controls: (PD: pear decline, AP: apple proliferation, ESFY: European stone fruit yellows).

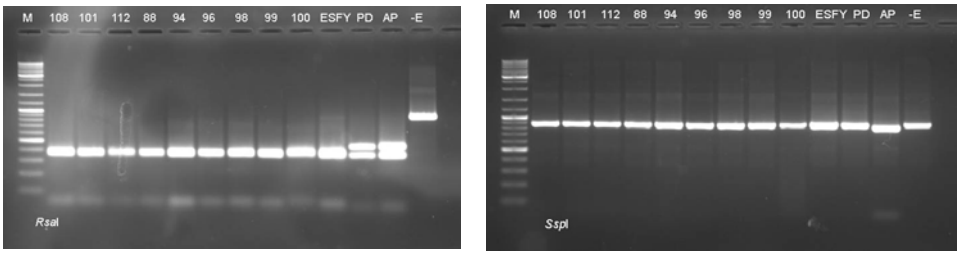

Figure 3. Results of RFLP analyses on fU5/rU3 nested PCR products from apricot (88, 101, 108, 112); plums (94, 96); almond (98, 100); peach (99) using *Rsa*I and *Ssp*I restriction enzymes. M: Marker, #SMO331 (MBI Fermentas), positive controls: (PD: pear decline, AP: apple proliferation, ESFY: European stone fruit yellows), -E: positive control uncut.

Figure 4. Abnormal flowering on cherry plants resembling phytoplasma infection.

Highest infection rate detected by PCR/RFLP analyses was found in apricot (12.65%), followed by plum (11.60%), almond (5.74%) and peach (2.43%) trees. No phytoplasma was found in wild *Prunus* species (Figures 2 and 3).
Recently phytoplasma-like symptoms were observed on cherries. Symptomatic cherry trees showing proliferation of branches, off season and abnormal flowering, and declining were sampled and, when tested by nested PCR, resulted positive (7 out of the 15 symptomatic samples tested) (Figure 4). RFLP analyses of 16R758f/16R1232r (=M1/M2) amplicons (Gibb *et al.*, 1995) showed identical restriction profiles referable to aster yellows. One of these samples was sequenced in both direction after amplification with primers R16(I)F1/R1 and the sequence showed a 99% identity with 16S rDNA from phytoplasmas related to aster yellows group ('*Ca.* P. asteris'). The sequence obtained was deposited in Genbank under the accession number KF476062 (Çağlayan *et al.*, 2013).

Phytoplasma diseases in pome fruit trees

The first suspicious symptoms for the presence of PD in Bursa province, located in Marmara Region of Turkey, were observed in 2005. The main symptoms observed in pear orchards were small, leathery leaves with up-rolled margins, leaves become abnormally red in the autumn and drop prematurely (Figure 5). The presence of PD was confirmed by PCR/RFLP analyses in Bursa samples (Ulubas Serce *et al.*, 2006) and in one sample in the east Mediterranean region of Turkey (Sertkaya *et al.*, 2005). An extensive survey was conducted for estimating PD spread in Bursa province in 2006. Out of the 116 tested pear samples, 52.58% was found to be infected. The incidence of the disease in tested Deveci, Santa Maria, Comice and William pear cultivars was detected as 58.06%, 48.57%, 44.44% and 40%, respectively. RFLP profiles obtained using of *Rsa*I, and *Ssp*I restriction enzymes on amplicons from nested PCR with R16F2n/R2 primer pair, showed that phytoplasmas belong to 16SrX group (Gazel *et al.*, 2007).

Figure 5. Reddening of pear trees cultivar Deveci in autumn due to pear decline disease.

Apple proliferation disease has been not as widespread as ESFY and PD in Turkey. First molecular characterization of '*Ca.* P. mali' was reported by Canik and Ertunç (2007) and then by Dağtekin (2009). The typical symptoms of infected trees were autumn blossom, rosette growing of shoots and sometimes enlarged stipules (Figure 6). Most of the apple trees found infected by '*Ca.* P. mali' was symptomless (K. Çağlayan *et al.*, unpublished data).
According to surveys in central and west Mediterranean regions, out of 201 samples, 8 samples from cultivars Starkrimson, Krimson, Starking, Granysmith and Gloster gave positive results by using PCR/RFLP analyses. The results up to date showed that AP disease in Turkey has only been detected in limited locations and the local cultivars are free from the disease.

Phytoplasma diseases in other fruit trees

Recently phytoplasma-like symptoms, mainly yellowing and reddening, were observed on pomegranate, persimmon (kaki) and olives (Figure 7). Although all symptomatic samples gave positive results by using universal phytoplasma primers P1/P7 followed by R16F2n/R2, phytoplasma characterization studies are still in progress.

Figure 6. Rosette growing of apple branches associated with the detection of apple proliferation phytoplasmas.

Figure 7. Early reddening of pomegranate (left and middle) and yellowing on olive leaves (right) associated with phytoplasma detection.

Vector studies

Seventeen localities from seven provinces, where apricot, peach and plum growing is economically important in Turkey, were selected for surveys of *C. pruni* presence carried out during the springs 2010-2011. Psyllids were collected from wild (e.g. *P. spinosa, P. ceracifera*) or cultivated *Prunus* species and conifers. *C. pruni* was collected in provinces in which '*Ca.* P. prunorum' infections have been recorded in previous researches and the psyllid was found in both overwintered hosts (*Abies* sp.) and *Prunus* hosts (Serçe *et al*., 2012). Phytoplasma infected remigrants of *C. pruni* were detected in considerably high numbers. This situation clearly revealed the potential spread risk of '*Ca.* P. prunorum' by *C. pruni* in Turkey and the necessity of taking precautions. Molecular typing based on COI genes and an ITS region indicated that all *C. pruni* individuals were from the ecotype B.

'*Ca.* P. pyri' was detected by PCR/RFLP analyses in *C. pyri* individuals, from symptomatic pear orchards. Potential vectors of '*Ca.* P. mali' were also collected from hawthorn and apple trees in different provinces; *C. picta*, *C. affinis* and *C. melanoneura* were identified. Healthy pear and plum plants were inoculated with '*Ca.* P. pyri' and '*Ca*. P. prunorum' by using *C. pruni* and *C. pyri,* respectively (Serçe *et al*., 2012; K. Caglayan *et al*., unpublished data). Experimental transmission trials of '*Ca.* P. mali' is in progress.

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Phytoplasma infections in *Rhododendron hybridum*

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Abstract

The survey of the health status of azaleas and rhododendrons on production areas in the Czech Republic started in 2006. Symptoms of unknown aetiology on *Rhododendron hybridum* cultivar Cunningham's White were observed in 2010. The infected plant had malformed leaves, with irregular shaped edges, mosaic, leaf tip necrosis and multiple axillary shoots with smaller leaves. Transmission electron microscopy showed phytoplasma-like bodies in phloem cells of the symptomatic plant. Phytoplasma presence was confirmed by polymerase chain reaction using phytoplasma-specific, universal and group specific primer pairs. Restriction fragment length polymorphism analysis of 16S rDNA enabled classification of the detected phytoplasma in the aster yellows subgroup 16SrI-C. Sequence analysis of the 16S-23S ribosomal operon of the amplified phytoplasma from the infected rhododendron plant (1,724 bp) confirmed the closest relationship with the Czech *Echinacea purpurea* phyllody phytoplasma. These data suggest *Rhododendron hybridum* as new host for the aster yellows phytoplasma subgroup 16SrI-C in the Czech Republic and worldwide. Using a nested PCR assay, phytoplasmas belonging to aster yellows group were detected in two azalea bushes. Mosaic and yellowing were the main symptoms of the disease.

Key words: Czech Republic, electron microscopy, PCR, phytoplasma, sequencing.

Introduction

Rhododendron sp. is a favorite shrub, often evergreen, that is grown worldwide as a garden or potted ornamental plant, especially for showy flowers. A phytoplasma disease of *Rhododendron* sp. plants was first observed in Ukraine in azalea showing symptoms of yellowing (Onishchenko *et al*., 1988). Rhododendron plants with symptoms of leaf malformation and variegation were observed in the Czech Republic in 1997. "Stolbur"-type phytoplasmas were detected using nested polymerase chain reaction (PCR) and identified by restriction fragment length polymorphism (RFLP) analyses (Mertelík *et al*., 2006). The natural occurrence of '*Candidatus* Phytoplasma trifolii' in *Rhododendron hybridum* plants was revealed by transmission electron microscopy (TEM) and PCR/RFLP analysis (Přibylová *et al*., 2009). A phytoplasma associated with little leaf disease in azalea was detected in China; based upon results from RFLP analysis of multiple loci, this phytoplasma, named azalea little leaf (AzLL), was enclosed in a new 16SrI-T subgroup, in the aster yellows phytoplasma group (Wei *et al*., 2011). Similarly, symptoms of witches' broom disease were observed on Kanehirai azalea (*R. kanehirai*) in Taiwan, and phytoplasmas were detected by TEM in sieve elements (Wang, 1994).

Material and methods

A plant of *Rhododendron hybridum* cultivar Cunningham's White with unusual symptoms was found in a germplasm collection of rhododendron in central Bohemia in May 2010. This plant exhibited malformed leaves with irregular edges, showing also mosaic and leaf tip necrosis, leading to rolled and fragile leaves. Axillary shoots with smaller leaves were also present (Figures 1a and b). The symptomatic plant (approximately 20 cm in height) was taken from the field, replanted and maintained under glasshouse conditions for further tests. An asymptomatic rhododendron plant was taken as a negative control. Previously identified phytoplasmas maintained in periwinkle plants (*Catharanthus roseus*) served as positive controls: aster yellows subgroups 16SrI-B (Přibylová *et al*., 2001) and 16SrI-C (clover phyllody) (Fránová *et al*., 2004). Samples of midribs from symptomatic leaves of the diseased and asymptomatic plants were examined with TEM.

DNA was extracted from midribs of both asymptomatic and symptomatic plants, and from leaves of positive controls. A healthy *C. roseus* plant (raised from seed) was used as negative control. A chloroform/phenol method followed by isopropanol precipitation was employed for DNA extraction (Lee *et al*., 1991). In direct PCR universal, phytoplasma-specific primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) were used for amplification of a 1.8-kbp product and fU5/rU3 (Lorenz *et al*., 1995) yielding a product of approximately 800 bp. In nested PCR assays group-specific primers R16(I)FI/RI (Lee *et al*., 1994) were used. Tubes with the reaction mixture devoid of DNA template, with DNA from the asymptomatic rhododendron and from the healthy *C. roseus* were included in each experiment as negative controls.

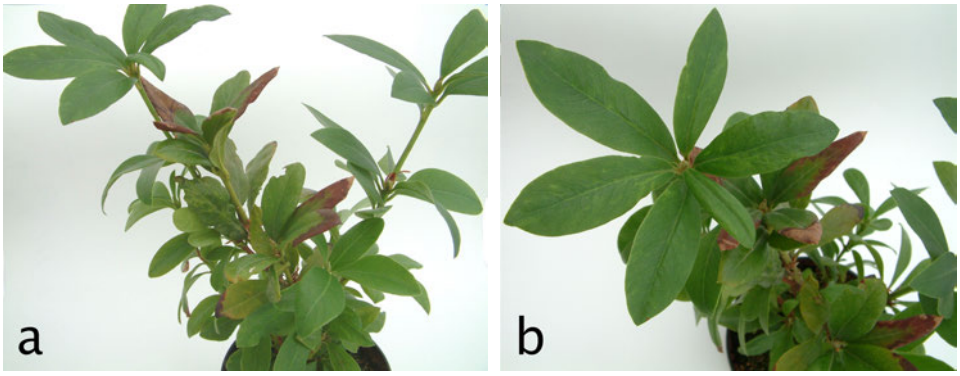

Figure 1. *Rhododendron* plant cultivar Cunningham´s White infected by a phytoplasma of the 16SrI-C group showing symptoms of mosaic, necrosis and witches´ broom growth (a). Detail of malformed leaves with mosaic and necrosis (b).

The nested PCR products (1.1 kbp) of phytoplasma 16S rDNA sequence, amplified with the primer pair R16(I)F1/R1, were subjected to RFLP analysis; 3 µl were digested overnight at 37°C with the restriction endonuclease *Mse*I (NE Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. The digest was resolved on an 8% polyacrylamide gel. The approximate molecular weight and size of the resulting DNA bands were estimated using a 100-bp DNA Ladder (Thermo Scientific, Vilnius, Lithuania).

A set of overlapping PCR products from the symptomatic rhododendron was generated by amplification with primers P1/U3 and 16R758/P7. PCR products were sequenced using a BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK) from both directions. Sequencing was performed in an ABI PRISM 310 sequencer (PE Applied Biosystems, Foster City, CA, USA). The sequence was compared with the phytoplasma sequences available in the GenBank database using the BLASTn network service available at the National Center for Biotechnology Information. Multiple sequence alignments were performed using CLUSTALW (Thompson *et al*., 1994).

Symptomatic plants of azaleas showing mainly mosaic and yellowing of leaves were also analyzed. Fourteen selected azalea plants of different cultivars were tested using nested PCR assays. DNA was extracted from mixed samples of midribs and phloem. PCR assays were performed with P1/P7 primers for the initial amplification. The diluted (1: 30) PCR product from the first amplification (1 µl) was used as template in nested PCR with internal primer pairs R16F2/R2 (Lee *et al*., 1995) and fU5/rU3, tests were carried out in the same way as mentioned above.

PCR products amplified with primers R16F2/R2 were sequenced as above and the sequences were compared with those in the GenBank database.

Results and discussion

Examination of ultrathin cross sections of leaf midribs from symptomatic leaves with a Jeol 100 MB TEM revealed that some phloem cells of the symptomatic rhododendron contained irregular ovoid and pleomorphic phytoplasma-like bodies ranging from 60 x 80 nm to 270 x 390 nm in diameter (average 159 x 240 nm). These were present in low numbers and were scattered within the cells among residual cytoplasm (Figure 2). No bodies were found in the asymptomatic rhododendron samples.

Polymerase chain reaction assays were carried out to detect and identify the phytoplasma. Amplification with the primer pair fU5/rU3 yielded a PCR product of the expected size. The nested PCR assay produced a PCR product of 1.1 kbp with primer pair R16(I)F1/R1. No PCR products were obtained from the asymptomatic plant and negative controls. When the restriction profile of the PCR product amplified with R16(I)F1/R1 primers digested with the *Mse*I, was compared with those obtained from the positive controls, the rhododendron-infecting phytoplasma profile was undistinguishable from the profile of clover phyllody, a member of the ribosomal subgroup 16SrI-C (data not shown).

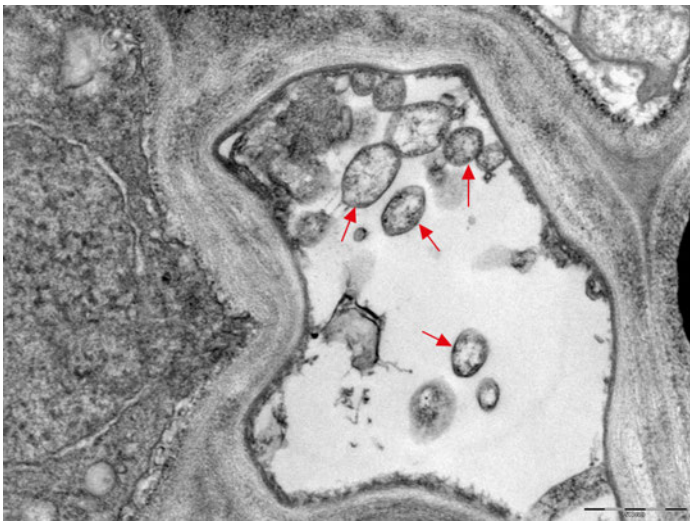

Figure 2. Phytoplasma-like particles (red arrows) in a leaf midrib phloem cell of the diseased *Rhododendron* plant (*bar* = 500 nm).

The sequence of 1,724 bp obtained from the phytoplasma infecting the rhododendron plant was deposited in the GenBank database (accession number KC009838). The detailed sequence comparison revealed the closest relationship of the rhododendron phytoplasma with the Czech *Echinacea purpurea* phyllody phytoplasma (EF546778), a member of ribosomal subgroup 16SrI-C (Fránová *et al*., 2009). The two sequences form a pair slightly different from the 10 closely related sequences (accession numbers AB551736, AB693125, AB693124, GQ249410, FJ824597, AY566302, AY101386, AB693126, AB558132, HQ589186). Twelve differences in sequences were found between the two sequences mentioned above and these 10 closest sequences available in GenBank database. The adenine 'A' at position 569 complements a restriction site and is a unique substitution, which discriminates the rhododendron sequence from all others, including the Czech *E. purpurea* phyllody phytoplasma.

By nested PCR assays, the phytoplasma was detected in two out of fourteen azalea samples. No visible PCR products were obtained from symptomatic azalea plants with the P1/P7 primers, and PCR products of 1.2 kbp were amplified by R16F2/R2 primers. No PCR products were obtained from the negative controls.

Comparison of the phytoplasma 16S rDNA sequence obtained from the azalea cultivar Cecille (1,091 bp) and from an unknown cultivar (1,116 bp) revealed the closest relationship with members of aster yellows group. Although there were some differences between the two sequences, clear relationship to aster yellows group was confirmed. BLAST analysis of these sequences revealed that the 16S rDNA sequence from cultivar Cecille as well as that from the unknown cultivar shared 99.9% homology with those of phytoplasmas belonging to 16SrI group (accession numbers HM067755 and KC283218, respectively).

The rhododendron and azalea plants, like those described in two previous studies (Přibylová *et al*., 2009; Mertelík *et al*., 2006), originated from a breeding programme at the Silva Tarouca RILOG Průhonice. The phytoplasma diseases found in the Czech Republic differed from one another, as they induced different symptoms. Stunting, yellowing and absence of flowers were the main symptoms of those infected by '*Ca.* P. trifolii' (Přibylová *et al*., 2009). The "stolbur"-type rhododendron plants exhibited leaf malformation and variegation (Mertelík *et al*., 2006). The aster yellows type here described on the rhododendron was characterized by witches' broom growth, malformation, mosaic and necrosis of leaves. Throughout three years of observations, the plant produced no flowers, which was a feature common with the plants infected by '*Ca.* P. trifolii'. The aster yellows disease observed on azaleas, inducing mosaic and yellowing symptoms, originated from the same plantation where later the rhododendron infected by *'Ca*. P. trifolii*'* was found (in 2010).
Although the aster yellows group (16SrI), '*Ca.* P. asteris', is the group of phytoplasmas infecting the widest range of plant hosts wordlwide, the *R. hybridum* plant infected by a phytoplasma belonging to the aster yellows subgroup 16SrI-C is the first of its kind ever found in the Czech Republic or worldwide.

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Maize redness disease: current situation in Bosnia and Herzegovina

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Abstract

Maize redness (MR) induced by "stolbur" phytoplasma was reported for the first time in 2012 in Semberija region of Bosnia and Herzegovina. In addition to symptomatic maize plants, "stolbur" phytoplasma also was identified in johnsongrass plants and in *Reptalus panzeri* specimens. Monitoring and sampling in 2013 were extended to Brčko District and Posavina region. Collected samples were molecularly analyzed for the phytoplasma presence. "Stolbur" phytoplasma was detected in symptomatic maize plants in all surveyed regions.

Key words: "Stolbur" phytoplasma, Semberija, Brčko District, Posavina, corn.

Introduction

Maize redness (MR) is a disease which develops characteristic symptoms of maize leaf discoloration from mid July until end of August. For 50 years, the disease was a phenomenon of unknown etiology. The disease symptoms were described and reported in 1957 in Banat region of Serbia (Marić and Savić, 1965). Main symptoms are central midrib, leaves and stalk redness, the plants often express abnormal ear development, cob deformations and maturation disorders. Later symptoms were described in other regions of Serbia as well as in maize fields of Romania and Bulgaria (Šutić *et al.*, 2002). In the last two decades of the 1900, MR appeared sporadically with low percentages. However, at the beginning of the 21 century, in fact in 2002 and 2003, MR induced serious losses in maize production ranging from 40% to 90% in fields of South Banat region (Bekavac *et al*., 2011).

Figure 1. Map of maize sampling sites in Bosnia and Herzegovina.

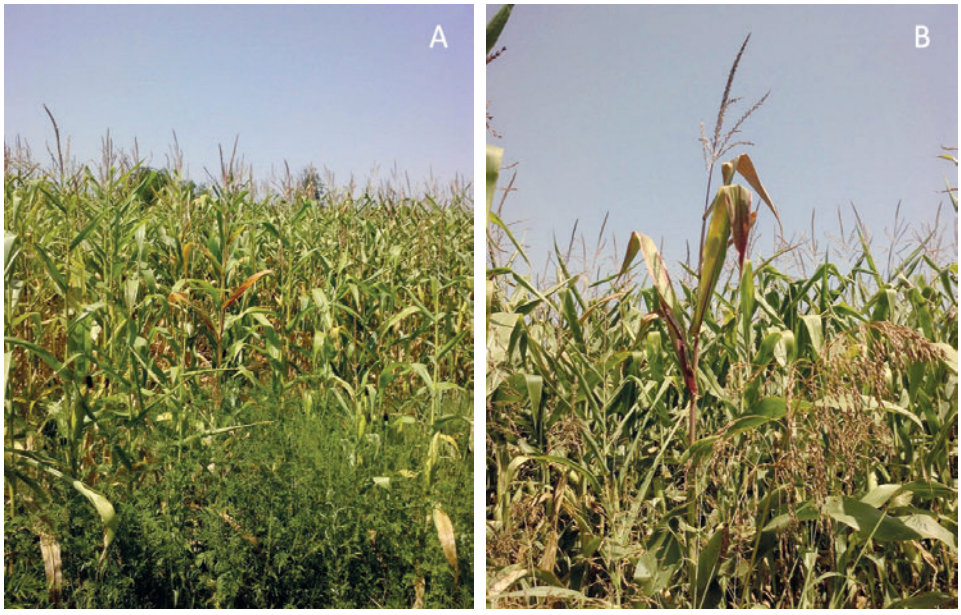

Figure 2. MR symptomatic corn plants in Brčko district (A) and Posavina (B) regions.

Figure 3. MR symptomatic corn plants in Semberija region.

After the first report of the disease, numerous studies have been conducted with the aim to determine the causal agent attributed to *Fusarium* spp., fastidious bacteria or to the occurrence of abiotic factors such as drought, lack of some micro- or macro-elements (magnesium, zinc, phosphorus) or even shock-effect due the temperature change (Bekavac *et al*., 2011). Nevertheless, in 2006 applying molecular diagnostic tools "stolbur" phytoplasmas (subgroup 16SrXII-A, '*Candidatus* Phytoplasma solani') were found to be associated with MR disease in maize plants collected from South Banat

Serbia (Duduk and Bertaccini, 2006). Epidemiological studies in Serbia showed that cixiid *Reptalus panzeri* (Löw) is a vector of "stolbur" to maize as well as that johnsongrass (*Sorghum halepense* L.) and overwintering wheat (*Triticum aestivum* L.) plays important role in the disease cycle (Jović *et al*., 2007). Phytoplasma infected adult *R. panzeri* emerge in mid June and transmit "stolbur" phytoplasma during feeding. At end of July, females lay eggs on infected maize roots, and nymphs living on these roots acquire the phytoplasma from infected maize. Hereafter, the nymphs overwinter on the roots of johnsongrass and wheat grown into maize fields in the autumn (Jović *et al*., 2009; 2011). So far molecular analyses confirmed presence of "stolbur" phytoplasma associated with MR disease in Hungary (Acs *et al*., 2011) and Italy (Calari *et al*., 2011). Moreover, in Italy phytoplasmas belonging to 16SrI (aster yellows) and 16SrIII (X disease) groups were also identified in MR symptomatic maize (Calari *et al*., 2010; 2011; Mejia *et al*., 2013). Considering that maize is an important crop in European regions and the potential of MR spreading, in 2012 the EPPO Panel on Phytosanitary measures put "stolbur" phytoplasma associated with maize redness disease to the Alert List (EPPO, 2012).
In last five years, MR-like symptoms were observed in Semberija region of Bosnia and Herzegovina. In 2012, the first survey for the MR agent and vector identification was conducted. Laboratory analyses showed presence of the "stolbur" phytoplasma in symptomatic maize plants, johnsongrass and *R. panzeri* specimens collected from several locations in Semberija region (Kovačević *et al*., 2014).
Since maize production has a significant place in Bosnia and Herzegovina agriculture in 2013 was carried out an extended survey to verify the disease presence in larger areas.

Surveys for maize reddening detection in 2013

Three important centres for maize production (Semberija, Brčko District and Posavina) were surveyed for the occurrence of reddening symptoms in maize fields during first week of August 2013. Plants expressing symptoms such as midrib, leaf and stalk reddening were collected from nineteen sampling sites in northeast Bosnia and Herzegovina (Figure 1). In addition, in all sampling sites symptomatic plants percentage was estimated. Maize leaves and roots were sampled from symptomatic plants. All samples were kept at -20°C until DNA extraction.

Detection of "stolbur" phytoplasma in maize

Total DNA extraction

DNA was extracted from MR symptomatic maize plants according to previously reported modified Dneasy® Plant Mini Kit-a (QIAGEN) protocol (Green *et al*., 1999). Leaf midribs and adventitious roots (0.5-0.6 g) were homogenised in 3% CTAB buffer (3% CTAB, 100 mM Tris-HCl pH 8, 10 mM EDTA, 1.4 M NaCl, 3%

polyvinylpyrrolidone (PVP-40) and 0.2% ß-mercaptoethanol). The homogenate (0.5 ml) was transferred to a 2 ml microcentrifuge tube and mixed with RNase (20 mg/ml) then incubated at 65°C for 30 minutes. Following DNA extraction procedure was according to the kit manufacturer instructions.

Molecular detection of phytoplasma in maize

Extracted nucleic acids were analyzed by nested polymerase chain reaction (PCR) amplification of ribosomal DNA using phytoplasma universal primers. P1 (Deng and Hiruki, 1991) and P7 (Smart *et al.*, 1996) primer pair was used in direct-PCR procedure. For nested-PCR reaction R16F2n and R16R2 primers (Lee *et al.*, 1998) were employed. Nested-PCR products in which phytoplasma presence was detected were submitted to RFLP analyses with *Rsa*I (Fermentas) and *Alu*I (BioLabs) restriction enzymes. Digested products were run with electrophoresis on 2% MetaPhore (Cambrex) agarose gel in 1X TBE buffer. Gel was stained with ethidium bromide and visualized under UV transiluminator.
For "stolbur" phytoplasma characterization DNAs from infected samples were amplified in nested-PCR with fTuf1/rTuf1 (in direct-PCR) and fTufAy/rTufAy (nested-PCR) (Schneider *et al.*, 1997) primer pairs. Nested-PCR products were digested with *Hpa*II (Fermentas) restriction enzyme. RFLP profiles were observed with electrophoresis in 2% MetaPhore (Cambrex) agarose gel in 1 X TBE buffer.

Results of the 2013 survey for maize reddening

In early August 2013, symptoms of maize redness were observed in all three surveyed regions, but in different percentage. In Brčko district and Posavina regions, MR symptoms ranged from 10% to 30% (Figure 2). However, the high percentage (40%-80%) of the symptomatic plants was observed in Semberija region (Figure 3) where MR appearance in the last three years was epidemic. Nevertheless, most of the plants in the surveyed fields of Posavina and Brčko were dry because high drought was present during summer period in 2013. In total 28 samples with MR-like symptoms were sampled for laboratory analyses.

Results of laboratory analyses

Nested-PCR and RFLP analysis of DNAs from the collected maize plants indicated that 16 out of 28 tested samples (57%) were positive for "stolbur" phytoplasma (Table 1, Figure 4). Relatively high number of plants with MR-like symptoms showing absence of phytoplasma infection could be in correlation with various biotic and/or abiotic unidentified factors. On the other hand, in Brčko District and Posavina regions monitoring of *R. panzeri* wasn't performed, thus it is hard to estimate relationship of disease severity with presence and number of this possible vector. During the MR

monitoring in Semberija region in 2012 *R. panzeri* was found to be abundant in the maize fields where subsequently high percentage of "stolbur" positive plants were identified (Kovačević *et al*., 2013). It is worth to mention that most of the tested maize plants were quite dry that could influence quality and yield of extracted DNA and lead to phytoplasma negative results.

Table 1. Surveyed locations and presence of "stolbur" phytoplasma in maize in 2013.

N.	Location	Symptom description	Phytoplasma presence
	Semberija		
1.	Govedari	midrib redness and leaf necrosis	"stolbur"
2.	Gunjevac	leaf necrosis and slight redness	negative
3.	Drinsko polje	strong necrosis and slight leaf redness	negative
4.	Smiljevac	leaf and stalk violet colour, strong necrosis	"stolbur"
5.	Gospavići	leaf and stalk violet colour, strong necrosis	"stolbur"
6.	Barinovac	leaf and stalk violet colour, strong necrosis	"stolbur"
7.	Duga brazda	leaf and stalk violet colour, strong necrosis	"stolbur"
8.	Amajlije	slight leaf violet colour and strong necrosis	"stolbur"
9.	Popovi	slight leaf violet colour and strong necrosis	negative
10.	Dvorovi	strong violet colour of leaf and stalk and necrosis	"stolbur"
11.	Janja ustava	strong violet colour of leaf and stalk and necrosis	"stolbur"
12.	Janja ustava	slight leaf violet colour and strong necrosis	negative
	Posavina		
13.	Tursinovac	leaf chlorosis	negative
14.	Tursinovac	violet midrib, leaf chlorosis/strong necroses	negative
15.	Crkvina	midrib redness, leaf chlorosis	"stolbur"
16.	Crkvina	midrib redness, leaf chlorosis and necrosis	negative
17.	Obudovac	violet leaf and chlorosis	negative
18.	Obudovac	violet colour of leaf midrib and slight chlorosis	negative
19.	Obudovac	violet leaf and strong necrosis	"stolbur"
20.	Pelagićevo	violet leaf and strong necrosis	"stolbur"
21.	Pelagićevo	violet leaf and strong necrosis	"stolbur"
22.	Pelagićevo	violet leaf and slight necrosis	"stolbur"
	Brčko District		
23.	Bosanska Bijela	violet leaf, strong chlorosis and necrosis	"stolbur"
24.	Pantića Brod	violet leaf and slight chlorosis	"stolbur"
25.	Pantića Brod	violet leaf and strong necrosis	"stolbur"
26.	Pantića Brod	violet midrib	negative
27.	Sambića Brdo	violet midrib	negative
28.	Sambića Brdo	violet midrib	negative

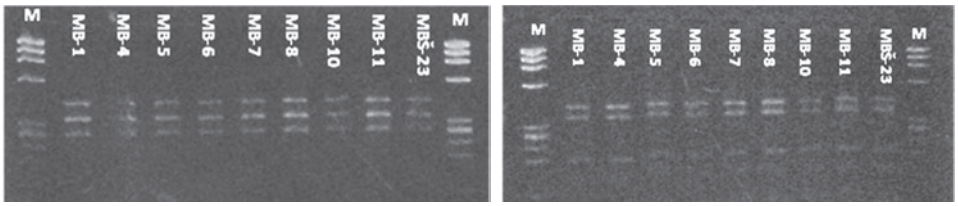

Figure 4. RFLP analysis of R16F2n/R16R2 PCR products digested with *Rsa*I (left) and *Alu*I (right) visualized after electrophoresis on 2% MetaPhore (Cambrex) agarose gels in 1XTBE buffer. Lane M, ΦX174 ladder (Fermentas).

Samples providing positive results in nested PCR assays with R16F2n/R16R2 primer pairs digested with *Rsa*I and *Alu*I restriction enzymes showed restriction profiles identical to each other, and referable to those of "stolbur" phytoplasma (Figure 4) and to other members of the 16SrXII group as defined previously (Lee *et al*., 1998; 1998a). Further molecular characterization of "stolbur" phytoplasma agent of maize redness disease was performed on elongation factor (*tuf* gene). Restriction analysis carried out with *Hpa*II endonuclease showed RFLP profiles identical to each other and having a tuf-type b profile (Figure 5), which is the most common type of "stolbur" phytoplasma which infects grapevine in Europe (Langer and Maixner, 2004).

Intensive wheat/maize crop rotation leads to the increased maize redness disease incidence because it favors the development of the vector *R. panzeri* larvae in high numbers (Jović *et al*., 2009). Moreover, stolbur tuf-type b was found to be less host specific and identified in many herbaceous plants (Belli *et al.,* 2010). Therefore several plant species acts as food hosts for vectors as well as "stolbur" inoculum such as winter wheat and johnsongrass in the case of MR disease. During surveys in 2012 and 2013 the highest incidence of MR disease was in fields where maize/wheat crop rotation was a common agriculture practice. According to this, further studies should be carried out to identify plants and vector populations source of the "stolbur" phytoplasma in maize fields in larger area of Bosnia and Herzegovina. However, it is possible to speculate that change of the plants in crop rotation or introduction of three-field rotation could significantly decrease the disease severity.

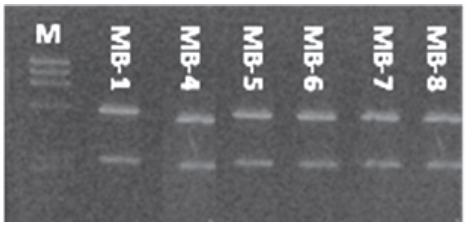

Figure 5. RFLP analysis of ftufAy/rTufAy PCR products digested with *Hpa*II visualized after electrophoresis on 2% MetaPhore (Cambrex) agarose gels in 1XTBE buffer. Lane M, ΦX174 ladder (Fermentas).

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Molecular diversity of phytoplasmas infecting *Rubus* spp. plants in Poland

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Abstract

Rubus spp. plants with severe symptoms of stunting, short and thin shoots were observed in central Poland both in commercial plantations and in natural environments. Shoot samples were collected from 13 plants of red raspberry, blackberry and hybrids of *Rubus* spp. The RFLP, sequence and phylogenetic analyses were carried out based on nucleotide sequences of 16S rRNA and *secY* genes in 11 and 9 detected phytoplasma strains respectively. Most of the strains were placed in the same clade with the reference strain RuS classified as '*Candidatus* Phytoplasma rubi' and belonging to the elm yellows group (16SrV). Results of these analyses also showed that a strain from loganberry belonged to X-disease group (16SrIII) and the phytoplasma infecting one of the wild blackberry was classified in aster yellows group, 16SrI ('*Ca*. P. asteris'). The comparative genomic analysis of the *secY* gene showed that nine Polish isolates were phylogenetically closely related to each other and to rubus stunt reference strain.

Key words: *Rubus* sp., phytoplasma, PCR-RFLP, sequencing, phylogenetic analysis.

Introduction

Poland is one of the leading producers of fruits in Europe and raspberry is one of the most important species cultivated: its productivity has doubled over the past five years. Fungal and viral diseases, although varying in expression, severity, and frequency, limit fruit quality, yield, and stand longevity. Phytoplasma diseases can be other potential threat for raspberry cultivation as they cause serious damages including stunting, small leaves, short internodes, enlarged sepals, phyllody, shoot and flower proliferation, and fruit malformations (van der Meer, 1987; Mäurer and Seemüller, 1994). These diseases are associated with presence of phytoplasmas belonging to several 16Sr groups: elm yellows (Marani *et al*., 1977; Mäurer and Seemüller 1994; Bertaccini *et al*., 1995; Marcone *et al*., 1997; Davies, 2000; Vindimian *et al*., 2004), X disease (Davies, 2000; Davis *et al*., 2001), aster yellows (Borroto Fernández *et al*., 2007; Fahmeed *et al*., 2009; Reeder *et al*., 2010) and "stolbur" (Borroto Fernández *et al*., 2007, Bobev *et al*., 2013). '*Candidatus* Phytoplasma rubi' (Malembic-Maher *et al*., 2011), member of the elm yellows group (16SrV), is the most common phytoplasma detected in *Rubus* spp. plants. It affects wild and cultivated raspberry, blackberry, and crosses of these species throughout Europe, north-eastern USA and Turkey (Mäurer and Seemüller, 1994; Converse *et al.*, 1982, Davis *et al*., 2001; Sertkaya *et al*., 2004).

The aim of this study was to identify and classify phytoplasmas occurring in *Rubus* sp. plants in Poland and to characterize them with molecular analysis of *16S rRNA* and *secY* genes.

Materials and methods

Rubus spp. plants with severe symptoms of stunting, short, and thin shoots were observed on raspberry and blackberry plantations as well as in natural environments in several localities in central Poland. Leaf samples were collected from 13 plants including red raspberry (Canby, Polka, Veten cultivars); blackberry (Loch Tay and Darrow cultivars), four wild blackberry (*Rubus fruticosus*) growing in forest (WB3, WB6, WB8, WB10); as well as in hybrids tayberry and 'Tummel' (*R. loganobaccus* x *R. idaeus*), and in two loganberry (*R. loganobaccus*) plants. Some of the plants showed distortion and premature reddening of the leaves, enlarged sepals, phyllody, flower proliferation, fruit malformation, and dieback.

Total DNA was extracted from veins of leaves using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA was subjected to PCR with P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by nested universal primers R16F2n/R16R2 (Gundersen and Lee, 1996): primer pairs R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1 (Lee *et al*., 1994) specific to 16S rDNA of aster yellows (16SrI), X disease (16SrIII), and elm yellows (16SrV) phytoplasma groups, respectively were also employed. DNA from a sample of healthy raspberry was included as negative control. RFLP and sequence analyses of 16S rDNA fragment amplified with universal primers R16F2n/R16R2 were conducted to identify the phytoplasmas. PCR products were digested using *Hpa*II, *Hha*I, *Rsa*I, and *Bfa*I restriction enzymes. AY1 (16SrI), CX (16SrIII), and RuS (16SrV) strains, kindly provided by A. Bertaccini (DipSA, Plant Pathology, University of Bologna, Italy), were employed as reference strains. The resulting RFLP patterns were also compared with published profiles of representative phytoplasma strains (Lee *et al*., 1998; 2004).

SecY gene was analysed to detect a possible finer differentiation among 10 strains of '*Ca.* P. rubi'. Amplification of the entire translocase (*secY*) gene and portion of the ribosomal protein L15 (*rpl15*) gene was conducted using two sets of 16SrV-group specific primer pairs: FD9f2/FD9r followed by FD9f3/FD9r2 (Angelini *et al*., 2001; Daire *et al*., 2001). RFLP analysis was conducted after digestion of the nested PCR products with restriction enzymes: *Taq*I, *Mse*I, *Alu*I, *Hpa*II, *Hh*aI, and *Ssp*I. Phylogenetic analysis was carried out on nucleotide sequences of 16S rRNA and *secY* genes from 11 and 9 phytoplasmas respectively, and reference strains obtained from GenBank. The partial nucleotide sequences of 16S rRNA and *secY* genes were compared using the BLAST algorithm (http://ncbi.nlm.nih.gov/BLAST). Multiple alignment was made using ClustalW of the DNASTAR's Lasergene software (DNASTAR Inc., Madison, WI, USA). Phylogenetic and molecular evolutionary analyses were carried out by the neighbor-joining algorithm implemented in ClustalW of the genetic analysis software MEGA, 4.02 (Tamura *et al*., 2007).

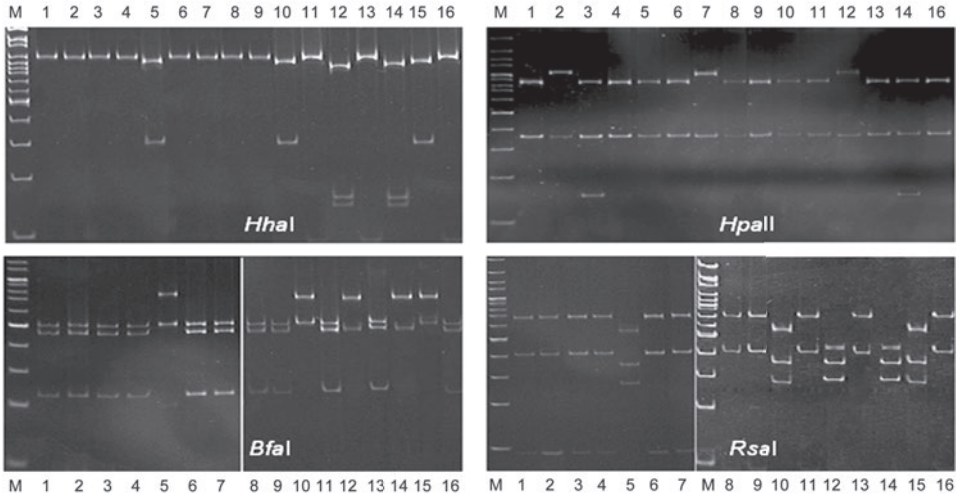

Figure 1. Polyacrylamide gels showing restriction fragment length polymorphism (RFLP) profiles obtained on R16F2n/R16R2 amplicon of phytoplasma-infected samples from *Rubus* spp. plants after digestion with *Hha*I, *Hpa*II, *Rsa*I, and *Bfa*I restriction enzymes. M - GeneRuler 100 bp Plus DNA Ladder (Fermentas, Lithuania). Samples: 1. Veten, 2. Polka, 3. Loch Tay, 4. Tummel, 5. loganberry 6. tayberry, 7. Darrow, 8. Canby, 9. WB3, 10. Loganberry, 11. WB6, 12. WB8, 13. WB10. Reference strains: 14. AY-1 (aster yellows, 16SrI-B), 15. CX (X-disease, 16SrIII-A), 16. RuS (rubus stunt, 16SrV-E).

Results and discussion

PCR products with R16F2n/R16R2 primers were obtained for all 13 tested samples, but for two of them (loganberry and WB8) no specific products were amplified when primers specific for elm yellows phytoplasma group were used. On the basis of RFLP analyses with *Hpa*II, *Hha*I, *Rsa*I, and *Bfa*I restriction enzymes and comparative analysis of the nucleotide sequence of the 16S rRNA gene, it was shown that *Rubus* spp. plants were infected by phytoplasmas belonging to three different ribosomal groups. Most of the samples were infected by '*Ca.* P. rubi' belonging to the elm yellows (EY) phytoplasma group, 16SrV (Figure 1). RFLP analysis of amplicons from cultivars Veten, Polka, Loch Tay, Tummel, Tayberry, Darrow, Canby, and three wild blackberry plants (WB3, WB6, WB10) yielded patterns indistinguishable from those of strains RuSR19, RuS400, RuS971, and RuS classified to the EY group (Lee *et al*., 2004). It was reported that this phytoplasma has also occurred in wild and cultivated raspberry and blackberry in Germany, France, Italy, the UK, and Poland (Mäurer and Seemüller, 1994; Bertaccini *et al*., 1995; Lee *et al*., 1995; Marcone *et al*., 1997; Davies, 2000; Vindimian *et al*., 2004, Cieślińska, 2011). The R16F2n/R16R2 products from two loganberry plants digested with four endonucleases resulted similar to the reference strain of X-disease phytoplasma group (16SrIII). X disease phytoplasma was previously identified in loganberry in the

UK (Davies, 2000) and in black raspberry (*Rubus occidentalis*) in USA (Davis *et al*., 2001). Digestion patterns of a 16S rDNA fragment from the wild blackberry (WB8) growing in natural environment resulted identical to the one of the reference strain of '*Ca*. P. asteris' (16SrI-B). Although this phytoplasma infects *Rubus* spp. plants rarely it was also identified in wild raspberry and blackberry growing in Austrian forests (Borroto Fernández *et al*., 2007), as well as in blackberry found in Pakistan (Fahmeed *et al*., 2009), and in the UK (Reeder *et al*., 2010).

The analysis of nucleotide sequence of 16S rDNA fragment of 11 selected strains confirmed the results of PCR-RFLP analysis. A phylogenetic tree derived by analysis of 16S rRNA gene sequences delineated three distinct clusters among analyzed phytoplasmas. It was shown that nine of them (from cultivars Veten, Polka, Loch Tay, Tummel, Tayberry, Darrow, Canby, WB3, and WB6) showed a 99.7-99.9% sequence similarity to each other and formed a monophyletic cluster with the reference strain RuS (GenBank accession no. Y16395) from *Rubus fruticosus* from Italy (Figure 2).

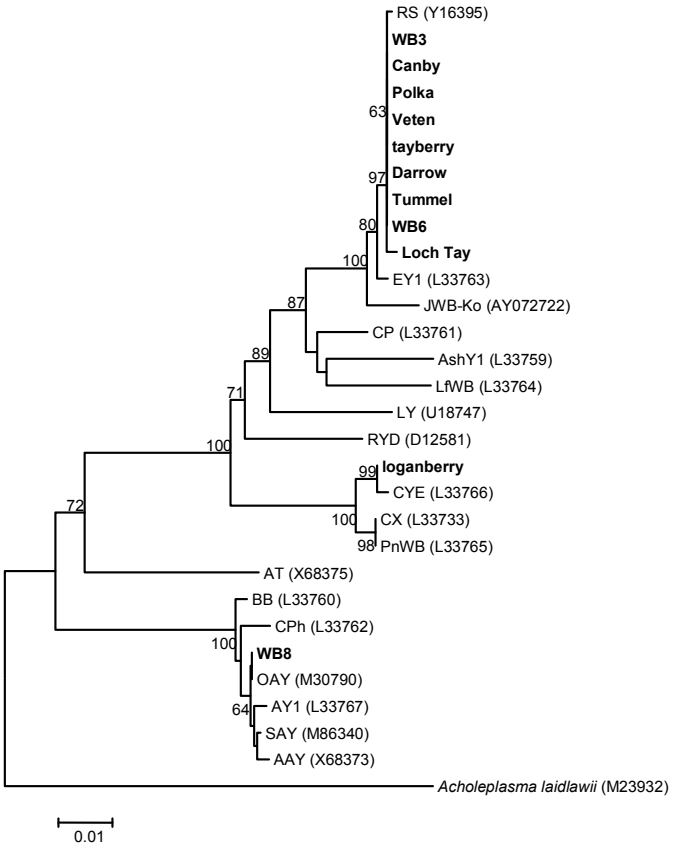

Figure 2. Phylogenetic tree constructed using neighbor-joining algorithm of 16S rDNA sequences of the phytoplasmas infecting *Rubus* spp. plants and representative phytoplasma strains in the aster yellows, X disease, and elm yellows groups. *Acholeplasma laidlawii* (M23932) is included as an outgroup. Sequences were aligned with ClustalW (DNASTAR Lasergene software, Madison, WI, USA). Bootstrap values are shown on branches.

Based on the phylogenetic analysis results the strain from loganberry plant shared 99.8% sequence similarity with CYE reference strain from Canada assigned to X diseases phytoplasma group (GenBank accession no. L33766). X disease phytoplasma was previously identified in loganberry in the UK (Davies, 2000) and in black raspberry (*Rubus occidentalis*) with witches' broom symptoms in Oregon (Davis *et al*., 2001). Multiple alignments revealed that the 16S rDNA nucleotide sequence of WB8 strain from wild blackberry shared 99.9% similarity with the sequences of OAY reference strain of *'Ca.* P. asteris' (GenBank accession no. M30790) belonging to aster yellows phytoplasma group. The partial sequences of the 16S rDNA region of the five strains (from cultivars Canby, Polka, Veten, loganberry, and WB8) of phytoplasmas detected in *Rubus* spp. plants were deposited in the GenBank database under accession numbers: GU125723-27.

Results of the PCR-RFLP analyses on the entire *secY* gene and fragment of *rpl15* gene confirmed that ten *Rubus* spp. plants were infected by '*Ca*. P. rubi'. Although the *secY* gene is one of the most variable among the markers used for differentiation of phytoplasma strains especially from the same 16S rRNA group (Daire *et al*., 2001; Martini *et al*., 2007; Lee *et al*., 2010), the restriction profiles of Polish '*Ca*. P. rubi' strains were indistinguishable from each other and from the RuS reference strain (Figure 3).

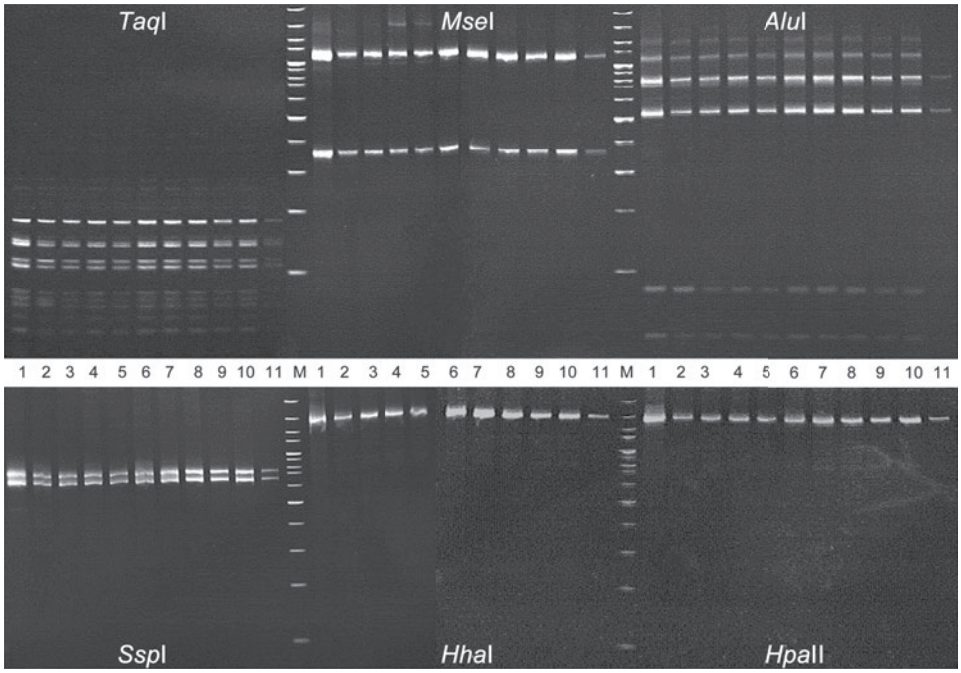

Figure 3. Polyacrylamide gels RFLP profiles obtained on FD9f3/FD9r2 amplicon from '*Ca.* P. rubi'- infected samples of *Rubus* spp. plants after digestion with *Taq*I, *Mse*I, *Alu*I, *Ssp*I, *Hh*aI, and *Hpa*II restriction enzymes. M - GeneRuler 100 bp Plus DNA Ladder (Fermentas, Lithuania). Samples: 1. Veten, 2. Polka, 3. Loch Tay, 4. Tummel, 5. tayberry, 6. Darrow, 7. Canby, 8. WB3, 9. WB6, 10. WB10, 11. RuS reference strain as in Figure 2.

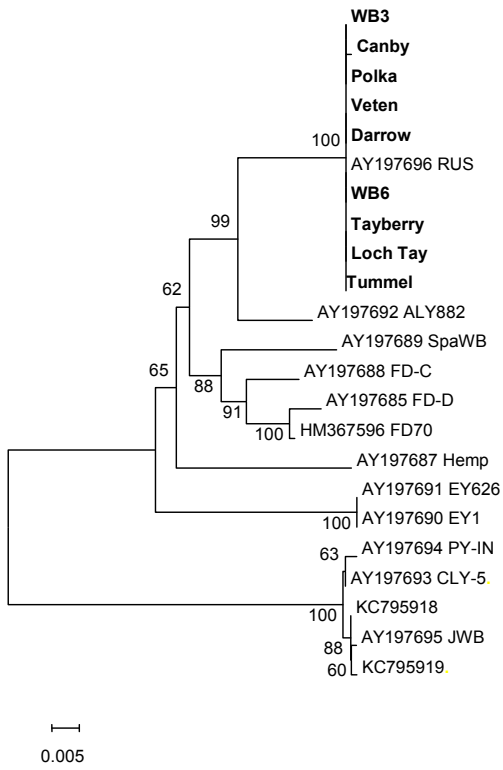

Figure 4. Phylogenetic tree constructed using neighbor-joining algorithm of the entire *secY* gene and a portion of *rpl15* gene sequences of the Polish '*Ca.* P. rubi' strains and representative phytoplasma strains in the elm yellows group (16SrV). Sequences were aligned with ClustalW (DNASTAR Lasergene software, Madison, WI, USA). Bootstrap values are shown on branches.

Phylogenetic analysis confirmed the PCR/RFLP results and indicated that these strains grouped together with the RuS reference strain (Figure 4). It was previously reported that *secY* gene was a reliable marker to differentiate some of the "flavescence dorée" strains from Italy, Serbia, and France (Martini *et al*., 2002; Paltrinieri *et al*., 2011). Although Polish strains of '*Ca*. P. rubi' originated from several localities in central Poland, they seemed to form a genetically homogenous group.

Four of the phytoplasmas found in Poland were detected in *Rubus fruticosus* growing in forest. Although the phytoplasmas are transmitted mainly with infected plant material, *Rubus* spp. plants growing in natural environments can become a reservoir of these pathogens. After detecting aster yellows and "stolbur" phytoplasmas in wild raspberries and blackberries, Boroto Fernández *et al*. (2007) suggested that infected plants growing in a natural environment may play an important role in their spreading.

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Identification of phytoplasmas associated with *Rubus* spp. as prerequisite for their successful elimination

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Abstract

A high number of plants showing symptoms attributed to phytoplasma infection was observed in *Rubus* spp. in the field. In order to obtain a reference sequence from *Rubus* plants a 1,852 bp fragment amplified from the 16S rRNA-23S rRNA region was cloned and sequenced, and the phytoplasma was determined as belonging to group 16SrV. Nested PCR assays using the universal primers allowed the detection of phytoplasmas in 35/133 (26.3%) samples, corresponding to 28/39 plants (71.8%). Nested PCR using group V specific primers showed that in 21/34 samples a phytoplasma belonging to the group 16SrV was present, and this was further confirmed by RFLP analyses. The presence of a phytoplasma belonging to 16SrIII was also confirmed in one *Rubus* cultivar. Explants of phytoplasma-positive plants were established *in vitro* for sanitation by *in vitro* thermotherapy and meristem preparation.

Key words: raspberry, rubus stunt, cloning and sequencing, PCR/RFLP, X disease.

Introduction

Phytoplasmas belongs to the class Mollicutes and are obligate intracellular plant-pathogenic bacteria, restricted to the phloem (Weisburg *et al*., 1989). They infect many economically important plants worldwide, including small fruit species like *Rubus ideaus* (red raspberry) and *Rubus fruticosus* (blackberry) in the Rosaceae family (Lee *et al*., 1995; Mäurer and Seemüller, 1995; Marcone *et al*., 1997; Davies, 2000; Vindimian *et al.*, 2004; Borroto-Fernández *et al*., 2007; Valiunas *et al*., 2007; Cieslinska, 2011). Phytoplasma infections induce symptoms on host plants which are often non-specific to a particular group of phytoplasma (Tolu *et al.*, 2006). Typical phytoplasma symptoms in infected plants include: stunting, shoot proliferation, small leaves, short internodes, enlarged sepals, phyllody, flower proliferations and fruit malformations (Valiunas *et al*., 2007; Bertaccini, 2007; Bertaccini and Duduk, 2009; Cieslinska, 2011). Phytoplasma infected fruits are of small sizes and have poor taste (Heinrich *et al.*, 2001; Laimer, 2009) which interferes with quality traits desired by the consumer. So far, symptoms referable to the above in *Rubus* spp. have been associated with phytoplasmas belonging to the groups of elm yellows (16SrV), X disease (16SrIII), aster yellows (16SrI) and "stolbur" (16SrXII) (Lee *et al*., 1995; Bertaccini *et al*., 1995; Mäurer and Seemüller, 1995; Marcone *et al*., 1997; Davies, 2000; Jarausch *et al*., 2001; Ermacora *et al*., 2003;

Sertkaya *et al*., 2004; Vindimian *et al*., 2004; Borroto Fernández *et al*., 2007; Valiunas *et al*., 2007; Oberhänsli *et al.*, 2011; Cieslinska, 2011).
It was proposed that *Rubus* stunt phytoplasma (16SrV) represent a novel distinct candidate taxon: '*Candidatus* Phytoplasma rubi' within the 16SrV group due to its distinct biological niche and genomic differentiation (Malembic-Maher *et al*., 2011).
Phytoplasmas and are transmitted from plant to plant by grafting and other vegetative propagation techniques and by phloem feeding insects, particularly leafhoppers, plant hoppers, and psyllids (Bertaccini and Duduk, 2009). It is therefore of importance to avoid spreading of phytoplasma diseases in field crops. Furthermore, for plant breeders it is important to avoid infected plant material for grafting, and to have healthy parental material for breeding work. To achieve this, an early detection of infected plants and an efficient sanitation procedure are prerequisites (Oberhänsli *et al*., 2011). Polymerase chain reaction (PCR) based methodologies are mainly used for phytoplasma detection (Gundersen and Lee, 1996). Primers design on phytoplasma specific DNA-probes or 16S rRNA gene sequences and restriction fragment length polymorphism (RFLP) of the same gene are used for identification and classification of phytoplasma groups (Lee *et al*., 1998).
A high number of field growing *Rubus* spp. plants showing symptoms attributed to phytoplasma infection were observed in Italy and Germany. For this reason PCR, RFLP and molecular cloning were employed to detect and identify phytoplasma infecting this plant species. *In vitro* methods established for the elimination of virus and phytoplasma from fruit trees were adapted to produce healthy *Rubus* spp. planting material (Laimer, 2009).

Material and methods

Plant samples and DNA extraction

From a high number of field growing *Rubus* spp. plants showing symptoms referable to phytoplasma infection a representative sample of 39 plants (32 *Rubus idaeus* and 7 *Rubus fruticosus*), originating from Italy and Germany, were used. Total genomic DNA was extracted from 133 samples of leaf veins, bark scrapings, flowers, flower buds, fruits, sepals, stipules, roots and petioles with DNeasy Plant Mini Kit (QIAGEN, Germany) according to the supplier's instructions.

Partial cloning and sequencing of a reference strain

For the cloning and sequencing of a reference strain, the universal primer pair P1/P7 was used to amplify a 1,852 bp fragment covering the entire 16S rRNA gene, the intergenic spacer region and the 5'end of the 23S rRNA gene. Amplification was performed in 25 µl final reaction volume containing 2.5 µl of 10x *Ex Taq* buffer, 2 µl of dNTPs mixture (2.5 mM each), 0.125 µl *TakaRa Ex Taq*™ HotStart, 1 µl of each primer (10 pmol), and 2 µl of total genomic DNA. PCR was performed for 35 cycles under the following conditions: 30s denaturation at 94°C (10s at 98°C for the first cycle), 30s annealing at 55°C and 1 min extension at 72°C. A final step of 10 min at 72°C ended the cycle.

A subsequent nested PCR assay was performed using different primer pairs (P1/RubR, PA2F/PA2R, and RubF/P7) (Table 1) to obtain a set of overlapping PCR products covering the entire region. The PCR mix and cycles were as before, except for the annealing temperature of 60°C for primers P1/RubR and RubF/P7. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

The P1/RubR, PA2F/PA2R, and RubF/P7 amplicons from *Rubus ideaus* were purified using the QIAquick gel extraction kit (QIAGEN). Products were ligated into pGEM®-T Easy Vector System (Promega) and transformed to competent cells of *E. coli* JM109 following the supplier's instructions. Transformants were selected on LB agar containing ampicillin (100 μg ml^{-1}), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (80 mg ml^{-1}) and isopropyl β-D-1-thiogalactopyranoside (0.5 mM). The transformed bacterial colonies were isolated using the alkaline-lysis method, digested with NotI-HF (New England BioLabs), visualized on agarose gels to confirm the presence of an inserted fragment and sequenced. The obtained sequences were analyzed using the DNASTAR Lasergene software and compared with phytoplasma sequences from GenBank using BLASTn (http://ncbi.nlm.nih.gov/BLAST/).

Table 1. List of primers used in this study.

Primer	Primer sequence 5´ to 3´	Source
R16mF2	CATGCAAGTCGAACGGA	Gundersen and Lee, 1996
R16mR1	CTTAACCCCAATCATCGAC	Gundersen and Lee, 1996
R16F2nM	GAAACGGTTGCTAAGACTGG	this study
R16R2	TGACGGGCGGTGTGTACAAACCCCG	Gundersen and Lee, 1996
R16VF1	TTAAAAGACCTTCTTCGG	Lee *et al*., 1994
R16VR1	TTCAATCCGTACTGAGACTACC	Lee *et al*., 1994
P1	AAGAGTTTGATCCTGGCTCAGGATT	Schneider *et al*., 1995
P7	AAGAGCCGATGAAGGACG	Schneider *et al*., 1995
RubF	GTGGTGCATGGTTGTCGTCAG	this study
RubR	CTAACATCTCACGACACGAACTGA	this study
PA2F	GCCCCGGCTAACTATGTGC	Heinrich *et al*., 2001
PA2R	TTGGTGGGCCTAAATGGACTC	Heinrich *et al*., 2001

PCR analyses with general and group specific primers

Nested PCR was performed using different primer sets (Table 1) to allow general or group specific phytoplasma detection. Nested PCR assays were carried out by using the universal primers R16mF2/R16mR1 amplifying approximately 1,500 bp, followed by the universal primers R16F2nM/R16R2 or by 16SrV specific primers R16(V)F1/

R16(V)R1 amplifying 1,250 bp and 1,100 bp respectively. The first PCR was carried out according to Gundersen and Lee (1996), except for an annealing temperature of 55°C. The amplicons were diluted in 1: 40 and used as a template in nested PCR. PCR mix and cycle conditions were similar to those used in the first PCR round except for an annealing temperature of 64°C and 56°C for R16F2nM/R16R2 and R16(V)F1/R16(V)R1, respectively.

Restriction Fragment Length Polymorphism (RFLP) analysis of PCR products

PCR products from primer R16F2nM/R16R2 were excised from the gel, eluted using QIAquick gel extraction kit (QIAGEN) and subjected to digestion with the restriction enzyme *Mse*I (New England Biolabs) following the manufacturer's instructions. Digested products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and resulting patterns compared with profiles of the restriction patterns of reference strains (Lee *et al*., 1998), sequence was compared in the interactive online phytoplasma classification tool iPhyClassifier (Zhao *et al*., 2009).

In vitro culture for sanitation

About 20 - 30 buds from actively growing plants were used for the shoot establishment. Every bud was treated like an individual clone. Following the surface disinfection procedure, the plantlets were propagated on MS (Murashige and Skoog, 1962) based medium supplemented with growth regulators and kept under at 24°C under 16/8 (light/dark) hrs photoperiod. Separated cultures of each clones were produced for the pathogen detection.

Results

Cloning and sequencing of nested PCR products allowed the assembly of a 1,852 bp fragment extending from the 5′end of the 16S rRNA gene to the 5′end of the 23S rRNA. Blastn analyses confirmed the identity of the phytoplasma, with 99% sequence similarity, to *Rubus* stunt isolate (accession number: Y16395) and virtual RFLP analyses allow to show identity with reference phytoplasmas in 16SrV group (Figure 1).

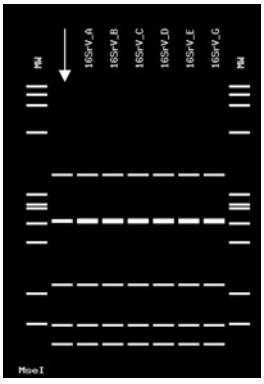

Figure 1. *Mse*I RFLP virtual patterns of the 16SrV group phytoplasmas. The phytoplasma sequence cloned from the cultivar Tulameen 4 was used as input sequence (arrow) for the iPhyClassifier online tool. ΦX174DNA-*Hae*III digested is used as molecular marker (MW).

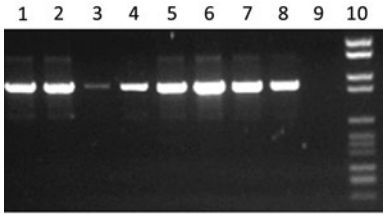

Figure 2. Results of nested PCR assays using 16SrV group specific primers yielding a fragment of 1,100 bp. 1: Glen Ample, 2: Tulameen 4, 3: Amira, 4: Tulameen W, 5-6: Pokusa, 7-8: Tulameen R, 9: VR01, 10: Molecular Marker VI (Roche).

Positive samples produced the expected 1,250 bp fragment of phytoplasma 16S rDNA after nested PCR using general primers R16F2nM/R16R2. From a total of 39 different plants, 28 (71.8%) were positive. In general, from the 133 samples collected from different plant parts, 35 (26.3%) were positive of which 14 out of 56 (25%) were leaf veins, 10 out of 49 (20.4%) bark scrapings, 4 out of 9 (44.4%) sepals, 4 out 7 flowers (57.1%), and 1 out of 3 (33%) fruits. In addition, 1 out of 2 samples (50%) analysed from the roots and petioles were positive while no products were amplified from flower buds and stipules. Furthermore, nested PCR with primers R16(V)F1/R16(V)R1 specific for group 16SrV phytoplasmas gave positive results in 21 out of 34 (61.8%) samples (Figure 2). The presence of phytoplasmas belonging to 16SrIII (X disease phytoplasma) was confirmed in one *Rubus* cultivar (data not shown).

In vitro culture for sanitation

Explants of positive plants were established as *in vitro* cultures and maintained in the Wien collection of fruit pathogens (Table 2). Sanitation experiments by *in vitro* thermotherapy and meristem preparation are currently underway.

Discussion

Phytoplasmas are present in low titres and are often unevenly distributed between different host plant tissues (Lee *et al*., 1995), which makes their detection rather difficult (Nejat *et al*., 2009).

The most reliable diagnostic methods encompass the collection of samples from different parts of an individual plant to be tested. This was quite relevant in the current study as detection showed that not all samples collected from an infected plant yielded positive results. Furthermore, utilization of sensitive techniques such as PCR and nested PCR are important in phytoplasma identification, with the RFLP analysis of PCR products providing additional evidence of differentiation into phytoplasma ribosomal group (Bertaccini, 2007). In the present study, cloning and sequencing of a partial sequence resulted affiliation of the detected phytoplasma to group 16SrV-E. Nested PCR assay using the group 16SrV specific primer pair on *Rubus* plants showed that 61.8% of samples were associated with a phytoplasma belonging to this group. The

presence of phytoplasmas belonging to 16SrIII (X disease) was confirmed in one *Rubus* cultivar, in agreement with previous findings that showed the presence of group 16SrIII and 16SrV in *Rubus* spp. (Davies, 2000; Borroto-Fernández *et al*., 2007; Cieslinska, 2011; Malembic-Maher *et al*., 2011).

In conclusion, the presence of phytoplasmas infecting *Rubus* spp. is a serious threat to the cultivation of this economically important fruit species. The presence of these phytoplasmas is connected to their transmission with infected plant material during vegetative propagation. This therefore calls for a rapid elimination strategy to be utilized in the production of propagation and breeding plant material.

Table 2. *In vitro* collection of *Rubus* species maintained in Wien collection of fruit pathogens.

Species	Cultivar or clone	Mericlone
Rubus idaeus	Rosalia Nr. 9a	R.i.9a
	Amira red	AMR 1.1 – AMR 1.9
	Amira white	AMW 1.1 – AMW 1.3
	Cowichan	CO 1.1 – CO 1.17
	Erika	ER 1.2 – ER 1.3
	Glen Ample	GLA 1.1 – GLA 1.25
	Heritage	HE 1.1 – HE 1.11
	Himbotop	HI 1.1 – HI 1.23
	Kweli	KW 1.1 – KW 1.10
	Meeker	ME 1.1 – ME 1.10
	Polka	PO 1.1 – PO 1.19
	Schönemann	SC 1
	Sugana	SU 1.1 –SU 1.16
	Tadmor	TAR 1.1 – TAR 1.12
	Tulameen Dieffenbach	TD 3.1 – TD 3.17
	Tulameen van Dessel	TS 2.1 – TS 2.4
	Tulameen Nr. 15	TV 1.1 – TV 1.25
	Tulameen Canada	TC 1.1 – TC 1.8
	Verona	VR 1.1 – VR 1.4
	Willamette	WI 1.1 – WI 1.5
Rubus fruticosus	Chester	CHS 1.1 – CHS 1.26
	Rosalia Nr. 8	R.i. 8
Rubus hochstetterorum	Azores	RAZ 21

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Fruit tree phytoplasmas and their insect vectors in pome fruit growing in Belgium: research efforts

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Abstract

Apple proliferation (AP) and pear decline (PD), two diseases associated with '*Candidatus* Phytoplasma mali' and respectively '*Ca.* P. pyri', have been reported in Belgium. Sporadically apple trees with clear symptoms of witches' broom and pear trees with symptoms of pear decline were reported in the past. Recently a limited survey was carried out and samples were analysed using molecular tools. Root samples of apple trees with and without symptoms were collected and analysed by real time PCR which confirmed the presence of AP in both symptomatic and asymptomatic trees. PCR analysis of leaf samples of pear trees showing early discoloration revealed PD presence. As *Cacopsylla* spp. are able to transmit '*Ca.* P. mali' from infected to healthy trees within and between orchards, the dynamic of the psyllid population was studied in apple orchards and hawthorn hedges from March to August. Several insect species were identified: *C. mali*, *C. melanoneura, C. picta, C. peregrina,* and *C. affinis.* Molecular analysis of individuals indicated the natural infection of *C. mali* and *C. peregrina* with AP, however preliminary transmission trials on an artificial substrate could not prove the vector ability of *C. mali*.

Key words: apple proliferation, pear decline, *Cacopsylla*, vector, control.

Introduction

Phytoplasmas, polymorphic bacteria lacking a cell-wall, infect the phloem of many plants (Lee *et al*., 2000) and, probably due to the lack of important genes, have a parasitic way of life in order to survive and multiply. With their size of about 500 nm in diameter and their genome ranging from 530 to 1,350 kilobases they belong to the smallest microbes among the class of Mollicutes (Marcone *et al*., 1999). In European top fruits two phytoplasmas are known: '*Candidatus* Phytoplasma mali' (apple proliferation, AP, in apple) and '*Ca.* P. pyri' (pear decline, PD, in pear), both member of the 16SrX apple proliferation group (Bertaccini, 2007). They are mentioned on the EPPO A1/A2 list and are present in 25 (AP) and 23 (PD) European countries (EPPO, 2013).

Although symptoms vary depending on variety, age, vigour of the tree, and climatic conditions, specific symptoms of apple proliferation include witches' broom, enlarged stipules, shortened internodes, elongated peduncles and undersized fruit (Kunze, 1989; Lee *et al*., 2000; Seemüller *et al*., 2002). Less specific symptoms are phyllody, leaf yellowing and reddening, leaf curl, premature autumn discoloration and increased sensitivity to powdery mildew (*Podosphaera leucotricha*). The infection of '*Ca*. P. pyri' is not easy to be recognized, as symptoms of early leaf discoloration can also be caused by rootstock/scion incompatibility, variety, or physiological disorder.

Transmission of AP phytoplasmas is established by propagation of infected budwood and/or rootstocks (Kartte and Seëmuller, 1991), by root contact between trees (Ciccotti *et al*., 2007) and by insects (Weintraub *et al*., 2010). Both fruit tree phytoplasmas are transmitted by psyllids belonging to the genus *Cacopsylla* (Hemiptera, Sternorrhyncha, Psylloidea) in a propagative, persistent manner (Marzachì *et al*., 2004). Within this genus two species *C. melanoneura* (Foerster) (Frisinghelli *et al*., 2000; Tedeschi and Alma, 2004; Pedrazzoli *et al*., 2007; Mattedi *et al*., 2008) and *C. picta* (Foerster) (Jarausch *et al*., 2003; 2004; Carraro *et al*., 2008) were thus far found to be able to transmit '*Ca*. P. mali'. Both psyllid species were also found in orchards in the southern part of Belgium (Fassotte *et al*., 2010).

Transmission of pear decline has been described for *C. pyricola* (Foerster) in England (Davies *et al*.,1992) while in other parts of Europe *C. pyri* (L.) was identified as the main vector (Garcia-Chapa *et al*., 2005). The pear sucker is the most common pest in pear orchards in Belgium (Bangels *et al*., 2008) and morfological determination of specimens collected in orchards in the Flemisch part of Belgium indicated that *C. pyri* was the dominant species in 4 provinces; only in one province (near the North Sea) *C. pyricola* was in the majority (Vandijck, 1987). According to monitoring data of the past decades collected by pcfruit vzw, the mean date for the start of activity is February 4th, soon followed by egg laying. At about the 1st of April, the first larval stages emerge in the field. Larvae develop through 5 instars into a winged imago and over time become less mobile and secrete more honeydew. This cycle is repeated several times per year, resulting in 3 to 5 succeeding generations. The development stages of the first two generations can be distinguished easily, while from third generation on, in summer, they largely overlap. For the efficient control of pear suckers we developed and tested optimal IPM strategies based on well thought-out treatments of insecticides and alternative products (e.g. kaolin, potassium bicarbonate and mineral oils) (Belien *et al*., 2013).

In 1981, apple proliferation was reported for the first time in Belgium (Maroquin *et al*., 1981). Until recently symptomatic trees had been observed sporadically and found infected, but they were subsequently destroyed. In September 2009, two apple trees close to each other were sampled by the Federal Agency for the Safety of the Food Chain (FASFC) in a nursery in the south-west of Belgium and tested positive by PCR (Olivier *et al*., 2010). Infected trees, as well as adjacent trees, have been uprooted and burned

to prevent disease spread. Symptoms of pear decline had been observed in the past in some orchards, but the presence of PD was never confirmed by molecular tools. To gain information on the status of both diseases in Belgium a research project funded by the FASFC was started. Besides this diagnostic survey the psyllid population in a few apple orchards and hawthorn hedges was also investigated as well as the natural infectivity and vector ability of the psyllids.

Material and methods

Detection of '*Ca*. P. mali' in roots of apple trees

In 11 apple orchards, 3 private and 8 commercial, distributed over 5 fruit growing area in Belgium root samples were collected by digging up small pieces at a distance of 20 cm from the trunk of the tree and taken at a depth of 20-40 cm. In production farms samples of symptomless trees were taken from every 15th tree, as well as from other trees showing any suspicious symptom(s) of apple proliferation.

Roots were cleaned with water and 200 mg of phloem tissue was collected by scalpel scraping, then transferred into a plastic bag and stored on ice. Two ml cold extraction buffer (137 mM NaCl; 8 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 2.7 mM KCl; 80 mM Na_2S0_3; 3 mM NaN_3; 0.05% Tween 20; pH 7.2) was added followed by homogenization with a Homex (Bioreba, Reinach, Switzerland). The suspension was centrifugated (13,000 x rpm, 20 min, 4°C) and the supernatans - also referred to as 'crude extract'- were stored at -20°C until further use (Aldaghi *et al*., 2009). The crude extracts of asymptomatic samples belonging to the same orchard were analysed in groups: each group consisted of 50 µl of 10 samples in a total volume of 500 µl. This mix was diluted 10 or 100 times with sterile distilled water while extracts of symptomatic samples were also diluted but analysed individually.

Two different real time PCR programs were used to detect an infection with AP. Reactions were performed in a total volume of 25 µl containing either 1x qPCR MasterMix plus (Eurogentec), 400 nM of each primer (q-AP16S-R: CCAGTCTTAGCAGTCGTTTCCA, q-AP16S-F: CGAACGGGTGAGTAAC ACGTAA; Baric and Dalla-Via, 2004), 200 nM TaqMan MGB probe (FAM CTGCCTCTTAGACGAGG NFQ; Aldaghi, 2007), 5 µl crude extract, water or 1x SYBR Green qPCR Mix 2x (Eurogentec), 200 nM of each primer (P1: AAGAGTTTGATCCTGGCTCAGGATT, R16F1r: CATCTCTCAGCATAC TTGCGGGTC; Torres *et al*., 2005), 5 µl crude extract and water. Program of the TaqMan MGB PCR was 2 min 50°C; 10 min 95°C and 45 cycles (15 sec 95°C, 1 min 60°C) (Aldaghi *et al*., 2009) and of the SYBR Green PCR 10 min 95°C; 45 cycles (15 sec 95°C, 30 sec 60°C, 30 sec 72°C) followed by a melting curve at 15 sec 95°C, 1 min 60 °C, 15 sec 95°C (Torres *at al*., 2005). Both programs were performed on a StepOne™ Real-Time PCR System (Applied Biosystems) or a 7500 Real Time PCR System (Applied Biosystems).

Identification and population dynamics of *Cacopsylla* spp. in apple and hawthorn

In 2009 the population dynamics of *Cacopsylla* spp. was studied in 4 apple orchards (from March till October) and in 5 hawthorn hedges (from March till June) using the beating tray method and yellow sticky traps on a 2-weekly basis. Psyllids sampled by the first method were aspirated into a collection tube and transferred to the lab where they were identified (species, sex, life stage) and counted. Determination of the species was carried out according to Burckhardt *et al*., 2008; Hodkinson and White, 1979 and Ossiannilson, 1992. The psyllids on the yellow sticky plates were counted in the field using a magnifying glass (15x).

Natural infection with '*Ca*. P. mali' of psyllids in apple trees and hawthorn hedges

From June till September psyllids *C. mali* were collected in an apple orchard showing symptoms resembling those associated with '*Ca.* P. mali'. The surrounding hawthorn hedge was also sampled in the same period for *C. peregrina*. Samples were taken on a weekly basis and consisted of >100 individuals. After a night at 10°C total DNA of 10 insects was extracted using the QIAamp DNA Mini Kit and examined by real time PCR for AP presence (Baric and Dalla-Via, 2004; Aldaghi, 2007; Aldaghi *et al*., 2009) (see Detection of '*Ca*. P. mali' in roots of apple trees).

Transmission of '*Ca*. P. mali' by *Cacopsylla mali*

In July, August and September adult psyllids of *C. mali* were collected in the same orchard mentioned above and kept at 10°C for a night. Each insect was transferred to an individual rearing unit - a microcentrifuge tube with 200 µl nutritional medium (5% sucrose, 0.5% sorbitol, 9.4 mg/l NCTC 135) applied in the lid and sealed with Parafilm M – and kept under controlled conditions (16°C, 14/10h L/D). At different time intervals (1 till 120 hours after transfer) 10 insects were removed and examined under stereo microscope for mortality and sex. Both media and psyllids were used for DNA extraction (Zhang *et al*., 1998; Jarausch, 2010) singly and examined by real time PCR for AP presence (Baric and Dalla-Via, 2004; Aldaghi, 2007; Aldaghi *et al*., 2009) (see Detection of '*Ca*. P. mali' in roots of apple trees).

Results

Detection of '*Ca*. P. mali' in roots of apple trees

Table 1 shows the results of PCR analyses and indicates that in all sampled orchards, located in different areas, the AP presence was detected, regardless cultivation method (production or private) or presence of symptoms. A large number (70%) of the individually tested symptomatic trees proved to be positive. The asymptomatic trees were tested in groups of 10 at first and results revealed the presence of AP within several groups (21/36). Additional analysis of individual samples taken at 5 locations indicated

that the infection rate of these 'suspicious' orchards varied between 5% (see results 5/100 positives) and 50% (see results 25/50 positives).

Table 1. Results of real-time PCR performed on root samples collected in apple orchards in Belgium.

Location (area)	Type of orchard	Sample size	Real-time PCR	Results (positive/tested)	
				Group	Individual
Roots of symptomatic trees, tested individually					
A (Limburg)	production	6 x 1	TaqMan MGB		4/6
S (Limburg)	production	5 x 1	TaqMan MGB		5/5
Z (Limburg)	private	6 x 1	TaqMan MGB		5/6
Al (East Fl.)	private	11 x 1	TaqMan MGB		5/11
AB (Liège)	production	10 x 1	TaqMan MGB		10/10
F (Namur)	private	10 x 1	Sybr Green		5/10
GD (Namur)	private	9 x 1	TaqMan MGB		6/9
Roots of asymptomatic trees, tested in groups of 10 and individually					
A (Limburg)	production	10 x 10	TaqMan MGB	3/10	5/100
S (Limburg)	production	4 x 10	TaqMan MGB	1/4	n.t.
M (Liège)	production	5 x 10	TaqMan MGB	3/5	6/50
AB (Liège)	production	5 x 10	TaqMan MGB	5/5	n.t.
R (Hainaut)	production	5 x 10	TaqMan MGB	2/5	7/50
SD (Namur)	production	5 x 10	TaqMan MGB	5/5	25/50
H (Namur)	production	2 x 10	TaqMan MGB	2/2	6/20

n.t. = not tested

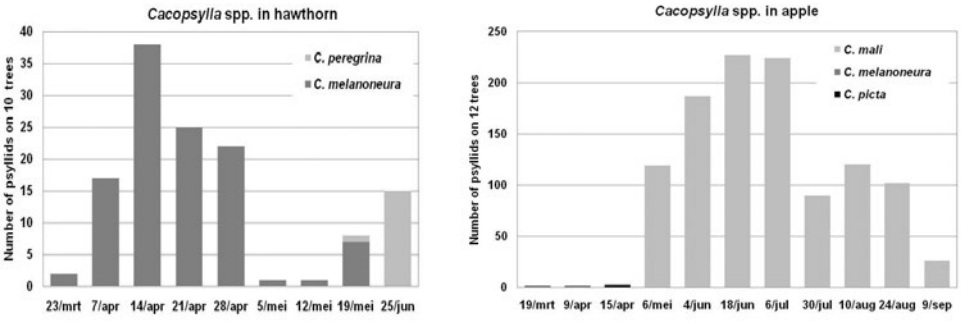

Figure 1. Adult *Cacopsylla* species and their population dynamics in an apple orchard (left) and in a hawthorn hedge (right).

Identification and population dynamics of *Cacopsylla* spp. in apple and hawthorn

Five different *Cacopsylla* species were identified: *C. mali, C. melanoneura, C. picta, C. peregrina* and *C. affinis*. In apple trees (Figure 1) *C. mali* was the most abundant species: before bloom the overwintering eggs (on shoots) hatch into young nymphs (March) and migrate into the flower buds (April). After blossoming (early May) the first adults appear and remain in the orchard until September-October when they start laying eggs whereupon they die. Adult psyllids of the overwintering generation of *C. meloneura* returned into the orchard during February-March in a very small number. By the time *C. melanoneura* had left the apple trees (April) few adults of *C. picta* occurred and only for a few weeks. In hawthorn hedges (Figure 1) the number of returning *C. melanoneura* was much higher and adults were dominantly present in the months March and April. After egg laying adults of the next spring generation appeared during May and emigrated back to their shelters by end of June. At the same time *C. peregrina* psyllids were noticed (May) and their number increased gradually from June onwards. Specimen of *C. affinis* were found sporadically in March (apple) and April (hawthorn). During summer (July) adults of *C. mali* may disperse from apple trees to adjacent hawthorn hedges.

Natural infection with '*Ca.* P. mali' of psyllids in apple trees and hawthorn hedges

Adults of *C. mali* and *C. peregrina* proved to be naturally infected with '*Ca*. P. mali' as indicated by the PCR-results (Table 2). Detection of phytoplasma in the insect resulted positive mainly for samples collected during the month September for *C. mali* and July for *C. peregrina*.

Table 2. Detection of '*Ca.* P. mali' in psyllids (collected in the field) by real time PCR.

Species	Date	Host plant	PCR	Species	Date	Host plant	PCR
C. mali	26/06	apple	-	*C.* peregrina	23/06	hawthorn	-
C. mali	2/07	apple	-	*C.* peregrina	30/06	hawthorn	-
C. mali	16/07	apple	-	*C.* peregrina	2/07	hawthorn	-
C. mali	28/07	apple	+	*C.* peregrina	13/07	hawthorn	+
C. mali	4/08	apple	-	*C.* peregrina	27/07	hawthorn	+
C. mali	27/08	apple	-	*C.* peregrina	27/07	hawthorn	-
C. mali	11/09	apple	+	*C.* peregrina	3/08	hawthorn	-
C. mali	18/09	apple	+	*C.* peregrina	7/09	hawthorn	-
C. mali	25/09	apple	+				

Transmission of '*Ca.* P. mali' by *C.* mali

Molecular analysis revealed that although psyllids were infected with AP (caught in September), no transmission of phytoplasmas to NCTC could be established regardless the inoculation access period spent on the substrate (Table 3).

Table 3. Detection of '*Ca.* P. mali' in an artificial substrate (NCTC) by real time PCR after transmission by *C. mali.*

Psyllid species	Collection date of psyllids in orchard	Inoculation access period (in hours)	Number of insects	PCR results
C. mali	8/07	6-24-96-120	40	-
C. mali	14/07	1-2-3-4-5-6-24-32-72	90	-
C. mali	10/08	1-2-3-4-5-6-24-30-48-60	100	-
C. mali	10/09	1-2-3-4-5-6-72	70	-

Conclusion

Molecular analysis of root samples in apple trees and of leaf samples of pear trees collected during a limited survey in production orchards and in private gardens located at different fruit growing areas confirms the presence of '*Ca*. P. mali' and '*Ca*. P. pyri' in Belgium. In apple nearly 70% of the tested trees with symptoms were found to be infected with AP, whereas in asymptomatic trees the infection rate varied between 5 and 50%. In pear only symptomatic trees were tested, and 15% of them proved to be infected with PD (Olivier *et al*., 2012). As only suspicious orchards were sampled results are biased and care must be taken not to extrapolate to other orchards or even nurseries. As a consequence of these results, an extensive and systematic survey was carried out by the Federal Agency for the Safety of the Food Chain during 2011-2012 in 87 apple tree nurseries. Infected trees were destroyed and eradication measures were imposed in the nurseries concerned. The NPPO of Belgium is currently focussing its official inspections on the production of propagation material and fruit trees. A new research project has also been launched and funded by this Agency focussing on the presence of pear decline and its vector(s).

In apple orchards 3 psyllid species were found: dominantly *Cacopsylla mali*, fewer *C. melanoneura* and rarely *C. picta*. Early in the season (February-March) overwintering adults of *C. melanoneura* (few) and *C. picta* (lesser) entered the orchard, but after a short period left to their host plants. On the apple trees overwintering eggs of *C. mali*, hatched before blossoming and during flowering nymphs, migrated into the flowers. In May all immatures turned into adults and remained in the orchard until oviposition in September-October when they died.

On hawthorn hedges *C. melanoneura* was also present from March to July and *C. peregrina* from May onwards, *C. affinis* occurred sporadically in April (also in apple in March). During summer (July) adults of *C. mali* may disperse from apple trees to adjacent hawthorn hedges. Molecular analysis of *C. mali* and *C. peregrina* revealed a natural infection of both species with '*Ca*. P. mali' when collected in July or later. However transmission of AP by *C. mali* to an artificial substrate could not be established.

Efficient control of psyllids in apple and pear orchards relies on a perfect tuning of treatment schedules, taking into account efficacies of (at preferably) low-impact insecticides and side-(repellent)-effects of alternative products (e.g. kaolin, potassium

bicarbonate and mineral oils), the optimal positioning of these crop protection agents, and the best possible presence of beneficial predators (Belien *et al*., 2013). From a phytoplasma management viewpoint the economic threshold of especially *C. pyri* should be lowered if there are any indications of pear decline in the close environment. Indeed, a control treatment that eliminates low numbered, but phytoplasma infected psyllids will provide an economic return, possibly not on short term by lowering honeydew-linked damage, but certainly on long term by preventing new infections of healthy pear trees.

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Chapter 4

Molecular tools for phytoplasma detection and identification in COST FA0807


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IN SCIENCE AND TECHNOLOGY

Molecular tools in COST FA0807 Action

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Abstract

Following the application of molecular technologies the phytoplasma phylogeny was resolved and led to the new trivial name of phytoplasma and to the designation of a new taxon named '*Candidatus* phytoplasma'. Because of the highly conserved nature of the 16S rDNA, many biologically or ecologically distinct phytoplasma strains, which warrant designation of a new taxon may fail to meet the requirements necessary for their differentiations. Some additional tools for phylogenetic analyses and finer strain differentiation such as *rp*, *secY*, *tuf*, *groEL* genes, and the 16S-23S rRNA spacer region sequences were used as supplementary tools or developed to provide useful and reliable taxonomic information in combination with 16S rDNA.

Key words: molecular differentiation, markers, *groEL* gene, phytoplasma population, RFLP analyses.

Introduction

The phytoplasma classification scheme established using 16S ribosomal groups is based on 16S rDNA properties. This tool for the phytoplasma differentiation has become the most comprehensive and widely accepted classification system allowing phytoplasma strain differentiation into groups and subgroups (Lee *et al*., 1998). However the highly conserved double copy 16S rDNA that has been used for more than a decade as the primary marker for classification of phytoplasmas is affected by the not uncommon presence of 16S rDNA interoperon sequence heterogeneity (Table 1), and it is not able to differentiate many closely related strains, ecologically or epidemiologically different (Duduk and Bertaccini, 2011). During the last decade, the number of phytoplasma strains reported worldwide has increased exponentially. Without adequate information of newly reported phytoplasma strains, it becomes difficult to update the classification scheme in a timely manner. As an alternative approach has been developed an automated computer-simulated virtual gel RFLP analysis system, iPhyClassifier, made on the basis of sequence data available from GenBank for classification of an unknown phytoplasma. This allowed to expand and update the classification, which now comprises more than thirty 16Sr groups and 100 subgroups (Lee *et al*., 2010). On the other hand, the '*Candidatus* Phytoplasma' species description refers to 16S rRNA gene sequence with

a threshold <97.5% similarity to that of any previously described '*Ca*. Phytoplasma' species (IRPCM, 2004). However, because of the highly conserved nature of the 16S rDNA, many biologically or ecologically distinct phytoplasma strains, which may warrant designation of a new taxon may fail to meet this requirement. Additional unique biological properties such as antibody specificity, host range and vector transmission specificity, as well as other molecular criteria need to be included for speciation.

An accurate molecular distinction is therefore necessary for phytoplasma strain characterisation and epidemiological studies, so additional markers were developed and/or employed. At least the subgroup designation is necessary, in some cases for quarantine pathogens such as those associated with 'flavescence dorée' assignment is necessary, although not supported yet by official taxonomy. Multilocus sequence determination, using other genes such as ribosomal proteins, *sec*Y, *sec*A, *tuf*, *gro*EL, nitroreductase and rhodonase-like is now employed as supplementary tool providing useful and reliable information in combination with 16S rDNA for phytoplasma strains differentiation (Mitrović *et al*., 2011; Fránová *et al*., 2013).

As for most microorganisms, identification of phytoplasmas in field collected materials is carried out on populations rather than on individual clones. Examination of selected grapevine plants (that showed mixed phytoplasma presence in nested PCR assays) by deep amplicon sequencing reveals not only mixed phytoplasma infection of diverse 16Sr groups/'*Ca*. P. species', but also a high number of single nucleotide polymorphism. This demonstrates that phytoplasmas in individual plants are composed of populations and deep amplicon sequencing is an useful tool to study this aspect in epidemiological perspectives (Nicolaisen *et al*., 2011).

Table 1. Classification of phytoplasmas based on RFLP analyses and/or sequencing of 16S rDNA.

16Sr group and subgroups	Strain (acronym)	*Candidatus* sp.	Genbank number
	16SrI: Aster yellows		
I-A	Aster yellows witches' broom (AYWB)		NC_007716
I-B	Aster yellows (MAY)	'*Ca.* P. asteris'	M30790
I-C	Clover phyllody (CPh)		AF222065
I-D	Paulownia witches' broom (PaWB)		AY265206
I-E	Blueberry stunt (BBS3)		AY265213
I-F	Aster yellows apricot - Spain (A-AY)		AY265211
I-I	Strawberry witches' broom (STRAWB1)		U96614
I-K	Strawberry witches' broom (STRAWB2)		U96616
I-L	Aster yellows (AV2192)		AY180957
I-M	Aster yellows (AVUT)		AY265209
I-N	Aster yellows (IoWB)		AY265205
I-O	Soybean purple stem (SPS)		AF268405
I-P	Aster yellows from *Populus* (PopAY)		AF503568

16Sr group and subgroups	Strain (acronym)	*Candidatus* sp.	Genbank number
I-Q	Cherry little leaf (ChLL)		AY034089
I-R	Strawberry phylloid fruit (StrawbPhF)		AY102275
I-S	Mexican potato purple top phytoplasma (COAH10)		FJ914654
I-U	Mexican potato purple top phytoplasma (JAL6)		FJ914650
I-V	Mexican potato purple top phytoplasma (SON18)		FJ914642
I-W	Peach rosette-like disease (PRU0382)		HQ450211
I-Y	"Brote grande" of tomato	'*Ca.* P. lycopersici'	EF199549
	16SrII: Peanut witches' broom		
II-A	Peanut witches' broom (PnWB)		L33765
II-B	Lime witches' broom (WBDL)	'*Ca*. P. aurantifolia'	U15442
II-C	Faba bean phyllody (FBP)		X83432
II-D	Papaya mosaic (PpM)	'*Ca*. P. australasia'	Y10096
II-E	Pichris echioides phyllody (PEY)		Y16393
II-F	Cotton phyllody (CoP)		EF186827
	16SrIII: X-disease		
III-A	Peach X-disease (PX11CT1)	'*Ca*. P. pruni'	JQ044392/ JQ044393
III-B	Clover yellow edge (CYE)		AF173558
III-C	Pecan bunch (PB)		GU004371
III-D	Goldenrod yellows (GR1)		GU004372
III-E	Spiraea stunt (SP1)		AF190228
III-F	Milkweed yellows (MW1)		AF510724
III-G	Walnut witches' broom (WWB)		AF190226/ AF190227
III-H	Poinsettia branch-inducing (PoiBI)		AF190223
III-I	Virginia grapevine yellows (VGYIII)		AF060875
III-J	Chayote witches' broom (ChWBIII)		AF147706
III-K	Strawberry leafy fruit (SLF)		AF274876
III-L	Cassava frog skin disease (CFSD)		EU346761
III-M	Potato purple top (MT117)		FJ226074
III-N	Potato purple top (AKpot6)		GU004365
III-P	Dandelion virescence (DanV)		AF370119/ AF370120
III-Q	Black raspberry witches' broom (BRWB7)		AF302841
III-T	Sweet and sour cherry (ChD)		FJ231728
III-U	Cirsium white leaf (CWL)		AF373105/ AF373106
III-V	Passion fruit phytoplasma (PassWB-Br4)		GU292082
	16SrIV: Coconut lethal yellows		
IV-A	Coconut lethal yellowing (LYJ-C8)		AF498307
IV-B	Yucatan coconut lethal decline (LDY)		U18753
IV-C	Tanzanian coconut lethal decline (LDT)		X80117

16Sr group and subgroups	Strain (acronym)	*Candidatus* sp.	Genbank number
16SrV: Elm yellows			
V-A	Elm yellows (EY)	'*Ca*. P. ulmi'	AY197655
V-B	Jujube witches' broom (JWB-G1)	'*Ca*. P. ziziphi'	AB052876
V-C	"Flavescence dorée" (FD-C)		X76560
V-D	"Flavescence dorée" (FD-D)		AJ548787
V-E	Rubus stunt (RuS)	'*Ca*. P. rubi'	AY197648
V-F	Balanite witches' broom (BltWB)	'*Ca*. P. balanitae'	AB689678
16SrVI: Clover proliferation			
VI-A	Clover proliferation (CP)	'*Ca*. P. trifolii'	AY390261
VI-B	Strawberry multiplier disease (MC)		AF190224
VI-C	Illinois elm yellows (EY-IL1)		AF409069/ AF409070
VI-D	Periwinkle little leaf (PLL-Bd)		AF228053
VI-E	*Centarurea solstitialis* virescence (CSVI)		AY270156
VI-F	Catharanthus phyllody phytoplasma (CPS)		EF186819
VI-H	Portulaca little leaf phytoplasma (PLL-Ind)		EF651786
VI-I	Passionfruit (WB-Br4)	'*Ca*. P. sudamericanum'	GU292081
16SrVII: Ash yellows			
VII-A	Ash yellows (AshY)	'*Ca*. P. fraxini'	AF092209
VII-B	Erigeron witches' broom (ErWB)		AY034608
VII-C	Argentinian alfalfa witches' broom (ArAWB)		AY147038
16SrVIII: Loofah witches' broom			
VIII-A	Loofah witches' broom (LufWB)		AF086621
16SrIX: Pigeon pea witches' broom			
IX-A	Pigeon pea witches' broom (PPWB)		AF248957
IX-B	Almond witches' broom (AlWB)	'*Ca*. P. phoenicium'	AF515636
IX-C	Naxos periwinkle virescence (NAXOS)		HQ589191
IX-D	Almond witches' broom (AlWB)		AF515637
IX-E	*Juniperus* witches' broom		GQ925918
IX-F	Almond and stone fruit witches' broom (N27-2)		HQ407532
IX-G	Almond and stone fruit witches' broom (A1-1)		HQ407514
16SrX: Apple proliferation (Europe, America)			
X-A	Apple proliferation (AP)	'*Ca*. P. mali'	AJ542541
X-B	European stone fruit yellows (ESFY)	'*Ca*. P. prunorum'	AJ542544
X-C	Pear decline (PD)	'*Ca*. P. pyri'	AJ542543
X-D	Spartium witches' broom (SpaWB)	'*Ca*. P. spartii'	X92869
X-E	Black alder witches' broom [BAWB(BWB)]		X76431
16SrXI: Rice yellow dwarf			
XI-A	Rice yellow dwarf (RYD)	'*Ca*. P. oryzae'	AB052873
XI-B	Sugarcane white leaf (SCWL)		X76432
XI-C	Leafhopper-borne (BVK)		X76429

16Sr group and subgroups	Strain (acronym)	*Candidatus* sp.	Genbank number
	16SrXII: Stolbur		
XII-A	Stolbur (STOL11)	'*Ca*. P. solani'	AF248959
XII-B	Australian grapevine yellows (AUSGY)	'*Ca*. P. australiense'	L76865
XII-C	Strawberry lethal yellows (StrawLY)		AJ243045
XII-D	Japanese hydrangea phyllody	'*Ca*. P. japonicum'	AB010425
XII-E	Yellows diseased strawberry (StrawY)	'*Ca*. P. fragariae'	DQ086423
XII-F	"Bois noir" (BN-Op30)		EU836630
XII-G	"Bois noir" (BN-Fc3)		EU836647
XII-H	Bindweed yellows (BY-S57/11)	'*Ca*. P. convolvuli'	JN833705
	16SrXIII: Mexican periwinkle virescence		
XIII-A	Mexican periwinkle virescence (MPV)		AF248960
XIII-B	Strawberry green petal (SGP)		U96616
	16SrXIV: Bermudagrass white leaf		
XIV-A	Bermudagrass white leaf (BGWL)	'*Ca*. P. cynodontis'	AJ550984
XIV-B	Bermudagrass white leaf Iran		EF444485
	16SrXV: Hibiscus witches' broom		
XV-A	Hibiscus witches' broom (HibWB)	'*Ca*. P. brasiliense'	AF147708
XV-B	Guazuma witches' broom (GWB)		HQ258882
	16SrXVI: Sugarcane yellow leaf syndrome		
XVI-A	Sugarcane yellow leaf syndrome	'*Ca*. P. graminis'	AY725228
	16SrXVII: Papaya bunchy top		
XVII-A	Papaya bunchy top '*Ca*. P. caricae'		AY725234
	16SrXVIII: American potato purple top wilt		
XVIII-A	American potato purple top wilt	'*Ca*. P. americanum'	DQ174122
	16SrXIX: Chestnut witches' broom		
XIX-A	Chestnut witches' broom	'*Ca*. P. castaneae'	AB054986
	16SrXX: Rhamnus witches' broom		
XX-A	Rhamnus witches' broom	'*Ca*. P. rhamni'	AJ583009
	16SrXXI: Pinus phytoplasmas		
XXI-A	Pinus phytoplasma (PinP)	'*Ca*. P. pini'	AJ310849
	16SrXXII: -		
16SXXII-A	Coconut lethal yellowing-Nigerian Awka disease (LDN)		Y14175
	16SrXXIII: -		
16SXXIII-A	Buckland valley grapevine yellows		AY083605
	16SrXXIV: -		
16SXXIV-A	Sorghum bunchy shoot		AF509322
	16SrXXV: -		
16SXXV-A	Weeping tea witches' broom		AF521672
	16SrXXVI: -		
16SXXVI-A	Sugarcane phytoplasma D3T1		AJ539179

16Sr group and subgroups	Strain (acronym)	*Candidatus* sp.	Genbank number
16SrXXVII: -			
16SXXVII-A	Sugarcane phytoplasma D3T2		AY539180
16SrXXVIII: -			
16SXXVIII-A	Derbid phytoplasma		AY744945
16SXXIX: Cassia witches' broom			
16SXXIX-A	Cassia witches' broom (CaWB)	'*Ca*. P. omanense'	EF666051
16SXXX: Salt cedar witches' broom			
16SXXX-A	Salt cedar witches' broom	'*Ca*. P. tamaricis'	FJ432664
16SXXXI: Soybean stunt			
16SXXXI-A	Soybean stunt (SoyST1c1)	'*Ca*. P. costaricanum'	HQ225630
16SXXXII: Malaysian periwinkle virescence and phyllody			
16SXXXII-A	Malaysian p. viresc. (MaPV)	'*Ca*. P. malaysianum'	EU371934
16SXXXII-B	Malayan yellow dwarf phytoplasma (MYD)		EU498727
16SXXXII-C	Malayan oil palm phytoplasma (MOP)		EU498728
16SXXXIII: *Allocasuarina muelleriana* phytoplasma			
XXXIII-A	Allocasuarina phytoplasma	'*Ca.* P. allocasuarinae'	AY135523

- the ribosomal group has no designation since only Genbank sequences were employed for its determination

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DNA barcoding of phytoplasmas: a tool for their fast identification

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Abstract

In the frame of the EU FP7 project QBOL the traditional phytoplasma classification based upon molecular phylogeny of the 16S rRNA was employed to generate a barcode system that was implemented by adding two more genes *tuf* and *secA*. Sequences from a wide range of phytoplasmas representing the most relevant '*Candidatus* Phytoplasma species' were generated and deposited in a dedicated database (Q-Bank). Phylogenetic analysis of all the genes used for barcoding has revealed full congruence and concordance with the one obtained by conventional 16S rDNA, indicating that the system can be used for a cost effective and fast phytoplasma identification.

Key words: phytoplasma, detection, PCR, sequencing, 16SrDNA, *tuf* gene, *secA* gene.

Introduction

Phytoplasmas infect a high number of plant species. Based on the 16S ribosomal gene sequence, over 30 ribosomal groups have been established within the '*Candidatus* Phytoplasma' taxon, some of which are of quarantine status. Currently, phytoplasma molecular identification and taxonomy are mostly based on 16S ribosomal DNA gene comparison, however some other genes, such as translaton elongation factor Tuf, protein translocase subunit secY, ribosomal protein operon rp, 16-23S intergenic sequence and protein translocase subunit Sec A (Botti and Bertaccini, 2003; Hodgetts *et al*., 2008; Lee *et al*., 2006; 2012; Marcone *et al*., 2000) are also in use for discrimination of phytoplasmas belonging to the same 16Sr DNA group. Based on the at least 97.5% sequence identity within 16S ribosomal sequence rule, over 30 '*Candidatus* Phytoplasma' taxons were designed (IRPCM, 2004; Bertaccini and Duduk, 2009).

The basic phytoplasma identification methods is based on RFLP analysis of a 1.2 kbp region of the 16S rDNA, however, this method is not easy to set up and it only considers a part of the available phytoplasma sequence information. As part of QBOL project (Bonants *et al*., 2010), a universal DNA barcoding based tool for phytoplasma identification was developed, and the system was validated for relevant quarantine phytoplasmas. The phytoplasma *secA* and *tuf* genes were employed as additional genes in combination with 16SrDNA for phytoplasma strains differentiation (Makarova *et al*., 2012; 2013; Hodgetts *et al*., 2014).

Materials and methods

Sets of primers amplifying a fragment of the *tuf* gene, a fragment of the *secA* gene and a fragment of the 16S rDNA gene, respectively, were designed and their potential as DNA barcodes was verified using both experimentally infected samples in periwinkle and naturally infected samples from original host species (Makarova *et al*., 2012; 2013; Hodgetts *et al*., 2014). The amplicons produced were sequenced, aligned and submitted to Q-Bank, sequences for the most relevant quarantine phytoplasmas were also submitted to Genbank.

Results and discussion

While the full 16S ribosomal gene is commonly used for phytoplasma taxonomy, its use as a barcode is limited by the relatively long size and associated difficulties with sequencing. Nevertheless, it was possible to identify a variable region of approximately 600 bp in the 3' of the 16Sr gene, that resulted to be useful for barcoding. This region was amplified from all phytoplasma strains tested, and construction of a phylogenetic tree indicated its capacity to distinguish phytoplasmas at 16Sr group level.

A conserved region within the *tu* gene, present in all phytoplasmas, was also studied as barcode by using a cocktails of primers (Makarova *et al*., 2012; 2013), and a 420-444 bp long barcode was amplified from all phytoplasma strains tested. Tuf barcode sequence alignments and NJ tree construction showed that the tuf-based tree was highly congruent with 16S rDNA derived trees and therefore useful as well as barcode.

The tuf and 16S ribosomal barcodes could separate main ribosomal groups and most of their subgroups. The phylogenetic tree, which was constructed based on these sequences, followed branching patterns of that constructed with full 16S sequences, suggesting that the tuf barcode enables fine discrimination of phytoplasmas on both group and subgroup levels. Phytoplasma tuf barcodes were deposited in the NCBI GenBank and to the newly developed Q-bank (http://www.q-bank.eu/Phytoplasmas), a freely available online identification tool for plant pests and pathogens under quarantine status in the EU.

Successful amplification and sequencing of more than 150 phytoplasma strains in total, and ability to separate various phytoplasma strains to '*Candidatus* species' level, 16S ribosomal group and subgroup level, suggest that these barcodes are efficient phytoplasma identification tools.

Since it has been recently demonstrated that also the *secA* gene provides a phylogenetic analysis which is congruent with the standard 16S rRNA gene (Hodgetts *et al*., 2014), it is possible to use the *secA* gene as a diagnostic barcode tool. The three barcode regions were up to now amplified from 37 strains and deposited under Genbank (Table 1).

The obtained sequences can thus be used by plant health services and researchers for online phytoplasma identification. As DNA sequencing is now routine in most countries and the sequencing costs have substantially dropped: this allows DNA

barcoding to become a viable identification technique, as indicated by a recent move for DNA barcode based identification of EU-regulated organisms (van de Vossenberg *et al*., 2013).

Table 1. Selection of phytoplasmas available in Q-Bank for which three barcode sequences were produced.

16Sr Group	Phytoplasma (acronym)	Accession number			Original plant host species	Country of origin
		***16S rRNA* gene**	***tuf* gene**	***secA* gene**		
I-A	Chrysanthemum yellows (CHRYM)	AY265214/ HQ589187	JQ824240	KJ462009	*Chrysanthemum frutescens*	Germany
I-A	New Jersey aster yellows (NJ-AY)	HM590622	JQ824265	KJ462010	*Callistephus chinensis*	USA
I-B	Maryland aster yellows (AY-1)	AF322644	JQ824205	KJ462011	*C. chinensis*	USA
I-B	Aster yellows (AY-J 24126)	HM590616	JQ824215	KJ462012	unknown	France
I-B	Atypical aster yellows (AVUT)	AY265209 AY265211	JQ824285	EU168720	*C. chinensis*	Germany
I-C	Clover phyllody (KVE)	AY265217	JQ824248	KJ462014	clover	UK
I-C	Carrot yellows (CA)	HM448473	JQ824226	KJ462015	*Daucus carota*	Italy
I-F	Apricot chlorotic leaf roll (A-AY)	AY265211	JQ824251	EU168724	apricot	Spain
II-B	Witches' broom disease of lime (WBDL)	EF186828 MOU15442	JQ824276	KJ462017	*Citrus aurantifolia*	Oman
II-C	Faba bean phyllody (FBP)	EF193354 HQ589188	JQ824262	EU168725	*Vicia faba*	Sudan
II-C	Primula blue yellow (PrBY)	JQ868438	JQ824229	KJ462018	primula	UK
II-D	Tomato big bud (TBB-KG)	Y08173 JQ868448	JQ824250	KJ462021	tomato	Australia
II-D	Sweet potato little leaf (SPLL)	AJ289193 JQ868446	JQ824270	EU168728	*Ipomoea batata*	Australia
II-E	*Pichris echoides* phyllody (PEP)	Y16393	JQ824244	KJ462023	*Pichris echioides*	Italy
III-A	Peach X disease (CX)	HQ589202	JQ824211	KJ462024	peach	Canada
III-A	X disease (BF)	HQ589203	JQ824233	KJ462025	unknown	USA
III-A	Green valley X disease (GVX)	EU168790	JQ824252	EU168733	unknown	USA

16Sr Group	Phytoplasma (acronym)	Accession number			Original plant host species	Country of origin
		***16S rRNA* gene**	***tuf* gene**	***secA* gene**		
III-B	Plum leptonecrosis (LNI)	JQ868444	JQ824230	KJ462027	plum cv Orzark Premier	Italy
III-D	Goldenrod yellows (GRI)	GU004372	JQ824232	KJ462028	*Cornus racemosa*	USA
III-E	Spirea stunt (SPI)	HQ589206	JQ824281	KJ462029	*Spirea* sp.	USA
III-F	Vaccinium witches' broom VAC)	HQ589201	JQ824260	KJ462030	*Vaccinium myrtillus*	Germany
III-F	Milkweed yellows (MW1)	HQ589200	JQ824206	KJ402031	milkweed	USA
III-H	Poinsettia branching factor (JRI)	AF190223	JQ824267	EU168735	*Euphorbia pulcherrima*	USA
V-A	Elm yellows (EY1)	AY197655	JQ824225	KJ462034	elm	USA
V-A	Elm witches' broom (ULW)	X68376	JQ824259	EU168741	*Ulmus carpinifolia*	France
V-B	Jujube witches' broom (JWB)	AY197661	JQ824203	KJ462036	jujube	China
V-E	*Rubus* stunt (RuS)	AY197648	JQ824210	KJ462043	*Rubus* sp.	Italy
VI-A	Potato witches' broom (PWB)	AY500818	JQ824282	EU168742	potato	USA
VI-A	Clover proliferation (CP1)	AY390261	JQ824231	KJ462045	clover	Canada
VI-A	Brinjal little leaf (BLL)	EF186820	JQ824268	EU168743	*Solanum melongena*	India
VI-C	Catharanthus phyllody (CPS)	EF186819	JQ824293	EU168744	*Catharanthus roseus*	Sudan
X-A	Apple proliferation (AT)	CU469464	JQ824224	KJ462047	apple cv Golden Delicious	Germany
X-A	Apple proliferation (AP15)	DQ661859, AJ542541	JQ824216	EU168747	apple	Italy
X-B	Plum leptonecrosis (LNp)	JQ868450	JQ824235	KJ462048	plum cv Orzark Premier	Italy
XI	Napier grass stunt (NGS-BS)	JQ868440	JQ824249	KJ462053	*Pennisetum purpureum*	unknown
XII-A	From unknown insect (BA)	JQ868436	JQ824228	KJ462058	insect	Italy
XII-B	Australian grapevine yellows (AGY)	NC_010544	JQ824254	KJ462054	*Vitis* sp.	Australia

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Reliable detection of European stone fruit yellows phytoplasma in apricot and peach trees

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Abstract

The problem of European stone fruit yellows (ESFY) disease detection in apricot and peach trees grown in South Moravia, Czech Republic was studied during eight years. ESFY symptoms were evaluated visually. Biological indexing was carried out on the peach indicator GF-305 and different primer pairs were used for molecular detection by PCR. Thirty eight apricot trees were evaluated. Forty seven percent of trees infected with ESFY died during the time of evaluation. False positive reactions in nested PCR assays were obtained in samples from fifteen trees, but also the biological indexing was not reliable because of many negative results from symptomatic trees. Also ESFY detection in peach trees by biological indexing was found to be not very suitable, the presence of pathogen was proved only in 42.2% of symptomatic trees. The reliability of ESFY detection by nested PCR in peach trees was higher than in apricot trees. ESFY was detected in 82.2% of symptomatic peach trees and less false positive results were found. Three false positive results of PCR were proved in two ESFY free peach trees during the period of five years. The presence of specific and typical symptoms, interveinal yellowing, partial rolling and fragility of leaves is still the most reliable criterion for detection of ESFY in apricot and peach trees grown in the Czech Republic and further improvement of PCR procedures is necessary for its early and reliable detection in symptomatic apricot and peach trees.

Key words: phytoplasma, ESFY, stone fruits, detection, symptoms.

Introduction

The presence of European stone fruit yellows (ESFY) phytoplasma, '*Candidatus* Phytoplasma prunorum', (Seemüller and Schneider, 2004) in apricot trees was reported in the Czech Republic since 1990 (Navrátil *et al.*, 1998). The phytoplasma infected apricot trees died within two years after symptom appearance (Navrátil *et al.*, 2001). In 2002 ESFY phytoplasma was detected by PCR in symptomless apricot trees in the South Moravia: these plants were not dying and the results of nested PCR assays were completely different from the biological test (Polák *et al.*, 2007). False positive reactions were proved both in direct and nested PCR in preliminary tests of ESFY detection. Only the typical symptoms of ESFY, yellowing and leaf rolling of leaves, little leaves together with small number of leaves on shoots were used as reliable diagnostic criterion for ESFY disease (Polák *et al.*, 2008). Peach trees were also found to be ESFY infected in the same region as apricot trees.

Ahrens and Seemüller (1992), Heinrich *et al*. (2001), and Skrzeczkowski *et al*. (2001) have reported the possibility of false positive results in the diagnostics of phytoplasmas of fruit trees by PCR assays. Other authors published evidence that sets of primers used for detection of phytoplasmas in fruit trees can give inaccurate results (Barba *et al*., 1998; Brzin *et al*., 2003; Jarausch *et al*., 1999; Lorenz *et al*., 1995a); therefore experiments were established in apricot and peach orchards where typical symptoms of ESFY were observed. Results of eight years study of ESFY detection in apricot orchard and six years study in peach orchard are presented.

Detection of ESFY in apricot trees

A collection of samples from 38, twelve year old trees of four apricot cultivars (Bergeron, Goldrich, Velkopavlovická, and Boccuccia Liscia) that grew in orchard where ESFY symptoms appeared, was evaluated during eight years. The presence of ESFY was evaluated by inspection of symptoms, PCR detection, and indexing on the peach indicator GF-305. Visual evaluation of symptoms was performed at least three times during vegetation period from June to October in the years 2004-2011. All trees were tested by direct and nested PCR assays once per year. Shoots were sampled at the beginning of November and the detection of ESFY by PCR tests was carried out immediately after collection. Specific primers fAT/r PRUS were used in direct PCR assays (Lorenz *et al.,* 1995). Direct PCR was carried out with the primers R16F1/R16R0 (Lee *et al.,* 1995), and nested PCR was performed with two pairs of primers, R16F2/R16R2 and fU5/rU31 (Lee *et al.,* 1995; Lorenz *et al.,* 1995a). A modified protocol from Ahrens and Seemüller (1992) was used for DNA extraction.

Shoots sampled in November were stored at 4°C for biological indexing. Buds taken from sampled shoots of tested tree were grafted in March on the wooden indicator GF-305 in three replicates for each tested tree. Symptoms on indicator plants were evaluated for the first time one months after chip-budding, and then after five months at least once per month.

Collection was carried out in the part of the orchard with high presence of ESFY symptoms. Sixteen trees (42.1%) showed no ESFY symptoms during the whole eight year period of evaluation. Four trees died of *Monilia* sp. attack (10.5%). Eighteen trees (47.4%) showed ESFY symptoms and died during the eight years of evaluation. Six trees with ESFY symptoms in the first year of evaluation died by the end of the first vegetative period. Another twelve trees died during the next seven years. Apricot trees showing ESFY symptoms died in the year when ESFY symptoms appeared, or in the next one or two years. There were not significant differences among the four apricot cultivars as concerns ESFY infection.

The biological indexing on GF-305 was not reliable and is inapplicable because of many negative results of tested trees with ESFY symptoms. No positive results were obtained in five years and just only one or two positive results (GF-305 indicator plants died)

in 2008, 2009, and 2011. Results of ESFY detection by nested PCR were a little more reliable than results of direct PCR assays, therefore only results of nested PCR were included in Table 1.

Table 1. Results of evaluation of ESFY symptoms and nested PCR assays in apricot trees.

N. of apricot tree	ESFY symptoms								Nested PCR results							
		0	5	1	1	1	1	3								
	2004	2005	2006	2007	2008	2009	2010	2011	2004	2005	2006	2007	2008	2009	2010	2011
1	-	-	-	-	-	dm	nt	nt	-	-	-	-	+	nt	nt	nt
2	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+
3	-	-	-	-	-	dm	nt	nt	-	-	-	-	+	nt	nt	nt
4	+	+	d	nt	nt	nt	nt	nt	-	+	nt	nt	nt	nt	nt	nt
5	-	-	+	+	+	-	-	-	-	-	+	+	+	-	-	-
6	-	-	+	+	-	-	+	d	-	+	-	+	-	-	+	nt
7	-	-	+	d	nt	nt	nt	nt	-	-	nt	nt	nt	nt	nt	nt
8	-	-	-	-	-	-	+	d	-	-	+	+	-	+	+	nt
9	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+
10	+	+	d	nt	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	nt
11	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-
12	-	-	-	-	-	-	-	-	+	-	+	+	-	±	+	+
13	-	-	-	-	-	-	-	-	-	-	-	+	+	±	+	+
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
15	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
16	-	-	-	+dy	d	nt	nt	nt	-	+	-	+	nt	nt	nt	nt
17	-	-	-	-	-	-	d	nt	-	-	-	-	-	-	+	-
18	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	+	-	-	+	+	nt	-	+
21	-	-	-	-	-	dm	nt	nt	-	-	-	-	+	nt	nt	nt
22	-	-	d	nt	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	nt
23	-	-	d	nt	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	nt
24	-	-	-	-	-	d	nt	nt	-	-	-	-	±	nt	nt	nt
25	-	+	d	nt	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	nt
26	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
27	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
28	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
29	-	-	-	-	-	dm	nt	nt	+	-	-	+	+	nt	nt	nt
30	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+
31	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-
32	-	-	-	-	-	-	+	d	-	+	+	+	+	-	+	nt

-, negative result; +, positive result; d, tree died; nt, not tested; dm, tree died from *Monilia* wilt; ±, weak reaction in nested PCR; +dy, ESFY symptoms dying by the end of the vegetative period

A lot of false positive reactions were recorded in nested PCR assays in the sixteen trees with no ESFY symptoms during eight years of evaluation; negative results of nested PCR in healthy, asymptomatic trees were obtained during the eight years of evaluation only in one tree (6.25%). False positive reactions were obtained in 15 asymptomatic trees (93.75%).

Results obtained during the eight years in the commercial orchard of apricot trees with ESFY presence, proved the devastating effect of this phytoplasma disease; 47% of apricot trees showing ESFY symptoms died during the eight years of evaluation. One tree was showing ESFY symptoms for three years, and no symptoms in the other three years. The only reliable detection of ESFY resulted to be the visual evaluation of symptoms, *i.e.* the presence of specific symptoms in leaves and shoots of apricot trees.

Detection of ESFY in peach trees

A collection of 16 peach trees eight years old growing in orchard where ESFY disease was present was evaluated during 2007-2012. Four selected peach trees were healthy and 12 peach trees showed typical ESFY symptoms, interveinal yellowing and partial rolling of leaves, smaller leaves and sparse foliage with different intensity in the first year of the evaluation. The presence of ESFY was detected by indexing on the peach indicator GF-305, by PCR methods, and by evaluation of symptoms. The latter was performed at least three times (in June, August, October) during vegetation period in the years 2007-2012. Two-years old shoots were collected in November (2007-2011), and partly was immediately used for molecular tests, the second part was stored till March for biological indexing.

Three independent diagnostic methods were compared: PCR analyses, biological indexing on peach GF-305, and evaluation of ESFY symptoms. Molecular detection of ESFY was carried out by PCR. DNA was extracted using DNeasy Plant Mini Kit (Qiagen) from phloem scrapings taken from two-year old branches. From every sample, three different PCR reactions were run: primers ECA1/ECA2 under conditions according to Jarausch *et al*. (1998); primers fAT/rPRUS according to Smart *et al*. (1996), and nested PCR assay with primers R16R0/R16F1 followed by R16R2/R16F2 according to Lee *et al*. (1995).

Results of six years evaluation (2007-2012) on peach trees are presented in Table 2. Two peach trees remained without ESFY symptoms from the beginning of year 2007 till May 2012, and biological and PCR tests were negative. Two trees without ESFY symptoms included at the beginning of 2009 showed ESFY symptoms, the first in 2010, the second one in 2012, but ESFY was detected by PCR already in 2011. Two times ESFY symptoms were not present in diseased trees. The reliability of visual inspection and evaluation of ESFY symptoms in peach trees was 97%. Five peach trees with ESFY symptoms died: three of them in the second half of vegetation period in 2008, one during the winter 2008/2009, and the last one died during the winter 2010/2011.

Table 2. Evaluation of ESFY presence in selected peach trees, period 2007–2012.

Tree	2007			2008			2009			2010			2011			2012
No	PCR	BT	S	PCR	BT	S	PCR	BT	S	PCR	BT	S	PCR	BT	S	S
1	-	-	+	+	-	++	++	-	++	+++	-	+++	+++	-	+++	++
2	+	+	+	+	+	++	-	-	++	+++	+++	+++	+++	-	+++	+++
3	-	+	+	+	-	++	+	-	+	+++	-	+++	+++	+	+++	+++
4	-	+	+++	++	-	+++,D	nt	nt	D	nt	nt	D	nt	nt	D	D
5	++	+	+++	++	++	+++,D	nt	nt	D	nt	nt	D	nt	nt	D	D
6	-	++	+++	++	++	+++,D	nt	nt	D	nt	nt	D	nt	nt	D	D
7	±	++	+	+	+	+++	++	-	++	+++	-	+++	nt	nt	D	D
8	-	-	+	-	-	++	-	-	-	++	-	+	-	+	+++	++
9	-	-	+	-	+	++	-	-	-	++	+	+	-	+	+++	++
10	-	-	-	(+)	-	-	-	-	-	-	-	-	(+)	-	-	-
11	-	-	-	-	-	-	-	-	-	(++)	-	-	-	-	-	-
12	+	-	+++	++	+++	+++	++	-	+++	+++	-	+++	+++	+	++	+++
13	+	-	++	++	++	++	nt	nt	D	nt	nt	D	nt	nt	D	D
14	nt	nt	nt	nt	nt	nt	-	-	-	++	-	+++	+	+	+++	++
15	nt	nt	nt	nt	nt	nt	+	-	+	+++	-	+++	+++	+	+++	++
16	nt	nt	nt	nt	nt	nt	-	-	-	-	-	-	(++)	-	-	(++)

PCR, molecular detection by nested PCR; BT, biological detection on GF-305; S, symptoms; D, death of tree; nt, not tested. Intensity of PCR results and symptoms: –, not present; ±, dubious; +, weak; ++, medium severe; +++, severe

The presence of ESFY was proved by PCR tests in trees with symptoms. Results of all the three used PCR procedures were the same, but the bands of nested PCR (Figure 1) were stronger. The reliability of ESFY detection by PCR assay was 82.2%. Three false positive PCR reactions were recorded together with 15 negative and 37 positive results. The incidence of false positive results was 5.8%. Symptoms on the indicator plants appeared for the first time one month after the chip budding and plants were evaluated in the period of April to July. Wilting and dying of indicator plants have appeared within one and two months after the chip budding. The presence of ESFY was proved in nine symptomatic trees from thirteen symptomatic trees tested by indexing on the indicator plants peach GF-305. The reliability of ESFY detection by testing on indicator plants was 69.2%. The evaluation of symptoms and molecular detection based on PCR assays are a reliable criteria for ESFY detection in peach trees.

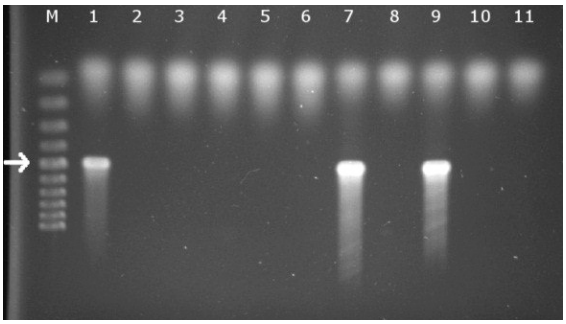

Figure 1. Detection of ESFY in peach trees by PCR with primers fAT – rPRUS. M - marker 100 bp (Fermentas); 1 positive control; 2 negative control; from 3 to 11 individual tested samples. Samples numbers 7 and 9 showed positive reaction, band with length ca 500 bp is marked with an arrow.

Conclusions

Obtained results of both, molecular detection and biological indexing of ESFY in peach trees are more reliable in comparison with reliability of ESFY detection in apricot trees. Peach trees showing ESFY symptoms can survive more than six years, but apricot trees not more than two, exceptionally 3 years. Detection of ESFY in apricot and peach trees by biological indexing is not suitable because of low reliability. The only advantage of biological testing is the absence of false positives. The visual inspection and evaluation of ESFY symptoms in apricot and peach trees is up to this time the most reliable diagnostic method.

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Chapter 5

Management of phytoplasma-associated diseases


cost
EUROPEAN COOPERATION
IN SCIENCE AND TECHNOLOGY

Management of phytoplasma-associated diseases

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Abstract

Working group 3 covered inside the COST action the aspect of phytoplasma control in crop systems. The network was mainly focused on economically important phytoplasma diseases of grapevine and fruit trees. Several aspects were covered such as identification and breeding of crop plant varieties that are resistant (or less susceptible) to the phytoplasmas; examination of the effects of biotic and abiotic environmental factors on disease and symptom development; improvement of vector control with special attention to low-impact insecticides, and treatment schedules as well as development of innovative, environmentally sustainable vector control strategies and recommendations for best practices in disease control verification. The interaction of endophytes with host plant and phytoplasma that could be used to control phytoplasma diseases and verification whether mild phytoplasma strains can effectively protect plants from infection by virulent (or severe) phytoplasma strains were also studied.

Key words: phytoplasmas, environment, plant resistance, endophytes, disease management.

Introduction

As phytoplasmas are phloem-limited plant pathogens which are spread by phloem-feeding insects in nature their control is difficult. It can be carried out theoretically either by controlling the vector or by eliminating the pathogen from the infected plants. The first requires the knowledge of the vector and its biology, and usually is achieved by repeated insecticide treatments. As European policy aims to reduce the impact of insecticides, one major objective was to develop and validate innovative environmentally friendly control strategies of phytoplasma vectors. Elimination of phytoplasmas from infected plants is almost impossible, and may only be achieved by repeated antibiotic (tetracycline) treatments which are usually not allowed in practical application. Therefore, another objective was the identification and use of natural resistance of plants to phytoplasmas. One of the best studied examples of plant resistance to phytoplasmas is apple. Natural resistance to '*Candidatus* Phytoplasma mali' has been found in *Malus sieboldii*, and is currently used to develop phytoplasma-resistant apple rootstocks. Studies aiming to elucidate the mechanism of this resistance indicate that general plant defense mechanisms are involved, and that some responses are similar to those found in susceptible *Malus* which show recovery, a more or less stable remission of symptoms.

As natural genetic resistance is rare – and engineered resistance neither developed nor accepted – a further objective was the identification of alternative control strategies based on biocontrol agents or plant resistance inducers. In this regard, endophytes have gained major interest, especially for the control of phytoplasma infections in perennial woody crops like grapevine and fruit trees. It could be shown that the microbial population in healthy, phytoplasma-infected and recovered grapevines or apple is different. The interaction of endophytes with phytoplasmas might be due to the induction of a systemic resistance. The histological, biochemical and molecular studies carried out to elucidate the phenomenon of recovery might be of great help also to analyse the possible biocontrol action of microorganisms.

Phytoplasmas live and multiply in two different hosts: plants and insects. They are highly dependent on host metabolites and their interaction with the environment is complex. A successful, sustainable and environmentally friendly phytoplasma control will have to be also complex: the plant defense to phytoplasmas needs to be strengthened, and the spread of the phytoplasma by its insect vector needs to be reduced. A smart combination of different approaches will reduce the need for a high efficiency of a single method.

Together with insect vectors, propagation material plays a main role in phytoplasma disease dissemination in woody host plants, especially for long-distance transmission and introduction of diseases in new geographic areas. Use of phytoplasma-free planting material is therefore a prerequisite for any control strategy.

Management of phytoplasma diseases through genetic resistance

Despite the numerous diseases associated with phytoplasmas on cultivated and wild plants worldwide, few resistant species or varieties have been obtained. Some studies performed on woody plants, like fruit or coconut trees are the exception. Indeed, exploitation of genetic resistance for phytoplasma disease management is most advanced in apple.

Identification of natural genetic resistance is often hampered by the lack of an efficient resistance screening system. A defined phytoplasma *inoculum* should be efficiently inoculated to the plant genotype to be tested, and the infected plant should be evaluated with a defined system. In this regard, important differences between fruit trees and grapevine have to be considered: whereas phytoplasmas can easily be maintained in fruit trees and can efficiently be transmitted by graft-inoculation, this is not the case in grapevine. Here, inoculation by the insect vector is most efficient. This makes the resistance screening of a large number of genotypes more difficult, and a distinction between a resistance to the phytoplasma or to the insect vector cannot easily be made. The evaluation of the resistance of the inoculated plant has become much more precise since the measurement of phytoplasma concentration by quantitative real-time PCR has been applied. In this regard it is important to note that resistance to phytoplasmas

in fruit trees and grapevine is defined as absence of symptoms and growth alteration, together with low titer of the pathogen, whereas tolerance is absence or mild symptoms but high titer of the pathogen.

Further research focus on the development of model systems to study the resistance and its molecular basis under controlled conditions in the laboratory. Here, plant tissue culture of fruit trees has been used.

A practical application of genetic phytoplasma resistance in fruit trees and grapevine is also largely dependent on the physiology of the plant, in particular the phloem renewal in scion (=cultivar). The complete replacement of the previous year's phloem by the new year's phloem takes place every year in late winter in pome and stone fruits. This leads to a natural elimination of the phytoplasmas from aerial parts of the trees, which is complete in apple and pear, but less complete in stone fruits. Phloem renewal in the root system is a constant process permitting the survival of the phytoplasmas in the roots of the infected trees. The resistance strategy for the management of fruit tree phytoplasma diseases is therefore based on the development of resistant rootstocks, which prevent a recolonisation of the aerial part of the trees by the phytoplasma in springtime. This strategy has the great advantage that the pomological traits of the cultivar remain unchanged. This resistance strategy can probably not be applied in grapevine as the phloem in the aerial parts of grapevine is not completely replaced every year. In this case, resistant cultivars are needed. However, not only a resistance to the phytoplasma must be considered but also a resistance to the insect vector.

Search for natural genetic resistance to '*Ca.* P. mali' within the taxa *Malus* has been extensively carried out in the eigthies and nineties of the last century. Satisfactory resistance was shown by trees on *M. sieboldii* and *M. sieboldii* hybrids. A classical breeding program has been started in 2001 to integrate the agronomic values of established apple rootstocks into these AP-resistant genotypes. However, this breeding program faced several obstacles which drastically reduced the output of selected genotypes: a high level of apomixis of *M. sieboldii* and its hybrids made it difficult to obtain a sufficient number of recombinant genotypes for further selection. The different degrees of polyploidy of the resistance genitors made it impossible to develop molecular markers for assisted selection, and finally, a high sensitivity of some genotypes to latent apple viruses was observed during the resistance evaluation of the breeding progeny. To date, several selected genotypes are available from the breeding program which are resistant to '*Ca.* P. mali', but tolerant to latent apple viruses and which have promising pomological values. These genotypes can easily be micropropagated and will now be assessed in further agronomic trials (Jarausch *et al.*, 2013).

As pear decline (PD) is a devastating disease not only in Europe but also in North America, screening for natural resistance to '*Ca.* P. pyri' has been first done in America by studying different *Pyrus* taxa and clonal rootstocks under natural infection conditions. In a more recent trial carried out in Germany progenies of 39 open pollinated genotypes belonging to 26 *Pyrus* taxa were graft-inoculated and observed for at least 18 years. Also

this study revealed considerable differences in PD resistance between and within the progenies. Although identification of genetic resistance to '*Ca.* P. pyri' is not as clear as for '*Ca.* P. mali', its potential for a durable management of PD disease is similar. As for AP the use of resistant rootstocks would be sufficient to reduce the impact of PD, because the winter elimination of the phytoplasmas in the aerial parts of *Pyrus* is similar to the conditions observed in *Malus* (Jarausch *et al.*, 2013).

Identification of natural resistance to '*Ca.* P. prunorum' is less advanced. It infects several cultivated stone fruit species and wild *Prunus* whose response to the infection ranges from susceptible (apricot, peach, Japanese plum) to tolerant (European plum) and highly resistant (cherry). Since '*Ca*. P. prunorum' is able to overwinter in the above-ground parts of *Prunus*, resistance of both rootstock and scion cultivars is required. However, interesting levels of resistance have been observed in several hybrids and γ-ray mutants of Reine Claude cultivars (Jarausch *et al.*, 2013).

Intraspecific (*V. vinifera* cultivars) and interspecific (hybrids and rootstocks) variability in plant susceptibility to the grapevine yellows (GY) diseases is well known from field experience and observations. Differences between cultivars in terms of symptom frequency and intensity, but also in the ability to recover were recorded for "bois noir" (BN) and "flavescence dorée" (FD). Rootstocks appeared as a promising source of resistance because only a few rootstock genotypes were found to be naturally infected, and some inoculated rootstocks showed few or even no symptoms of the disease. A study was recently initiated in order to evaluate the susceptibility to the FD disease of major *V. vinifera* cultivars, *Vitis* hybrids used as rootstocks, but also wild *Vitis* species originating from North American and Asian continents under controlled conditions by insect inoculation. Highly (e.g. cultivars Cabernet Sauvignon, Chardonnay, Grenache) and poorly susceptible (e.g. cultivars Merlot, Syrah) genotypes could be identified based on recording the symptoms, the percentage of infected plants and by measuring the mean phytoplasma titer in the whole plants by quantitative real-time PCR (Jarausch *et al.*, 2013).

Several studies carried out in the last few years aimed to find out genes and proteins up and down regulated during GY infection, and thus potentially involved in grapevine resistance to phytoplasmas. Qualitative and quantitative changes in the global gene expression profiles of BN-infected and healthy plants of cultivar Chardonnay were performed using microarray technology. Expression levels of a few hundred genes were altered in infected plants, and some genes related to defence pathways were induced or repressed specifically in only one cultivar. Another study investigated the proteome responses of moderately susceptible and susceptible varieties to FD phytoplasma, and identified some proteins which were exclusively expressed in one cultivar. Thus, all genes and proteins differentially expressed in varieties showing different susceptibility are potentially associated with the differential susceptibility to the phytoplasma, and could be candidate genetic traits for GY resistance (Jarausch *et al.*, 2013).

Management of phytoplasma diseases through induced resistance

Stimulation of plant defenses has become of great interest for researchers because it offers the possibility to contain phytoplasma diseases within an integrated approach, together with the applied control measures based on clean propagating materials, vector control and weed management. This can be particularly true where phytoplasma diseases limit the growing of particular species or cultivars in areas with high disease pressure. One of the few possibilities to contain the occurring of disease symptoms, is the use of resistance inducers. These molecules, called also elicitors, can be of abiotic or biotic nature and challenge the plant, leading to a reaction, often linked to the production of antimicrobial compounds and/or the elicitation of plant defense mechanisms.

Recovery has been reported in several plant species belonging to the *Rosaceae* family (apple and stone fruits) and in grapevine; it has been defined as the spontaneous remission of disease symptoms in plants that previously showed them. The phytoplasmas disappear from the aerial parts, but may persist in the roots. Recovered plants are re-infected in nature in a lesser extent than the never infected ones, indicating that a resistance could be involved in the phenomenon. Studies carried out during the last years have enabled new insights into the physiological basis of this phenomenon. Cytochemical analyses revealed that it is accompanied by biochemical changes in the phloem. Recovered plants are able to accumulate, in the sieve elements, H_2O_2, a stable reactive oxygen species whose antimicrobial as well as signaling roles are well known. The variation of sieve-element oxidative status leads to modifications of phloem protein (P-protein) conformation, and in phloem occlusion expression patterns. An anomalous accumulation of callose and protein, associated with the upregulation of callose synthase- and P-protein-coding genes, has been observed in the sieve elements of recovered apple trees, supporting the hypothesis that recovered plants are able to develop resistance mechanisms depending on Ca^{2+} signal activity. Recently, the activation of jasmonate (JA)-related defense mechanism, via JA gene upregulation, has also been demonstrated in apple trees recovered from apple proliferation disease (Musetti *et al.*, 2013).

Abiotic and biotic resistance inducers were studied for their potential to induce this recovery reaction. Abiotic resistance inducers are usually chemical compounds able to start a reaction of the plant. The most common elicitors used to control phytoplasma diseases are: benzothiadiazole (BTH), phosetyl-aluminium, prohexadione calcium, indole-3-butyrric acid (IBA), indole-3-acetic acid (IAA), chitosan, salicylic acid (SA), mixture of glutathione and oligosaccharines (GOs). Most studies were carried out on experimental hosts infected with a phytoplasma. SA was applied in crops of tomatoes infected with potato purple top phytoplasma. Trials on woody crops infected by phytoplasmas can be more difficult because it is not easy to find a high number of plants that after application allow getting significant differences in recovery induction. However, the treatment with resistance inducers can be successful in decreasing the number of infected plants, reducing the severity of symptoms, and delaying the

appearance of disease. So far, resistance inducers have been successfully applied in grapevine (Romanazzi, 2013).

The role of arbuscular mycorrhizal (AM) fungi in phytoplasma infection has been investigated in several pathogenic systems. In "stolbur" infection of tomato, agglutinations and degeneration of phytoplasma cells, coupled to reduced symptom expression, was seen in plants treated with AM fungi. Inoculation with *Glomus intraradices* increased tolerance to pear decline in infected pear trees. However, the evidence that *G. mosseae* BEG 12 inoculation does not decrease periwinkle tolerance to mild and severe '*Ca*. P. asteris' strains has indicated that the effects of AM fungi on phytoplasma infection are complex and probably dependent on a combination of host plant, AM fungus and phytoplasma strain (Romanazzi, 2013).

Endophytic bacteria promote plant growth and protect them against pathogen infections. Endophytes-plant and endophytes-pathogens interactions are poorly explored. In particular, the ability of endophytes to control pathogens that are not managed directly is still new. Recently, *Pseudomonas putida* S1Pf1Rif was tested, alone or in combination with the mycorrhyzal fungus *Glomus mosseae* BEG12, against chrysanthemum yellows (CY) phytoplasma infection of chrysanthemum.

A basic point for the success of sustainable management of plant diseases based on biocontrol agents is the study of endophytic bacterial community associated with plants. Recently endophytic bacteria associated with healthy and phytoplasma infected plants have been described, suggesting some putative biocontrol agents. Nevertheless, to be suitable for biocontrol a bacterium should not only produce secondary metabolites, but it should compete with indigenous microorganisms, and maintain the interactions with the host. To verify the ability of endophytic bacteria in phytoplasma control, model systems need to be developed (Bianco *et al.*, 2013).

Fungal endophytes establish mutualistic relationships with plants, also inducing physiological modifications in their hosts, making them more resistant against biotic or environmental stresses. Strains have been identified from grapevines and apple plants grown in areas where a recovery phenomenon was recurrent. Strains of *Epicoccum nigrum* and *Aureobasidium pullulans* were chosen for further research activities because they were extensively reported as biocontrol agents or resistance inducers. Using the model plant *C. roseus* infected with '*Ca*. P. mali', it was observed that reduction in symptom severity and lower phytoplasma titre in host tissues occurred when the plants were previously inoculated with an endophytic strain of *E. nigrum* (Bianco *et al.*, 2013).

Management of fruit tree phytoplasma diseases through vector control

As there is no applicable means to cure a phytoplasma infected fruit tree; insecticide treatments were the first measures to control the spread of fruit tree phytoplasma diseases whenever a vector species was identified. Although it is impossible to eliminate all vectors from the environment, well managed vector control strategies significantly

reduce the chance of an epidemic outbreak of phytoplasma diseases. However, important differences exist between the control of univoltine and polyvoltine psyllid vectors. Highly efficient univoltine vectors, e.g. *Cacopsylla picta*, the vector of apple proliferation disease, might be present in the orchards in very low abundance and appropriate and efficient insecticides might miss or might not be homologated in all countries. Therefore, control of univoltine vectors is not well studied as it is the case for *Cacopsylla pruni*, the vector of European stone fruit yellows. On the contrary, polyvoltine pear psyllids, vectors of pear decline, are pests on their own and, therefore, control strategies and efficacies of different insecticides are well studied.

Pear decline is associated with '*Ca.* P. pyri' which is transmitted by pear suckers (psyllids/psylla). In UK *Cacopsylla pyricola* is known as vector species of pear decline, while in other parts of Europe *Cacopsylla pyri* has been described as the main vector. Efficient control of pear suckers relies on a perfect tuning of treatment schedules, taking into account efficacies of (at preferably) low impact insecticides and side (repellent) effects of alternative products (e.g. kaolin, potassium bicarbonate and mineral oils), the optimal positioning of these crop protection agents, and the best possible presence of beneficial predators. From a phytoplasma management viewpoint the economic threshold of *C. pyri* should be lowered if there are indications of pear decline presence in the close environment. Indeed, a control treatment that eliminates low numbered, but phytoplasma infected psyllids will provide an economic return by preventing new infections of healthy pear trees (Belien *et al.*, 2013).

Two univoltine psyllids, *C. picta* and *Cacopsylla melanoneura*, are acknowledged vectors of '*Ca.* P. mali'. As both species hibernate on conifers in larger distances from the orchards, their control is possible only when the insects are present on cultivated plants. The control strategy aims to prevent the reproduction of both species on apple. A particular problem arose for the control of overwintered adults of *C. picta* in years when oviposition coincided with the period of blossom, when insecticides cannot be applied. In this case the strategy can be focused on the control of the development of the new generation. The results of the transmission trials showed that both generations of *C. picta* can transmit the phytoplasma. Consequently, in areas where the disease is present, both the remigrants and the new generation must be controlled. Therefore the precise prediction of the migration phase and the larval development is indispensable for an efficient control of the vectors of '*Ca*. P. mali'.

Only very few attempts have been undertaken to control *Cacopsylla pruni*, the vector of '*Ca.* P. prunorum' by classical means of spraying. *C. pruni* is an univoltine species strictly oligophaguous on *Prunus* species which hibernates on conifers. Also for this psyllid, possible control is restricted to the period when it reproduces on its host plant. However, the disease might be endemic (present on wild plants) and the populations of *C. pruni* might be abundant.

Basic research on several phytoplasma - vector - plant systems are currently carried out with the aim to develop innovative control strategies such as species specific traps for

monitoring, and mass trapping of different vector species of fruit tree phytoplasmas. The strategy is based on the chemically mediated interactions of the different phytoplasmas affecting pome and stone fruits, their host plants, and the vectors *C. picta, C. melanoneura* and *C. pruni*. By analyzing the complex chemically mediated interactions between '*Ca.* P. mali', its two vectors *C. picta* and *C. melanoneura*, and their host plants (reproduction host and overwintering host), it could be shown that this phytoplasma lures the highly adapted vector *C. picta* to infected apple plants by changing its odor. The phytoplasma induces apple trees to produce more ß-caryophyllene which preferentially attracts new generation adults of *C. picta* (emigrants) just before their emigration to the overwintering host. By feeding on infected plants, the probability of an acquisition of the phytoplasma increases. In contrast, the hawthorn psyllid *C. melanoneura* did not react to this sesquiterpene. After overwintering, the psyllids return to apple plants (remigrants), but prefer to lay their eggs on uninfected plants. By doing so they transmit the phytoplasma to previously healthy hosts. Which infochemical(s) may regulate the observed oviposition behavior still remains unknown. Based on these findings traps with attractive components like ß-caryophyllene were constructed, e.g. useful for monitoring programs. By this the adequate date for spraying can be determined and, thus, the amount of chemical insecticides can be reduced. Because in the AP system the infochemical produced by infected plants is attractive to both sexes of psyllids, it could also be possible to develop mass trapping systems for a sustainable control of these insects in the future. Also for *C. melanoneura* and *C. pruni* potentially behavior modifying compounds could be identified, but to date they are not species specific. Interestingly, potential repellent chemicals for emigrants of *C. pruni* were identified. This offers the possibility to combine attractive compounds to be used in traps as lures for monitoring and mass trapping purposes and with repellent compounds to be used in complex push-and-pull strategies (Eben and Gross, 2013).

Management of grapevine phytoplasma diseases through vector control

Two grapevine yellows due to phytoplasma infection are mainly affecting the European vineyards: "flavescence dorée" (FD) is a quarantine disease in Europe as it is epidemically transmitted by the grapevine leafhopper *Scaphoideus titanus*, an insect of North American origin now widely distributed in the vineyards of southern Europe. "Bois noir" disease (BN) is endemic in the Euro-Mediterranean area and is mainly transmitted by *Hyalesthes obsoletus,* a planthopper residing in weeds such as bindweeds *(Convolvulus arvensis)* and stinging nettles *(Urtica dioica)* which also act as plant reservoirs for the BN phytoplasma. Due to the biology of *S. titanus*, that is strictly associated with grape and has only one generation per year, the control of "flavescence dorée" spread mainly relies on the control of this leafhopper and *S. titanus* population level and disease spread are clearly correlated. The control of the vector is mandatory and detailed guidelines have been enacted for the different vine growing regions. An area wide and prompt

monitoring of the vector is the pre-requisite to design a rational control strategy. Monitoring has several different purposes: to detect the presence of the vector in a given area, to establish the developmental stage of the population in order to well time the insecticide application, to detect the population level to eventually reduce the number of insecticide applications, to check the application of compulsory insecticide treatments, and to evaluate the efficacy of the insecticide applications. Among the different control techniques, only insecticides and agronomic methods are generally applied. Biological control with predators and parasitoids, though naturally acting in the field, is not effective enough. Insecticides are generally applied twice a year in the areas characterized by a high incidence/prevalence of the disease. In the areas where the disease is under control only one application is suggested. In the very last years, the presence of an important number of *S. titanus* adults late in the season (late August and September) has been recorded. Often these leafhoppers harbor FD phytoplasmas and, therefore, can be infectious. Due to the risk of these incoming infected leafhoppers, a third insecticide treatment to the vineyard borders surrounded by wild vegetations or by abandoned vineyards is suggested in August. Actually, untreated areas represent refuges for the vector that can re-colonize the cultivated vineyards. As a long term perspective, symbiotic control and vibrational mating disruption strategies are under investigation. Obviously, these latter perspectives are far from the field application and, therefore, the optimization of chemical and agronomical control techniques is imperative to limit the spread of FD (Bosco and Mori, 2013).

"Bois noir" (BN) is associated with '*Ca.* P. solani', a phytoplasma which is endemic in the natural vegetation and transmitted from and to herbaceous plants by the cixiid planthopper *H. obsoletus*. Grapevine is infected through the occasional feeding of this vector. Since *H. obsoletus* does not acquire BN phytoplasmas from grapevine, it is considered a dead end host for the phytoplasma. Consequently, BN incidence does not influence the further disease spread and there is no need to remove infected vines from vineyards. Infection pressure is rather determined by the presence and infection of natural host plants, associated vector and their density and distribution patterns within and around the vineyards. This implies that attempts to reduce infection pressure need to overstep the vineyard borders and must focus on the natural phytoplasma-vector-host-systems.

H. obsoletus has one generation per year in Europe. Eggs are deposited in the soil where the five larval instars feed on the roots of their host plant, of which field bindweed and stinging nettle are the most important species. BN is acquired by the nymphs from the roots of infected hosts. The life cycle is completed with the emergence of adult planthoppers, which live on the aerial parts of a wide range of host plants and occasionally feed on grapevine, too. The presence of *H. obsoletus* is not restricted to vineyards, but depends on the occurrence and distribution of its natural plant hosts within and outside the vineyards. Infection pressure is therefore not only determined by the specific conditions of particular vineyards, but it depends also on the general biotic

and abiotic conditions on a larger scale. Light and permeable soils, sparse vegetation and high isolation resulting in a favorable microclimate are key factors for the presence of *H. obsoletus*. Consequently, strategies to control this planthopper focus on the nymphs on the roots of their host plants. Since bindweed and nettle harbor different strains of BN, typing of BN isolates from infected vines allows the identification of the locally predominant reservoir plants. While a well managed greencover can suppress bindweed in the vineyard interrows, it remains a problematic weed in the undergrowth of the grapevines. Mechanical or chemical weeding of the area is advisable to avoid a high infection pressure emerging close to the grapevine plants. Chemical weeding of nettles aims at depriving the root feeding *H. obsoletus* nymphs of their food source. Herbicide treatments proved to be equally effective when applied either in autumn or in early spring.

Alternative approaches beside host plant control have been evaluated with more or less success. Although they might be not practicable in general, they may provide solutions to specific problems. Insecticides that are translocated to the roots after foliar application on host plants, proved to kill the nymphs and to reduce the numbers of emerging adult *H. obsoletus* with an efficiency that was comparable or slightly lower than the early chemical weeding. The use of insecticides could be considered as an "emergency treatment" shortly before the start of the emergence of adult vectors when herbicide treatments are not efficient anymore (Maixner *et al.*, 2013).

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"Stolbur" phytoplasma strains in Austria and their association with grapevine, bindweed, stinging nettle and *Hyalesthes obsoletus*

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Abstract

Hyalesthes obsoletus is the vector of '*Candidatus* Phytoplasma solani' ("stolbur") from herbaceous hosts to grapevine. The associated disease "bois noir" (BN) is regularly found in Austrian vine growing regions. Previous findings showed only small numbers of *H. obsoletus* in these areas, particularly on bindweed. In contrast, recent survey revealed high population densities of *H. obsoletus* on stinging nettle, both frequently infected with "stolbur". Sequence analysis of the "stolbur" marker genes *secY*, *stamp*, *tuf* and *vmp1* revealed the presence of a single dominating genotype, named CPsM4_At1. This genotype was the only one found in stinging nettle and was predominant in *H. obsoletus* and in grapevine in different areas. Interestingly, although the sequences of the markers secY, stamp and vmp1 of CPsM4_At1 were mostly related to previously described nettle genotypes, CPsM4_At1 showed a tuf-type b pattern in *Hpa*II restriction analysis, which was previously attributed only to bindweed associated "stolbur" strains. Altogether, five nettle and nine bindweed associated genotypes could be described. Transmission experiments with *H. obsoletus* and with *Anaceratagallia ribauti* resulted in the transmission of five strains to *Catharanthus roseus*. During this investigation also '*Ca*. P. convolvuli' was identified in nettle and bindweed.

Key words: "bois noir", '*Candidatus* Phytoplasma solani', stamp, vmp1, secY, bindweed yellows, '*Candidatus* Phytoplasma convolvuli'.

Introduction

"Bois noir" (BN) is a grapevine yellows disease associated with "stolbur" phytoplasma ('*Candidatus* Phytoplasma solani') that is also found on a number of herbaceous plants and on diseased nightshade plants such as tomato and potato (Quaglino *et al*., 2013). Grapevine is believed to be a dead end host for '*Ca.* P. solani', since its vector, *Hyalesthes obsoletus* cannot survive on grapevine alone and feeds only erratically on that plant (Constable, 2010; Maixner, 2011).

The most important sources of "stolbur" phytoplasma for grapevine infection seem to be bindweed and nettle. In Germany, it has been shown that "stolbur" phytoplasmas have a nettle and a bindweed associated host race corresponding to the Tuf elongation

factor restriction patterns tuf-type a and tuf-type b, respectively, potentially with distinct severity of symptoms (Langer and Maixner 2004; Maixner 2011; Johannesen *et al*., 2012). BN is widespread in several vine growing regions in Austria and previous investigations between 2003 and 2008 revealed an absence or low population densities of *H. obsoletus* in most viticultural areas of Austria. The phytoplasma was frequently ascertained in bindweed and grapevines, whereas infections of nettles were very unusual. Previous analysis of tuf-types showed sole presence of tuf-type b (Riedle-Bauer *et al*., 2006; 2008; Tiefenbrunner *et al*., 2008). In addition to *tuf*, the gene *secY*, encoding for a major membrane unit of the secretory pathway, and the membrane protein encoding genes *vmp1* and *stamp* have been used to characterize the genetic diversity of '*Ca*. P. solani' in multilocus sequencing approaches (Cimerman *et al*., 2009; Fabre *et al*., 2011).

Apart from *H. obsoletus,* also the Cixiidae *Reptalus panzeri* can transmit BN to grapevine seedlings (Cvrković *et al*., 2014) as shown for in south eastern European populations. Moreover, transmission experiments with several Cicadellidae (Hemiptera, Auchenorrhyncha) species have shown that the leafhopper *Anaceratagallia ribauti* can transmit '*Ca.* P. solani' to *Vicia faba* (Riedle-Bauer *et al*., 2008), but so far transmission to grapevine has not been proven (M. Riedle-Bauer, unpublished). The aim of the present study was to collect updated data on the epidemiology of BN in Austria, its vector occurrence and to characterize "stolbur" strains occurring in Austria by the molecular marker genes *secY*, *stamp*, *tuf*, and *vmp1*.

Materials and methods

The investigations were carried out in more than 30 vineyards and their surroundings all over Austria in 2011 till 2013. *H. obsoletus* and *A. ribauti* were collected by vacuum sampling directly from *Urtica dioica* and *Convolvulus arvensis* using a modified garden blower-vac (Stihl, Dieburg, Germany). Samples were also taken from diseased grapevines and from the weeds on which the insects had been collected. DNA extraction and PCR/RFLP analyses were carried out as published earlier (Langer and Maixner, 2004; Riedle-Bauer *et al*., 2008). Phytoplasma DNA was amplified directly as previously described with the primer pairs stamp fw-0 and rv-0 (stamp; Fabre *et al*., 2011), fTUFAY and rTUFAY (tuf; Schneider *et al*., 1997), POSecF1 and POSecR1, TYPH10F and TYPH10R (secY and vmp1; Fialová *et al*., 2009), and for 16S with CTAATACATGCAAGTCGAACG (R16mF2m) and TGACGGGCGGTGTGTACAAACC (R16R2m) for 40 cyles, 30 sec 94°C, 45 sec at 58°C and 90 sec at 72°C with 10 min final extension (modified from Lee *at al*., 1998). The PCR products were single strand sequenced with stamp fw-0, TYPH10R, rTUFAY and R16R2m, respectively. Field collected *H. obsoletus* and *A. ribauti* were subjected to transmission trials with *Catharanthus roseus* (cultivar Sorbas Reinweiß, Austrosaat, Vienna, Austria) seedlings using 10 to 50 insects per experiment.

Results and discussion

H. obsoletus presence was ascertained at 23 out of 30 sampling sites. At the majority out of these sites, the planthopper was found solely on stinging nettle (*U. dioica*), only on one site (Klosterneuburg) on bindweed (*C. arvensis*). In nettles population densities were high, often numbers of collected individuals exceeded 10 per square meter of nettle vegetation, reaching mass occurrences in *U. dioica* stands with >1,000 caught individuals, e.g. in Styria. *H. obsoletus* were frequently infected with the "stolbur" phytoplasma and it was ascertained in 25% of the analyzed insect specimens, in 11% of the analyzed nettles, and in less than 3% of the analyzed bindweeds. Interestingly, in three nettle and in several bindweed samples 16S rDNA sequences corresponding '*Ca.* P. convolvuli', a recently described taxon associated with bindweed yellows (Martini *et al*., 2012) was also detected. From these samples also tuf sequences were obtained in samples Ud12_270 and Ca12_686, which showed highest (89%) similarity to '*Ca.* P. solani' sequences.

In contrast to earlier works (Maixner, 2011), the previously described association (Langer and Maixner, 2004) between tuf-type and herbaceous host plant in the epidemic cycle was not confirmed. Positive *H. obsoletus* individuals trapped from stinging nettles were infected with tuf-type b "stolbur" phytoplasmas as ascertained with restriction analysis with *Hpa*II. Only 5% of all "stolbur" positive *H. obsoletus* individuals and not a single *H. obsoletus* analyzed in 2011 and 2012 showed a tuf-type a profile (n, number of "stolbur" positive samples = 99). The tuf-type b was also ascertained in grapevines (n = 47), in stinging nettles (n = 11) and in bindweed (n = 2). Tuf-type a genotypes could not be confirmed in any of these plant samples.

Sequence analysis shows, however, that only the minority of these tuf sequences are "pure" tuf-type b as compared with the reference strains, but the majority (100% of the stinging nettle, 66% of the grapevine, 91% of *H. obsoletus*, but none of the positive bindweeds) has a G instead of an A on position 727 after the start codon. This base is shared with tuf-type a strains and was named tuf-type b2. For a better analysis of epidemiological cycles *sec*Y, stamp and vmp1 sequences were characterized and 14 "stolbur" subtypes were identified in Austria (Table 1).

Despite this diversity, allowing a clear presence of these different genotypes, tuf-type b2 was almost exclusively linked with a single secY, stamp and vmp1 genotype, making this combination by far the most common genotype named CPsM4_At1 in *H. obsoletus* (91%), stinging nettle (100%) and grapevine (64%). Also, as exemplified in three different vine growing regions in Austria (Figure 1), this genotype is also the most common in the different regions, despite variation of the accompanying types. Moreover, the *secY*, *stamp* and *vmp1* genes linked to tuf-type b2 are more related to previously described nettle associated genotypes (Johannesen *et al*., 2012), than to the bindweed types, which suggests that sequence analysis of several marker genes still allows a discrimination between bindweed and nettle types, but that tuf-type b restriction analysis with *Hpa*II is not sufficient for all strains of "stolbur" molecular characterization.

Table 1. Genotypes of '*Ca*. P. solani' and their corresponding marker gene annotaton. Representative strains/samples for each host plant and genotypes deposited in the database are indicated. Strain abbreviations show the host name, the sampling year and a sampling number. Vv: grapevine. Ud: *U. dioica.* Ca: *C. arvensis.* Ho: *H. obsoletus.*, Ar: *A. ribauti*. Cr: *C. roseus.* Tuf-type a and tuf-type b1 sequences correspond to reference strains (tuf-type a: 48061, *H. obsoletus* Kesten; 48078, *H. obsoletus* Hafen. Tuf-type b: 47740, *H. obsoletus* Flache; 47629, *H. obsoletus* Pfalzgraben; CA-1 92, StolPO 130, tuf-type b2 to the sequence found in the major genotypes.

Genotype	tuf	vmp1	stamp	secY	Strain/Sample
CPsM4_At1	tufb2	Vm_At1	St_At1	Se_At1	Vv12_273; Ho12_486; CrHo12_650; Ud13_720
CPsM4_At2	tuf b2	Vm_At2	St_At2	Se_At1	Vv12_274
CPsM4_At3	tuf a	Vm_At3	St_At3	Se_At1	Ho13_1006
CPsM4_At4	tuf a	Vm_At4	St_At4	Se_At2	Ho13_936; CrHo13_1183
CPsM4_At5	tuf a	Vm_At5	St_At5	Se_At3	Ho13_838
CPsM4_At6	tuf b1	Vm_At6	St_At6	Se_At5	CrHo12_601; CrAr12_722_1
CPsM4_At7	tuf b1	Vm_At8	St_At6	Se_At5	Ca13_RF; Vv12_Kn2
CPsM4_At8	tuf b1	Vm_At10	St_At6	Se_At5	Vv12_754
CPsM4_At9	tuf b1	Vm_At11	St_At6	Se_At5	Vv12_753
CPsM4_At10	tuf b1	Vm_At7	St_At7	Se_At5	CrHo12_721
CPsM4_At11	tuf b1	Vm_At9	St_At7	Se_At5	Vv12_Ill6
CPsM4_At12	tuf b1	Vm_At11	St_At7	Se_At5	Vv12_752; CrAr12_722_2
CPsM4_At13	tuf b1	Vm_At10	St_At8	Se_At5	Vv12_751
CPsM4_At14	tuf b1	Vm_At10	St_At9	Se_At4	Vv12_Kn6

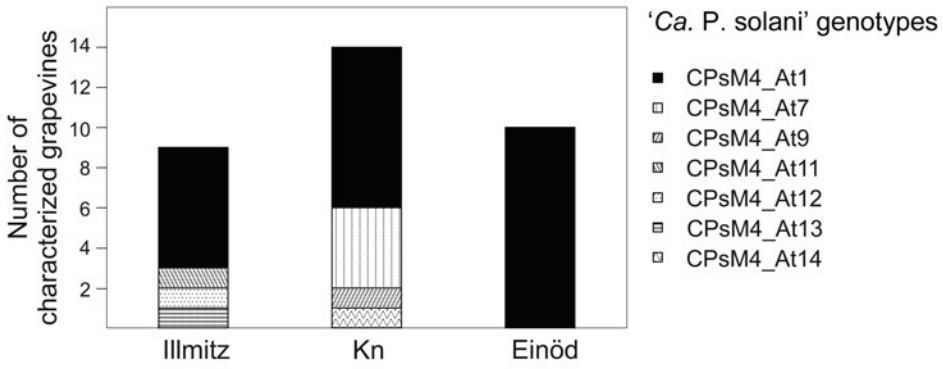

Figure 1. Occurrence of genotypes in different grapevine plants from three regions. Kn: Klosterneuburg, lower Austria. Einöd: Styria. Illmitz: Burgenland. Genotypes were defined on four sequenced marker genes. Genotype definitions are shown in Table 1.

Based on that, five of the 14 genotypes found in this study in Austrian vine growing regions belong to the nettle type (tuf-type a and tuf-type b2) and nine to the bindweed type (tuf-type b). Two of the nettle types including the major genotype CPsM4_At1, and three of the bindweed types could be also transmitted to *C. roseus*. Hereby, *H. obsoletus* was transmitting 4 different "stolbur" types including the major genotype, while *A. ribauti* only transmitted two types.

Based on the finding that *H obsoletus* is prevalent in nettle and the abundant occurrence of the same genotype CPsM4_At1 in stinging nettle, grapevine and *H. obsoletus,* it seems likely that the major Austrian "stolbur" genotype is transmitted in an epidemiological cycle including *H. obsoletus* and nettles. This also indicates a significant change in the epidemiology of BN in Austria within a few years. Whereas till around 2008 *H. obsoletus* and infections of nettle were rare (Riedle-Bauer *et al*., 2006; Tiefenbrunner *et al*., 2007; Riedle-Bauer *et al*., 2008), now high population densities of *H. obsoletus* and frequent infections of nettles are detected. A third of all BN infections of grapevine were associated with bindweed type, and it is not clear, where this type is originated from, or if it derives from previous infection cycles. Although the bindweed type "stolbur" was found in *H. obsoletus* and *A. ribauti* transmissions to *C. roseus*, this type was rarely detected, so further research is required to evaluate if other insects than *H. obsoletus* might play a role in BN infections also in Austria, especially by the bindweed type.

BN is a disease characterized by sudden outbreaks and different epidemiological cycles (Maixner, 2011). The reasons for such changes are not clear so far, but unexpected increases of *H. obsoletus* populations on nettles have also previously been observed in Germany and in South Moravia (Maixner *et al*., 2011, Šafářova *et al*., 2011). Shifts of the occurrence of "stolbur" types with an increase in bindweed type "stolbur" phytoplasmas have also been documented in south Tyrol (Baric and Dalla Via, 2007). It seems that this grapevine yellows is a dynamic disease, and it is possible that future outbreaks in Austria in grapevine may include also those tuf-types a found so far only rarely in *H. obsoletus* alone.

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Occurrence of *Hyalesthes obsoletus* and "stolbur" phytoplasma strains in grapevine and host plants in Spain

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Abstract

"Bois noir" disease (BN) is one of the most important grapevine yellows in Europe and is induced by '*Candidatus* Phytoplasma solani', belonging to the "stolbur" group (16SrXII-A). The disease is widespread in different vine growing regions in northeast of Spain such us La Rioja, Alava, Navarra, Catalonia and Aragón. Several surveys have been done and the BN incidence in affected plots of these regions varied with the geographic area and ranged from 3% to 75%. The main vector of "stolbur" phytoplasma in grapevine, the ciixid *Hyalesthes obsoletus* was identified in all areas, but the population was low in most of the sampled plots.

Key words: "Bois noir", '*Candidatus* phytoplasma solani', *Hyalesthes obsoletus*, *Urtica dioica*.

Introduction

"Bois noir" (BN) is one of the most important grapevine yellows in Europe and it is induced by *'Candidatus* Phytoplasma solani' (Quaglino *et al*., 2013). It is vectored by *Hyalesthes obsoletus* Signoret from vineyard weeds to grapevine, although other insects as *Reptalus panzeri* and *R. quinquecostatus* have also been reported as potential vectors (Palermo *et al*., 2004, Trivellone *et al*., 2005*).* Molecular characterization of strains in plants and insect vectors is an important tool to find out details about disease epidemiology such as the vectors and the host plants involved in the BN dissemination. In Spain, this disease was first observed in 1994 (Laviña *et al,.* 1995), but in recent years its incidence seems to have increased in different grapevine regions (Sabaté *et al*., 2007). According to the results obtained in previous studies, the strain tuf-type b was the prevalent in the regions studied in Spain (Sabaté *et al*., 2014). This "stolbur" strain was identified in all the *H. obsoletus* specimens and in most of the grapevine samples with the exception of the plants from "La Rioja Alta" and some plants from Navarre. The strain tuf-type b is associated with *Convolvulus arvensi*s while the tuf-type a, is associated with *Urtica dioica*. With the aim to determine the cause of the BN incidence increase, a new study was undertaken in several regions in the northeast of Spain.

Materials and methods

Samples of grapevine plants were taken in affected plots of different regions in "La Rioja", Navarre, Aragon and Catalonia. *H. obsoletus* was captured in these regions on

C. arvensis and *U. dioica*. The insects were captured weekly with a D-Vac aspirator, classified and stored in Eppendorf tubes at -20ºC until analysis. Samples of grapevine and *H. obsoletus* were analyzed by PCR technique.
The molecular characterization of "stolbur" strains was achieved using PCR-RFLP analyses with primers for *tuf* gene encoding the elongation factor tu and primers stol-1H 10, for the *vmp*1 gene encoding a membrane protein (Langer *et al*., 2004, Cimerman *et al.,* 2009).

Results and discussion

In the surveys conducted in Spain, tuf-type a and tuf-type b were identified in grapevine plants, however in the vector *H. obsoletus* only tuf-type b was identified (Batlle *et al.,* 2009). In 2013, *H. obsoletus* has been captured for the first time in *U. dioica* in Navarre and "La Rioja", with a higher population that appeared later than those captured on *C. arvensis*. (Table 1). The maximum population density in *U. dioica* was obtained in Navarre in August 6th, when as much as 30 individuals were captured in one aspiration. In *C. arvensis* only a maximum of 4 individuals were captured by aspiration in the same region in July 9th (Figure 1). In "La Rioja" the maximum population on *U. dioica* was obtained in August 14 th. The individuals captured on *U. dioica* are probably responsible for the spread of the strain tuf-type a in grapevine plants of "La Rioja" and Navarre. In Catalonia the strain tuf-type b continues to be the only one detected.

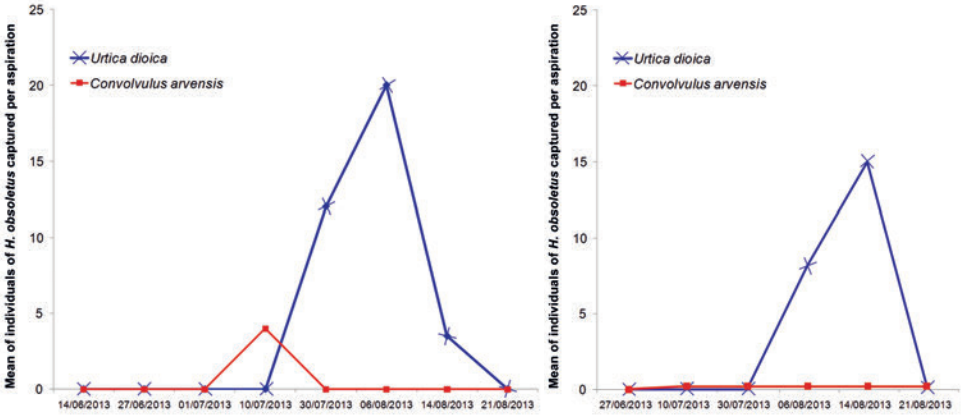

Figure 1. Individuals of *H. obsoletus* captured on *C. arvensis* and *U. dioica,* in 2013 in Navarre (left) and "La Rioja" (right).The maximum population density on *U. dioica* was obtained in August 6th in Navarre and 14th in "La Rioja".

Table 1. Total individuals of *H. obsoletus* captured in several regions on *C. arvensis* and *U. dioica* in 2013.

Locality	Total (% positives)	*Convolvulus arvensis*	*Urtica dioica*
Catalonia (Conca B.)	30 (40%)	30	0
Catalonia (Priorat)	18 (70%)	18	0
Extremadura	1	1	0
Galicia	0	0	0
La Rioja	31 (40%)	0	31
Navarre	80 (50%)	8	72
Valencia	1	1	0

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Mark, release and recapture experiments tracking the dispersal of *Cacopsylla pruni* (Hemiptera: Psyllidae), the vector of European stone fruit yellows in two model apricot orchards

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Abstract

During the last fifteen years European stone fruit yellows has become a major concern in Austrian fruit production. The disease is transmitted by the plum psyllid *Cacopsylla pruni*, a univoltine species overwintering on conifers and immigrating to *Prunus* spp. in early spring. In the present study dispersal of remigrant *C. pruni* in apricot orchards was tracked by aid of mark, release and recapture studies. Insects were collected in the field on *Prunus spinosa*, marked by fluorescent dyes and released in two model orchards. Movement of the marked insects and presence of naturally occurring insects were monitored by yellow sticky traps. In orchards without insecticide treatment, insects easily covered distances from tree row to tree row, or even farther, within 24 hours after release. After 8 days they were present in large parts of the model orchards. The mark, release and recapture experiments also allowed following the effects of a systemically acting insecticide that significantly reduced spatial and temporal spread of the marked insects.

Key words: '*Candidatus* Phytoplasma pruni', insect movement, population size, luminescent powder pigment, insecticide.

Introduction

European Stone Fruit Yellows (ESFY) associated with the presence of '*Candidatus* Phytoplasma prunorum' is currently present in all European and Mediterranean regions where apricots are cultivated (COST action FA0807, 2014). In apricots the disease is characterized by small-sized, conically rolled, chlorotic leaves, premature fruit fall, small tasteless fruits, decreased vitality of infected trees and eventually death of single branches or entire trees (Morvan, 1977; Marcone *et al.,* 2010).

ESFY is transmitted by the plum psyllid *Cacopsylla pruni* (Scopoli) (Carraro *et al.,* 1998) that is univoltine, overwinters on conifers and immigrates to *Prunus* spp. in early

spring. On their *Prunus* hosts the insects lay eggs and develop through five larval stages into adults which migrate to conifers in summer (Ossiannilsson, 1992; Thébaud *et al.,* 2009). Studies suggest that overwintered *C. pruni* returning to orchards in spring are the most efficient disease vectors. Secondary spread within a vegetation period seems to be less common (Carraro *et al.,* 2004, Thébaud *et al.,* 2009, Poggi Pollini *et al.,* 2010). Wild *Prunus* species are frequently tolerant hosts of the phytoplasma and suitable hosts for *C. pruni.* So these plant species could play a significant role as source of infectious vectors (Carraro *et al.,* 2002). In addition ESFY is transmitted via propagation material (Marcone *et al.,* 2010). Grafting experiments with ESFY-infected scions led to significant infection rates in the progeny (Riedle-Bauer *et al.,* 2013). Laxness or gaps in maintenance and testing of mother trees can therefore result in infection of new orchards.

The disease has become a major concern in Austrian fruit production during the last fifteen years. Investigations in 2011 and 2012 revealed high infection rates and an epidemic disease spread. The phytoplasma was ascertained in 9.8 to 63.3% of the analyzed apricot trees, in 20 to 40% of the plum trees and occasionally in blackthorns. Infection rates of the vectors were also considerable. Up to 11.5% of the overwintering generation insects and 3.44% of the springtime generation insects carried the phytoplasma (Maier *et al.,* 2013). As a consequence the development of well adapted management strategies is an urgent need.

Proposed management approaches include the use of healthy propagation material, the removal of rootstock suckers, uprooting of infected trees and application of insecticides against both the overwintering and the springtime generations (Marcone *et al.,* 2010). Clearing strategies, however, are likely to be insufficient or even inefficient in areas where the disease is present epidemically on wild *Prunus* hosts (Thébaud *et al.,* 2009). Actual effects of insecticides on disease spread are not yet clear. ESFY-transmission experiments in greenhouse revealed a minimum inoculation period of 1-2 days (Carraro *et al.,* 2001). In theory a fast acting, enduring insecticide should therefore kill the vectors before inoculation of the trees, and in the end reduce new infections. Insecticide applications in an experimental orchard, however, showed inconclusive effects on disease spread (Poggi Pollini *et al.,* 2007). Insecticide tests under controlled conditions proved significant effects of Abamectin on *C. pruni* eggs and larval stages (Jarausch *et al.,* 2010).

For the development of appropriate management strategies tracking vector presence and behaviour within an orchard seems very helpful. Mark, release and recapture experiments allow visualization of insect spread. Moreover they permit estimations of natural vector population sizes determining the rate between captured marked and unmarked insects. Our studies started with monitoring dissemination and population sizes of remigrant *C. pruni* in insecticide-free orchards. In a second step preliminary experiments focusing on the effects of a systemically acting insecticide were also carried out.

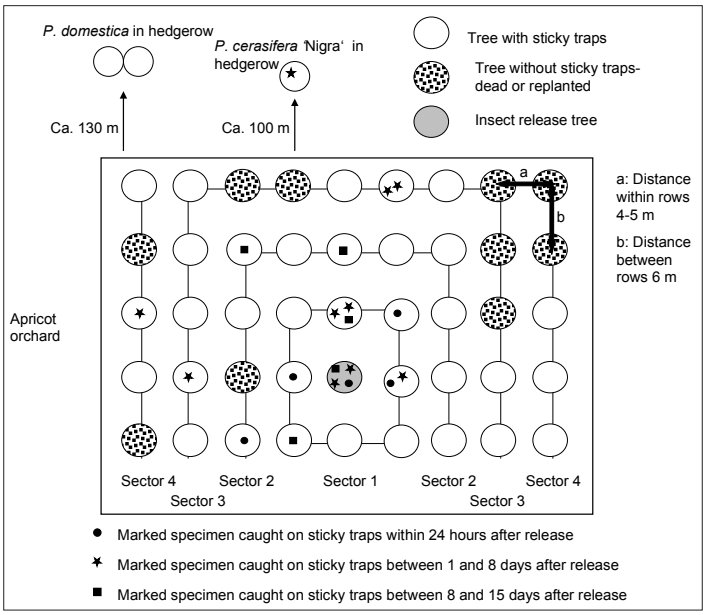

Figure 1. Mark and recapture experiment 1/2012 (Release 19th of April, end 3rd of May): size and shape of the model orchard, position of insect release tree, position of yellow sticky traps and number and position of marked insects recaptured on sticky traps (from Maier *et al.*, 2013).

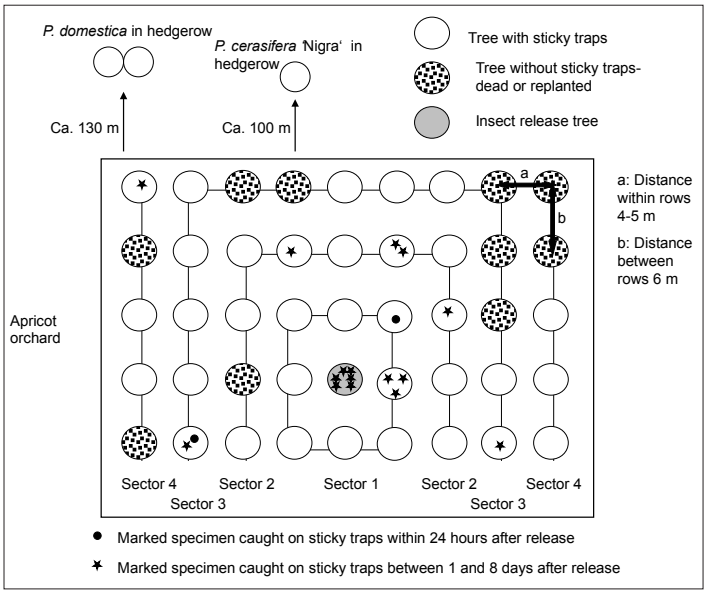

Figure 2. Mark and recapture experiment 2/2012 (Release 26th of April, end 3rd of May): size and shape of the model orchard, position of insect release tree, position of yellow sticky traps and number and position of marked insects recaptured on sticky traps (from Maier *et al.*, 2013).

Materials and methods

C. pruni remigrating to orchards in spring are regarded as the most efficient disease vectors, thus all experiments concentrated on this developmental stage.

Collection and marking of insects

Overwintered *C. pruni* adults were collected on *P. spinosa* by beating tray method. Marking was performed as previously described (Nakata *et al.,* 2008; Maier *et al.,* 2013). Pink (Karmin Tagesleuchtpigment, Artmaxx, Berlin, Germany) orange, green, white (all UV elements, Nordhausen, Germany) and light blue (Guardi, Prussian blue UV active, Boesner, Vienna, Austria) luminescent powder pigments were utilized. Stability of the dyes in the field was investigated by transferring 10 dead, stained specimens per dye to a yellow sticky trap exposed to outdoor conditions. Visibility of the stain in daylight and UV light was examined weekly. Effects of the pigments on insect survival were examined by a laboratory experiment with 10 individuals at a time. Statistical analysis (Kruskal-Wallis H Test) was performed by aid of SPSS 19.0 (SPSS Inc., Chicago, USA) (Maier *et al.,* 2013).

Field experiments 2012

The field experiments were carried out after flowering in an apricot orchard (1) located in Krems, lower Austria. Before and during the testing period no insecticides were applied. Trees had standard size, within row tree spacing ranged from 4 to 5 m, while between row distance was 6 m. Numbers and arrangement of the trees included in the experiments are illustrated in Figure 1 and Figure 2. Experiment 1 started on 19th and experiment 2 on 26th of April: 600 *C. pruni* per experiment were coated with luminescent pigment and freed on one single tree in the apricot orchard. For each release a different dye was used. Movement of stained insects and presence of naturally occurring insects within the orchard were monitored by yellow sticky traps (2 traps per tree including the release tree) from 19th of April (release of the first stained insects) until 3rd of May (8 days after the second insect release). The positions of the sticky traps are illustrated in Figures 1 and 2. During the experiment sticky traps were controlled at night using a black light torch (UV elements, Nordhausen, Germany). After the end of the experiment all traps were transferred to the laboratory and inspected under a binocular microscope and a standard UV transilluminator (Vilber Lourmat, Marne-la-Vallée, France) (Maier *et al.,* 2013).

Field experiments 2013

Procedures were identical to 2012 except that the experiments were carried out in two apricot orchards and insecticide applications were included in the study. Orchard 1 was the same as in 2012. Orchard 2 was located in Klosterneuburg (lower Austria), planted with spindle shaped trees; within row spacing was approximately 2 m, distance between rows 4 m and the test plot comprised 9 rows with 17 trees per row. Insect releases started on 18th of April in orchard 1, on 23rd of April in orchard 2. At this particular time the

orchards were insecticide-free. Thiacloprid (Calypso, Bayer Crop Science, Monheim, Germany) was applied according to the manufacturer's instructions on 25th of April in orchard 1 and on 29th of April in orchard 2. In order to estimate the effect of the insecticide on incoming remigrants, trees were allowed to air dry for 4 hours before new differently stained insects were freed. Insect survival and spread before and after insecticide application were monitored for two weeks (from the first release till one week after the second release).

Results

Stability of dyes and effects on insect survival

After exposure of marked insects to outdoor conditions for five weeks all dyes were clearly visible in black or UV light. Kruskal-Wallis H Test revealed no statistically significant differences between survival of unstained and stained insects and between survival of insects coated with the different pigments.

Field experiments 2012

The results of the two release experiments are illustrated in Figures 1 and 2. In the first trial 20 specimens (3.3%) were recaptured within 15 days (19 within the orchard, 1 in the adjacent *P. domestica* hedgerow), in the second trial 18 (3%) were trapped in the orchard within 8 days. In both experiments insect spread occurred rapidly. In the first trial marked insects were observed on trees adjacent to the release point and also in the next row of trees within one day after release. In the second trial marked specimens were ascertained on adjacent trees and on trees two rows farther within 24 hours. 53 unstained naturally occurring plum suckers were also captured during the test period. Relation between marked and unmarked insects on the traps allowed the rough estimation that approximately 1,700 individuals were naturally present in this orchard.

Field experiments 2013

Orchard 1. Fourteen marked and 40 unmarked individuals were captured within the test week on insecticide-free trees leading to a estimated natural population size of around 1,700 individuals. Some insects reached the farthest traps (distance approximately 24 m) already within 24 h after release and insect movement was observed during the entire test week. After insecticide treatment no more insects originating from the first release were trapped and the treatment decreased the number of recaptured insects from the second release to 11. Thiacloprid reduced the spatial dispersal as the vast majority of stained individuals was recaptured close to the release point. Movement of marked insects on insecticide treated trees was predominantly observed within the first 24 h after release. Moreover the treatment had a significant effect on naturally occurring insects. Within the second observation week only 18 individuals were ascertained on the traps.

Orchard 2. On untreated trees 37 marked and 128 unmarked *C. pruni* were captured within one week. Already after 24 h some marked specimens had reached the farthest traps. Insect movement continued during the whole observation period. The ratio of marked and unmarked insects on the traps allowed the estimation that around 2,000 *C. pruni* individuals naturally occurred in the test plot. After application of Thiacloprid no captures of insects originating from the first release were achieved. The number of recaptured insects from the second release decreased to 10 and the number of unstained individuals on the traps to 18. Spatial dispersal of stained individuals was reduced to the close vicinity of the release tree. Insects were mainly recaptured within 24 hours after release, after this period only two specimens were caught.

Discussion

The incidence of new ESFY infections in an apricot orchard depends on the quantity of vectors' present, on the infection rates of the insects as well as on insect mobility and numbers of trees that are visited by one individual. These tests showed that fluorescent dyes provide an easy and effective marking tool for field studies on psyllid density and movement. The selected pigments were stable for several weeks under outdoor conditions including rain. There was no detectable effect of the dyes on insect survival.

Recaptures of marked *C. pruni* on the sticky traps proved a fast and frequent tree to tree movement in insecticide-free orchards. Marked individuals easily covered distances up to 24 m within 24 hours after release. After 8 days they were present in a large part of the model orchards. All in all it seems likely that one infectious individual present in an orchard visits and infects several apricot trees. ESFY transmission experiments in the greenhouse revealed a minimum inoculation period of 1- 2 days (Carraro *et al*., 2001). In theory a fast acting, enduring insecticide should therefore kill incoming remigrants before inoculation of the trees, reduce insect numbers in orchards, downsize movement of the vectors from tree to tree and in the end diminish rates of new infections. Insecticide applications in experimental orchards, however, did not show conclusive effects on disease spread (Poggi Pollini *et al*., 2007). In general such experiments are hampered by the fact that several years can elapse between inoculation and development of visible disease symptoms, by the limits of the available diagnostic procedures to detect low phytoplasma titers, and by the possibility that weak infections of the starting plant material may remained undetected.

These preliminary mark and recapture experiments allowed a clear visualization of insecticide effects on psyllid survival and movement. Such experiments could contribute to the development of appropriate management strategies. Thiacloprid significantly reduced spread of marked insects and captures of unstained insects in both orchards. Especially in orchard 2 a striking effect was observed. The insecticide should therefore also reduce disease spread as long as its effect lasts.

The numbers of insects in these mark and recapture experiments were relatively small as compared to other release experiments with psyllids. Van den Berg and Deacon (1988) for example released a total of 25,000 *Trioza erytreae* in two experiments, Kobori *et al*. (2011) carried out two experiments with 11,000 marked *Diaphorina citri*. In these laboratory rearing experiments *C. pruni* produced high numbers of eggs which developed into numerous springtime generation adults. Migration to conifers and hibernation under artificial conditions, however, proved to be difficult (data not shown). Rearing significant numbers of remigrants seemed hardly possible. Consequently hibernated insects were captured in the field where their occurrence was limited. Nevertheless the obtained recapture rates in untreated orchards enabled to draw the conclusions outlined above although, also in these experiments, a higher number of released insects would have been desirable.

These results might represent a first step towards an appropriate sustainable management of *C. pruni* but there is still a long way to go. In Austria the flight period of the remigrants lasts at least for 6 weeks, starts in the first half of March and includes the blossom period. In order to overcome these 6 weeks a combination of several insecticides under consideration of bee activity will be required. Cold temperatures and early developmental stages of the trees at the beginning of March might adversely influence the effects of some insecticides. Authorization of insecticides and effects on beneficial insects must be kept in mind. Last but not least the effects of the control strategy on disease spread must be proven in field experiments.

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Isolation of potential biocontrol agents of '*Candidatus* Phytoplasma mali'

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Abstract

'*Candidatus* Phytoplasma mali', the causal agent of apple proliferation disease, is a quarantine pathogen controlled by chemical treatments against insect vectors and eradication of diseased plants. In accordance with the European guidelines, novel strategies should be developed for sustainable management of plant diseases by using resistance inducers (e.g. endophytes). A basic point for the success of this approach is the study of endophytic bacteria associated with plants. In the present chapter, endophytic bacteria living in healthy and '*Ca.* P. mali'-infected apple trees are described by cultivation-dependent and independent methods. The 16S rDNA sequence analysis showed the presence in apple of the groups *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Chlamydiae*, and *Firmicutes*. In detail, library analyses underscored 24 and 17 Operational Taxonomic Units (OTUs) in healthy and infected roots, respectively, with a dominance of *Betaproteobacteria*. Twelve and six isolates with different morphology were isolated and characterized from healthy and infected roots respectively. Different strains belonging to the genus *Pseudomonas* and *Lysinibacillus* showed a minimum of five beneficial traits related to mineral nutrition (phosphate solubilization, siderophores, nitrogen fixation), development (indolacetic acid synthesis), stress relief (catalase activity), disease control (siderophores). These strains will be tested for *in vivo* suppression of phytoplasma related diseases.

Key words: endophytic bacteria, apple proliferation, *16S rRNA* gene libraries, apple tree, plant growth promotion.

Introduction

Apple proliferation (AP), associated with the presence of '*Candidatus* Phytoplasma mali', is one of the most important phytoplasma diseases in Europe. AP is a quarantine pathogen vectored in nature by *Cacopsylla picta* and *Cacopsylla melanoneura* (Frisinghelli *et al*., 2000; Tedeschi *et al*., 2002). The management of AP mainly entails insecticide treatment against the insect vectors and eradication of diseased plants, both having a severe economic cost. Moreover, the new guidelines for the Common Agricultural Policy of the European Community (Dir 128/2009) require the use of sustainable agricultural practices with an eye on environmental safeguard. One of the most innovative and promising solutions is the substitution of the insecticide treatments with biotic (e.g. endophytes) and abiotic resistance inducers able to elicit the natural plant defense responses. Recently, endophytic bacterial community associated with healthy

and phytoplasma infected plants has been screened (Bulgari *et al*., 2009; Martini et al., 2009) to find putative biocontrol agents.
Endophytic bacteria are plant-associated microorganisms that live inside plant without inducing symptoms of disease. Endophytic bacteria seem to positively influence plant-host growth through mechanisms similar to those described for plant-growth promoting rhizobacteria (PGPR). Moreover, they can promote plant growth by reducing the deleterious effects of plant pathogens trough direct or indirect mechanisms (biocontrol) (Lugtemberg and Kamilova, 2009). In detail, they are able to suppress pathogens through the competition for an ecological niche, the production of allelochemicals (e.g. antibiotic, siderophores, lytic enzymes) or through the induction of a systemic plant defense response (ISR). The use of plant associated bacteria in plant protection is related to the understanding of bacteria-host interactions and to the ability to formulate and spread the bacteria under field conditions (Hallmann *et al.,* 1998). A basic point for the success of sustainable management of plant diseases based on biocontrol agents is the study of endophytic bacterial community living inside plants. In this work, the endophytic bacterial community associated with healthy and AP phytoplasma-infected apple roots is described and screened in order to find potential biocontrol agents.

Materials and methods

Plant material, DNA extraction and '*Ca*. P. mali' detection

Apple roots were collected from five asymptomatic and five AP-symptomatic plants during a field survey conducted in 2010 in north-western Italy. Roots were sterilized and the total DNA was extracted following the method describe by Doyle and Doyle (1990), with some modifications. Extracted DNA was used as template for '*Ca*. P. mali' identification by (PCR)-based amplification of ribosomal RNA genes. In detail, '*Ca*. P. mali' was detected by the use of primer pairs fAT/rAS specific for 16SrX phytoplasma group (Smart *et al*., 1996). PCR products were separated on 1% agarose gel and visualized by UV transilluminator.

Endophytic bacteria identification by cultivation-independent methods

Endophytic bacterial community associated with roots, from healthy and '*Ca*. P. mali'-infected apple, was described by *16S rRNA* gene library analyses. The bacterial 16S rDNA was amplified from total DNAs with bacterial universal primer pairs 799f/1492r (Chelius and Triplett, 2001). 16S rDNA bacterial specific bands were excised and purified from the gel with the QIAquick Gel Extraction Kit according to manufacturer's instructions. Purified products were cloned in the plasmid vector pCRII-TOPO (Invitrogen) and propagated in *Escherichia coli* as described (Shuman, 1994). The plasmid DNA of each clone was extracted from *E. coli* colonies with the QIAGEN Plasmid Mini kit (QIAGEN), and sequenced with an ABI 3730 sequencer

(Primm, Italy). Clone sequences were identified by comparison with National Center of Biotechnology Information (NCBI) GenBank database with the BLAST software (http://www.ncbi.nim.nih.gov/BLAST/). All the 16S rRNA gene sequences from clone libraries were clustered in Operational Taxonomic Units (OTUs) with the software Bioedit 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Nucleotide sequences sharing more than 97% identity were clustered in the same OTU. Nucleotide sequences of one representative clone of each OTU were deposed at NCBI GenBank at accession numbers from JQ291750 to JQ291790.

Isolation and characterization of endophytic bacteria from roots by cultivation

Endophytic bacteria were identified by cultivation on different artificial media. Partial volume of leaves homogenates (100 µl), serially diluted, was spread on Tryptic Soy Agar (TSA) and Luria-Bertani (LB) agar medium. The samples were incubated at 30°C for 5 days. After growth, bacterial colonies were selected on the basis of phenotypic characters. Bacterial density in the roots was calculated as CFU/g. Total DNA from bacterial colonies of root samples n. 1 and 4 (healthy plants), and n. 8 and 10 (infected plants) was extracted by microLYSIS (Microzone) according to the manufacturer's instructions. *16S rRNA* genes were amplified with the primers 27F and 1495R (Lane, 1991) following PCR conditions previously described (Bulgari *et al*., 2009), and were sequenced with an ABI 3730 sequencer (Primm). Sequences were identified by comparison with the NCBI GenBank sequence database with the BLAST software. Nucleotide sequences of representative isolated bacteria were deposited at NCBI GenBank database under accession numbers from JQ291734 to JQ291749. *16S rRNA* gene sequences were clustered in a Neighbour-Joining phylogenetic dendrogram bootstrapped 1,000 times with the software MEGA4 (Tamura *et al*., 2007).
The endophytic bacteria isolated on culture media were screened for plant growth promotion (PGP) and biocontrol abilities. In detail, qualitative estimation of phosphate solubilisation was carried out by observing a halo around colony growing on Pikovskaya's medium. Moreover, siderophore and indol acetic acid production and nitrogen fixation were estimated as reported in literature (Alexander and Zuberer, 1991; Husen *et al*., 2009; Melo *et al*., 2011).

Results and discussion

Detection of '*Ca*. P. mali' in apple roots

'*Ca*. P. mali' detection in root samples was carried out using primers fAT/rAS which target 16S rDNA and 16S-23S intergenic region of 16SrX phytoplasma group. Electrophoresis analysis showed a band of approximately 500 bp in all roots collected from symptomatic apple trees. PCR products were also found in root samples n. 2, 3 and 5 that are from asymptomatic plants (Table 1).

Table 1. Phytoplasma detection in roots of asymptomatic and symptomatic apple trees and correspondent CFU/g of endophytic bacteria isolated.

ID	Sanitary status	PCR[a]	CFU/g	
			LB Agar	TSA
1	asymptomatic	-	1.8×10^5	2.4×10^5
2	asymptomatic	+	2.8×10^4	3.6×10^4
3	asymptomatic	+	8.3×10^3	1.5×10^3
4	asymptomatic	-	1.1×10^5	1.6×10^5
5	asymptomatic	+	7×10^4	1.1×10^4
6	symptomatic	+	3×10^3	7.5×10^3
7	symptomatic	+	6×10^3	7×10^3
8	symptomatic	+	9×10^3	1.1×10^4
9	symptomatic	+	1.2×10^4	1.3×10^4
10	symptomatic	+	1×10^4	8.6×10^3

[a] Specific detection of phytoplasma group 16SrX by PCR-based amplification by primer pair fAT/rAS

Analysis of endophytic bacterial community by *16S rRNA* gene libraries

Endophytic bacterial community had been characterized in a wide range of woody and herbaceous plants (Lodewyckx *et al*., 2002), but no researches have described the microbial diversity associated with apple trees. In this study, *16S rRNA* gene libraries from infected and uninfected apple roots were analyzed to describe the endophytic bacterial community. A total of 120 clones were sequenced, 76 from the healthy roots and 44 from the infected ones. The coverage of libraries from uninfected and infected trees was 95% and 89% respectively.

On the basis of sequence similarity, clone sequences of the two libraries were organized in OTUs. These analyses allowed to estimate bacterial richness associated with healthy and phytoplasma-infected apple roots. Most of the clones showed ≥98% sequence similarity to the relative strains of GenBank. Twenty-four and 17 OTUs were identified in the libraries from healthy and infected roots, respectively. These data showed that bacterial diversity in healthy roots is higher than the one observed in infected ones. Also in previous work, clone library analysis of plant-associated bacteria in '*Ca*. Liberibacter asiaticus'-infected and uninfected citrus roots evidenced differences in the composition of their bacterial community (Trivedi *et al*., 2010). The influence of pathogen infection on endophytic bacterial community was also reported in healthy and phytoplasma-infected grapevine leaves (Bulgari *et al*., 2011). Such evidences, along with data from the present study, suggest that uncultured bacterial pathogens can modify the composition of endophytic bacterial communities associated with infected plants.

The sequences related to *Proteobacteria* represented the largest fraction of clone libraries (Figure 1). In detail, 7 and 5 OTUs of *Betaproteobacteria* group were identified in healthy and infected roots libraries, respectively. In healthy roots, the major number of

clones (19.6%) yielded the best matches with bacteria of the family Oxalobacteriaceae; in infected roots, 10.8% of clones showed the highest similarity with bacteria of the family Burkholderiaceae, genus *Burkholderia*. This bacterium has been reported as endophyte of pathogen-infected plants (Bulgari *et al*., 2011), and in various uninfected host plants such as potato, tomato, and grapevine (Bensalim *et al*., 1998; Onofre-Lemus *et al*., 2009). Several strains are known to enhance disease resistance in plants, contribute to better water management, and improve nitrogen fixation and overall host adaptation to environmental stresses (Compant *et al*., 2008).

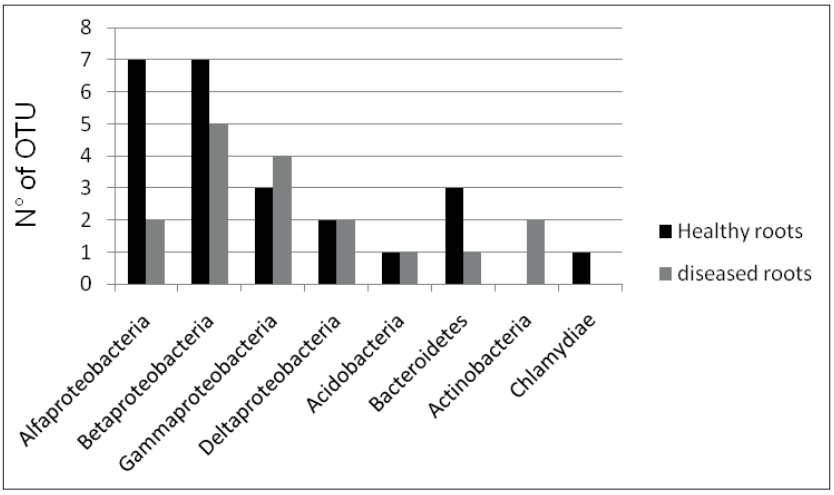

Figure 1. Composition of 16S rRNA gene libraries at Operational level.

The dominant OTU in healthy roots yielded best matches with *Gammaproteobacteria* group, Pseudomonandaceae family, *Pseudomonas fluorescens* (15.7% of clones). Interestingly, the genus *Pseudomonas* was the sole bacterium found by cultivation-dependent and independent methods in association with healthy and infected apple trees. *Pseudomonas* is one of the most frequently occurring genera in plants (Lodewyckx *et al*., 2002). Previous studies emphasized the potential of *Pseudomonas fluorescens* as biocontrol agents of several plant diseases through the production of antibiotics, and the activation of Induced Systemic Resistance (ISR) (Schouten *et al*., 2004; Verhagen *et al*., 2010).

Endophytic bacterial community described by cultivation-dependent methods

The diversity of microbiota associated with healthy and phytoplasma-infected apple roots was investigated both with cultivation-dependent and -independent methods in order to increase the range of diversity explored in a sample. Before '*Ca*. P. mali' detection in root samples, endophytic bacteria were isolated on TSA and LB from all

samples collected to calculate CFU/g. In healthy plants bacteria CFU/g were 10^5, while in diseased plants were 10^3-10^4 (Table 1). Interestingly, the asymptomatic plants positive to phytoplasma detection were characterized by a CFU comparable to symptomatic plants. These findings are in agreement with the data reported in other studies (Bulgari *et al*., 2011).

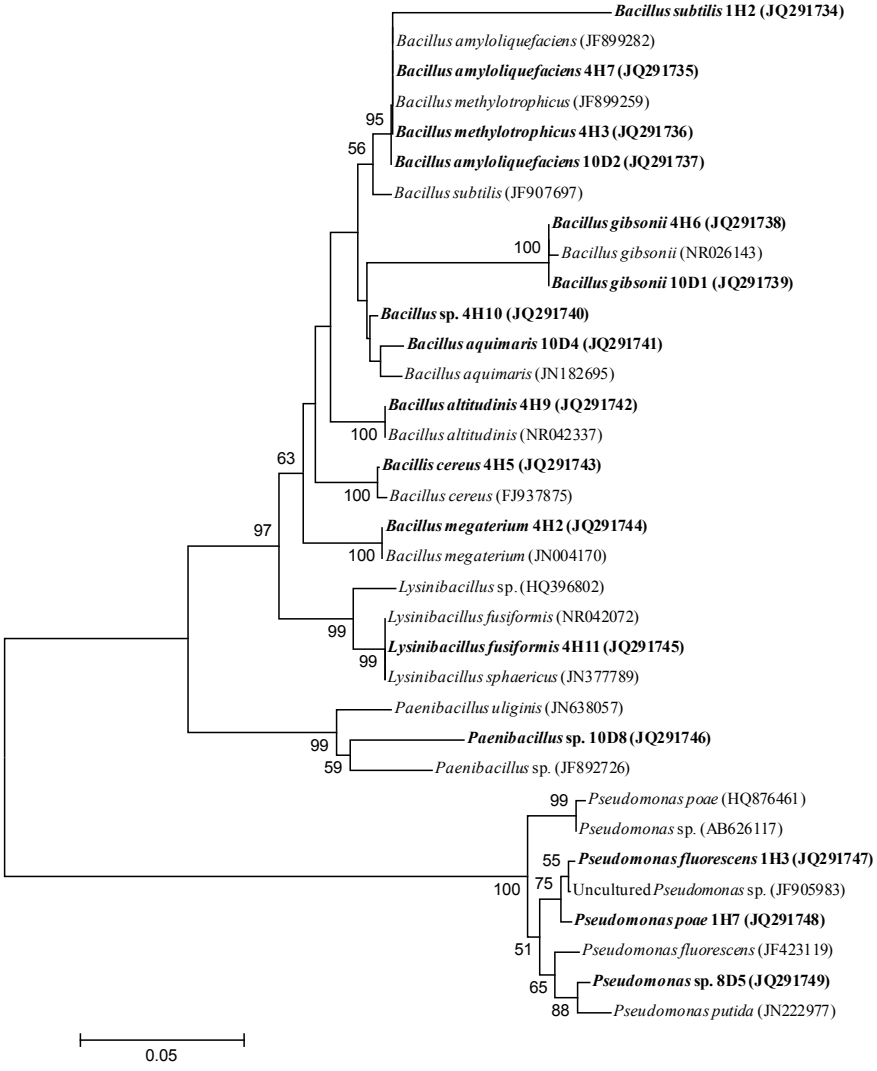

Figure 2. Phylogenetic relationships based on partial 16S rRNA gene sequences obtained from the endophytic bacteria associated with healthy and phytoplasma-infected apple roots and closely related sequences, retrieved from GenBank. Bootstrap values higher than 50% are displayed at tree nodes. GenBank accession numbers of nucleotide sequences are shown along with the name of the bacterial species. Bacterial strains isolated in this work are reported in bold.

Table 2. Plant growth promotion and biocontrol activities of endophytic bacteria isolated from healthy and phytoplasma-infected roots.

ID	Closest relative in GeneBank	P solubilization[a]	Siderophore[b]	N fixation	Catalase	IAA µg/ml
S1C1	*Bacillus subtilis*	(+)	(+)	+	-	13.168
S1C2	*Bacillus subtilis*	(+)	(+)	+	+	12.905
S1C3	*Pseudomonas fluorescens*	+	+	-	+	15.589
S1C4	*Pseudomonas* sp.	+	-	-	+	11.011
S1C6	*Gamma proteobacterium*	(+)	(+)	-	+	83.853
S1C7	*Pseudomonas poae*	+	(+)	+	+	21.274
S1C10	*Pseudomonas rhizospherae*	+	(+)	-	+	-
S1C11	*Pseudomonas* sp.	(+)	(+)	-	+	89.905
S1C12	*Pseudomonas* sp.	+	-	+	+	18.484
S4C2	*Bacillus megaterium*	-	-	-	-	10.063
S4C3	*Bacillus methylotrophicus*	(+)	-	+	+	13.747
S4C7	*Bacullis amyloliquefaciens*	(+)	+	+	+	11.274
S4C8	*Bacillus megaterium*	-	(+)	+	+	19.379
S4C9	*Bacillus altitudinis*	(+)	(+)	-	+	16.800
S4C11	*Lysinibacillus fusiformis*	-	(+)	-	+	68,168
S10C2	*Bacullis amyloliquefaciens*	(+)	-	-	+	-
S10C3	*Pseudomonas* sp.	(+)	(+)	-	+	21.800
S10C4	*Bacillus aquimaris*	-	(+)	/	/	9.011
S10C8	*Paenibacillus* sp.	-	-	/	/	50.011

[a] + = clear zone surrounding the colony; (+) = moderate clear zone surrounding the colony;
[b] + = orange fluorescence surrounding the colony; (+) = weak orange fluorescence surrounding the colony
/ = isolates not tested

Endophytic bacteria isolation was performed on healthy apple roots (sample n. 1 and n. 4) and on infected roots (sample n. 8 and n. 10). Twelve colonies with different morphology were isolated from healthy roots and six colonies from infected roots. Sequences of the *16S rRNA* gene identified *Firmicutes* of the genus *Bacillus*, *Lysinibacillus* and *Paenibacillus*; *Gammaproteobacteria* of the genus *Pseudomonas* (Figure 2). In detail, *Lysinibacillus* and *Paenibacillus* were isolated respectively only in healthy and AP-infected roots, while the other genera identified were shared by all samples analyzed. Six different *Bacillus* species were isolated from healthy apple trees

and, among these, *Bacillus amyloliquefaciens* and *Bacillus gibsonii* were found also in infected plants; *Bacillus aquimaris* was identified exclusively in phytoplasma-infected roots. Interestingly, bacterial strains, here isolated, belong to genera widely studied for developing biocontrol strategies to contain plant pathogens (Trivedi *et al.*, 2011).
All the bacterial isolates were screened for their PGP and biocontrol abilities using various qualitative and quantitative assays (Table 2). The major part of isolates showed the ability to solubilize phosphate and detoxify hydrogen peroxide. Interestingly, some strain e.g. *Lysinibacillus fusiformis* produced high amount of IAA.
In conclusion, some of the endophytic bacteria identified and characterized in the present study in healthy and phytoplasma-infected apple trees have been reported as biocontrol agents against different plant pathogens. This finding opens the possibility to in-depth investigate the action of those bacteria against '*Ca*. P. mali' with the aim to project novel strategies for AP disease containment.

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The role of vineyards not treated with insecticides on *Scaphoideus titanus* spreading

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Abstract

Symptomatic grapevines infected by "flavescence dorée" (FD) and its vector *Scaphoideus titanus* are more present in vineyards untreated with insecticides. Information on vineyard to vineyard mobility of vectors are needed to understand the risk posed by the vineyards in which there is no *S. titanus* control. To address this issue, a research was carried out in two localities in north-east Italy where a cultivated untreated vineyard contiguous to a cultivated treated vineyard are present. Results proved that the *S. titanus* can move from untreated vineyards to contiguous vineyards where insecticides are specifically applied for its control. The majority of adults were captured within 40-50 m from untreated vineyard, even if some individuals were captured at 80-100 m. The risk posed by grapevines untreated with insecticides against the *S. titanus* for FD spread in other vineyards is confirmed by the results obtained here. Moreover, results stress that insecticide applications for FD infections' risk reduction can be ineffective if untreated FD-infected grapevines are present in the surrounding areas.

Key words: "flavescence dorée", *Scaphoideus titanus,* vectors moving, untreated vineyards, insecticides.

Introduction

The "flavescence dorée" (FD) is a grapevine yellows disease causing severe damage in European vineyards. The phytoplasma agent of the disease, is transmitted also in Italy from grapevine to grapevine by the leafhopper *Scaphoideus titanus* Ball (Homoptera: Cicadellidae) (Bianco *et al*., 2001; Mori *et al*., 2002). In various surveys carried out on grapevines untreated with insecticides (cultivated or abandoned) high population levels of *S. titanus* were found (Pavan *et al*., 2005; 2012; Lessio and Alma, 2006; Lessio *et al*., 2007). The highest population of the vector and the highest percentage of symptomatic grapevines were found in cultivated vineyards where insecticides against *S. titanus* are not applied (Pavan *et a*l., 2005; 2012; Bressan *et al*., 2005). Therefore, infectious vectors moving from these vineyards can represent a potential risk for other vineyards. It should be noted that, insecticide treatments against the vector are compulsory only in grape-growing areas where FD is present (Barba, 2005; Pavan *et al*., 2005a). These areas are annually identified by the local governments, however in other areas, insecticides against *S. titanus* may not be applied despite the presence of the vector. Furthermore,

insecticides applied against other pests (e.g. grape berry mouth), may not be effective against *S. titanus*.
Information on vineyard to vineyard mobility of *S. titanus* are needed to understand the risk posed by the vineyards with no *S. titanus* control on other vineyards. To address this issue a research was performed in Italy to study the mobility of S. *titanus* adults between contiguous untreated and treated vineyards.

Materials and methods

The research was carried out in two localities in north-east Italy where a cultivated untreated vineyard contiguous to a cultivated treated vineyard are present.
Year 2011. Breganze location (45°42.43' N - 11°34.50' E, 117 m a.s.l.), Merlot and Cabernet Sauvignon cultivars, "espalier" training system at a distance of 3.5 m x 0.9 m. The treated (two applications /year organophosphate and neonicotinoid against young stages) and untreated vineyards were 1.92 and 1.35 ha, respectively. The rows were north-south oriented on the untreated vineyards, east-west on the treated one.
Year 2012. Lonigo location (45°24.05' N - 11°23.24' E, altitude 32 m a.s.l.), Garganega cultivar, "pergola" training system at a distance of 4.0 m x 1.0 m. The treated (two applications with organophosphate per year against young stages) and untreated vineyards were 1.04 and 1.06 ha, respectively. The vineyard rows were north-south oriented in both vineyards.
No other vineyards were present at the borders of the investigated fields. In both vineyards, the presence and flight dynamics of *S. titanus* were monitored every two weeks from July to October by using yellow sticky traps (SuperColor Giallo®, Serbios) positioned according to a regular grid design (approx. 30 m x 30 m). Each trap was geo-referred using GPS. All traps were analysed under dissection microscope in laboratory and captured *S. titanus* adults were counted.
Data were analysed using SADIE (Spatial Analysis by Distance IndicEs) red–blue methodology (Perry *et al.*, 1999), to detect spatial patterns in *S. titanus* cumulative captures. At each sampling point the local contribution to a group (cluster) of relatively high-density (patch) or to a group of zero or relatively small counts (gap) was assessed. Tests of non-randomness based on the overall index of aggregation (Ia) and on the average indexes of clustering into patch ($\overline{v}_i$) and into gap ($\overline{v}_j$) were performed ($\alpha = 0.05$) using kriging with SURFER (Golden Software Inc., CO), indexes of local aggregation (vi; vj) and catches were interpolated and used in the design of a two-dimensional map showing their spatial distribution.

Results and discussion

S. titanus adults were captured in both untreated and treated vineyards, however the captures were significantly higher in the untreated ones (Figures 1-3). Significant clustering into patch was detected in the distribution of *S. titanus* observed during the

two years ($p < 0.05$). *S. titanus* resulted aggregated in untreated vineyards, however also in treated vineyards, particularly in 2011 some patches were found. In both grape-growing areas a decreasing gradient of *S. titanus* captures was observed from untreated to treated grapevines. Results proved that the vector can move from untreated to contiguous vineyards where insecticides are specifically applied for its control. The majority of adults were captured within 40-50 m from untreated vineyard confirming the low mobility reported in previous surveys (Lessio and Alma, 2004; Beanland *et al*., 2006, Pavan *et al*., 2012), even if some individuals were captured at 80-100 m. The migration of *S. titanus* adults from untreated vineyards started at the beginning of August and the maximum number of captures on treated vineyard was registered at the end of August.

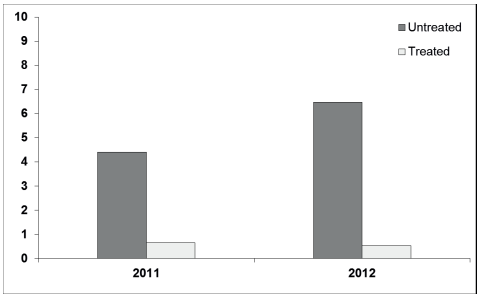

Figure 1. *S. titanus* captures in untreated and treated vineyards in 2011 and 2012.

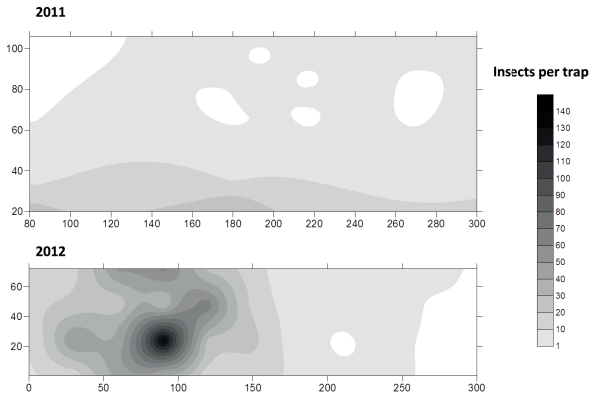

Figure 2. Map of interpolated *S. titanus* captures in untreated and treated vineyards during 2011 and 2012. Data were interpolated using kringing method. Values on axis indicate coordinates in meters.

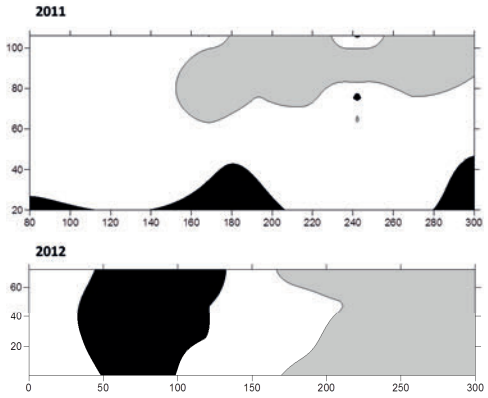

Figure 3. Map of local aggregation of *S. titanus* captures in untreated and treated vineyards during 2011 and 2012. Black areas are patches, with interpolated cluster index, vi > 1.5, grey areas are gaps, with interpolated cluster index, vj < -1.5. Values on axis indicate coordinates in meters.

The risk posed by grapevines untreated with insecticides against the *S. titanus* for FD spread in other vineyards is confirmed by the results obtained here. Results stress that insecticide applications for FD infections' risk reduction can be ineffective if untreated FD-infected grapevines are present in the surrounding areas.

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The role of grapevine arbours as overlooked sources of "flavescence dorée" and *Scaphoideus titanus* in southeastern vineyards of Austria

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Abstract

Field trials were conducted in 2011 to investigate the short distance spread of adult *Scaphoideus titanus* from grapevine arbours to either a commercial vineyard or to grapevine cultivar Chardonnay trap plants. In all locations the vector of "flavescence dorée" (FD) actively moved from the arbour to the Chardonnay trap plants and to the vineyard. The rate of the migrating population was between 0 and 7.1%, the maximum spread distance was 75 meters. In addition, 41 arbours in the municipality Tieschen in south-east Styria were tested for FD presence. The 5% of the grapevine arbours were positive. From these findings it is concluded that grape arbours act as a reservoir for both *S. titanus* and FD from which a small percentage of the vector population spreads to nearby vineyards. In areas where they are widespread, grapevine arbours are an important factor in the epidemiology of FD, therefore they should be considered in the management strategy.

Key words: phytoplasma, reservoir host plants, spread, vector, viticulture.

Introduction

In 2004, *Schapoideus titanus* was found for the first time in Austrian vineyards in southeast Styria. In 2009, single grapevine plants and some wild plant species in this area have been detected to be infected with the "flavescence dorée" (FD) phytoplasma. Since then the vector of FD has spread and is established in parts of Styria and Burgenland 2013. FD was detected hitherto only locally in south and southeast Styria (Zeisner, 2009; Reisenzein and Steffek, 2011; AGES, 2013).

Grape arbours and hedges are traditionally cultivated and commonly present in southeast Styria; American grapevines that are often used for grape arbours are usually not subject to insecticide treatments and therefore support high population levels of *S. titanus*. Consequently, such arbours may act as a source of both the vector and the FD phytoplasma. Furthermore, American grapevine usually do not show symptoms of this grapevine yellows disease (Lessio *et al.*, 2007).

The research project VitisCLIM was intitiated to model spread and impact of FD and its vector in Austria (Steffek *et al.*, 2011). Data from scientific literature on the epidemiology and the spatial spread dynamics of *S. titanus* were reviewed for the spread model. Moreover, field trials were conducted in summer 2011 to assess the percentage

and flight distance of *S. titanus* population that migrate from untreated arbours. In addition, the FD infection level of grapevine arbours was determined in a survey in the municipality Tieschen.

Material and methods

Short distance spread

The trials to study short distance spread of *S. titanus* were conducted in three locations in southeast Styria and Slovenia in 2011. A weather station was placed inside the vineyard to record rainfall, wind direction and speed (above the canopy) and temperatures (in the canopy) to interprete conditions of vector spread.

Location 1 consisted of a vineyard with an approximately 30 year old plot planted with the grapevine cultivars Welschriesling, Weißburgunder and Müller Thurgau, a four year old vineyard planted with cultivars Weißburgunder and Muskateller, and a new (2011) planted Chardonnay vineyard. A grapevine arbour was present 38 m air-line distance from the new planted vineyard and 75 m from the first row and upper edge of the old part of the vineyard. The arbour consisted of a mixture of wild *Vitis* sp. (*V. labrusca, V. riparia*) and *Vitis vinifera*.

Location 2 was an untreated arbour located in southeast Styria from which it was known that an established *S. titanus* population was present.

Location 3 consisted of an untreated arbour with high numbers of *S. titanus* close to grapevines in a private garden.

Field sampling of *S. titanus* nymphs

The number of *S. titanus* nymphs (L1 and L2) on five leaves of 40 plants was counted in the arbour and in the vineyard at four dates (30.05., 09.06., 21.06., 08.07.2011). At the beginning of the experiment 164 nymphs were counted mainly in the old vineyard. Due to an insecticide application (Chlorpyrifos-methyl) after the first assessment (30.05.) the nymphs in the vineyard dropped to very low numbers during the subsequent assessments (5, 2 and 3).

Maximum carrying capacity

The maximum carrying capacity of *S. titanus* in a grape arbour was determined for the spread model as it was assumed that adults spread when the maximum carrying capacity is reached.

The number of *S. titanus* nymphs was assessed in three untreated arbours by counting the first and second instars on an arbour surface area of 2500 cm^2 (date: 28.06.2012). The counts were conducted in various heights of the grape arbours and replicated 18 times. The average number of nymphs on 2500 cm^2 was extrapolated to 1 m^2. This value was used to calculate the maximum number of nymphs in an untreated grapevine in an arbour.

Figure 1. Principle set-up used to assess the short distance spread of *S. titanus* adults from grapevine arbours to trap plant groups (arrows). Location 2 grape arbour and two out of four cultivars. Chardonnay trap plant groups that were planted around the arbour in the main cardinal points. In each trap plant group and in the arbour one yellow sticky trap was placed.

Vector migration from arbours

To assess the percentage and flight distance of migrating populations of *S. titanus* from untreated arbours (source of the vector), trap plants groups of 5-6 one year old grapevines of cultivar Chardonnay were used. They were propagated in an insect proof greenhouse and planted in various distances from the source in early July, shortly before adults appear. Yellow sticky traps (yst) (Rebell® giallo) were placed in the grape arbours, in the cultivar Chardonnay trap plants, at the border of the 30 years old vineyard and randomly in both the 30 years and the 4 years old vineyard at location 1. The sides of the traps were labeled to trace if the catched *S. titanus* individuals were on the side facing towards the grape arbour.

Traps were changed on a weekly interval between 14th of July and 29th of September. The principle of the set-up in locations is shown in Figure 1. In order to detect a relationship between the location of trapped *S. titanus* and the arbour, results were analysed with the inverse distance weightening function (Shepard, 1968).

"Flavescence dorée" infection survey of grape arbours

In autumn 2011, a survey was carried out within a focus zone in southeats Styria to detect the infection level of grapevine arbours with the FD phytoplasma. The extraction of the phytoplasmas from plant samples was done according to Seemüller *et al*. (1994). A real-time PCR assay was used for FD detection (Angelini *et al.*, 2007).

Results

Maximum carrying capacity

The results of the counts in the arbours are presented in table 1. A grapevine in an arbour is estimated to cover a leaf area of 3.6 m^2 in average (http://www.rebschule-meier.ch/PDF/Reben_Haus_Garten.pdf). Based on this estimation and on the results of the larval counts in the arbour, the maximal carrying capacity of a plant in an arbour was set to 288 leafhoppers of larval stage L1.

Table 1. Number of *S. titanus* nymphs (1st and 2nd instar) in three untreated arbours on a surface area of 2500 cm^2.

Sampling height	Arbour 1	Arbour 2	Arbour 3
	Number of *S. titanus* nymphs		
0 cm	68	0	0
0-50 cm	29	6	0
50-100 cm	19	26	0
100-150 cm	57	33	26
150-200 cm	26	39	23
200-250 cm	30	6	2
average number of nymphs (rounded values)	37	17	9

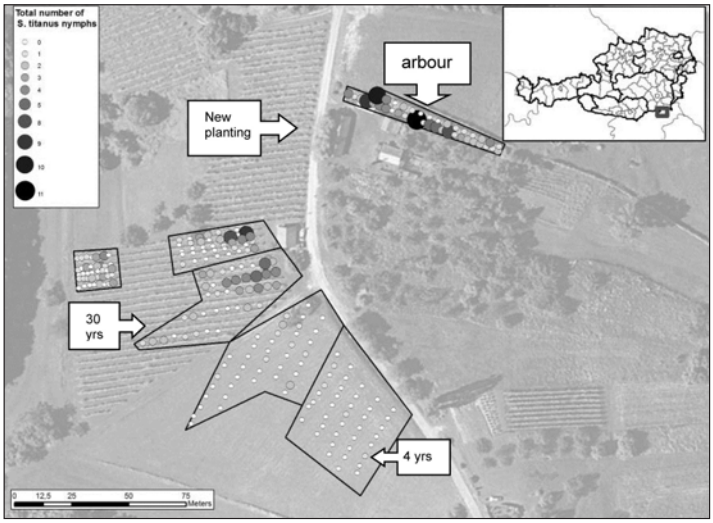

Figure 2. Total number of first and second instar of *S. titanus* per plant (four sampling dates) in the grapevine arbour, in 30 year and in four year old vineyards.

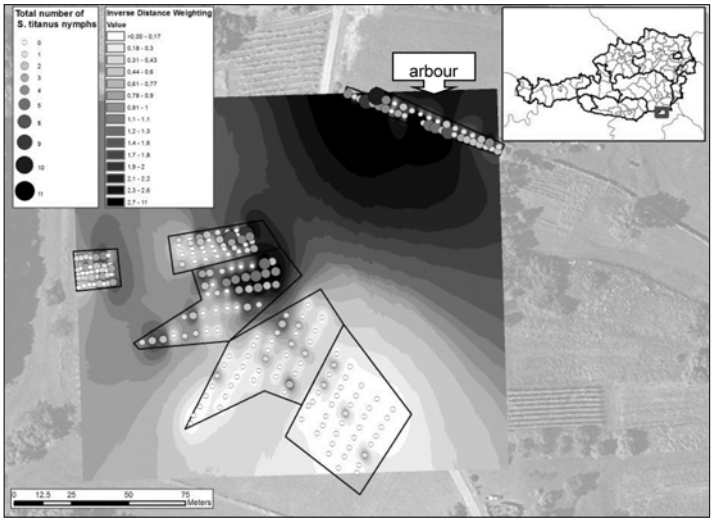

Figure 3. Total number of *S. titanus* nymphs per plant and inverse distance weighting of the counts in the grapevine arbour and in the vineyard. The arbour is assumed to act as the source for migrating *S. titanus* adults.

Sampling results

The distribution of *S. titanus* nymphs in the vineyard is aggregated with a higher density at the borders compared to inside the vineyard (Figure 2). Furthermore nymphs are distributed along rows. More larvae are present in the 30 years old vineyard (159) compared to the 4 years old vineyard (15).
Figure 3 shows the total counts of nymphs of the single sampled plants illustrated as inverse distance weighting.

Vector migration from arbours

At the end of July, adults of *S. titanus* were found for the first time in the cultivar Chardonnay trap plant group closest (2 m) to the grape arbour and at the border of the old vineyard. The maximum number of *S. titanus* catched was in the middle of August (8 specimens of *S. titanus* adults). Six of these individuals were present in the trap placed at the edge of the old vineyard and one individual was recorded in the trap plant group located 2 m away from the arbour. All adults in the old vineyard and the trap plants were catched on the trap side facing towards the grape arbour.
In total, 329 *S. titanus* adults were caught in the grape arbour, 2 in the cultivar Chardonnay group which was two meters away from the arbour, 26 in the 30 years old vineyard and 3 in the four year old vineyard.
The results of migrated *S. titanus* adult in the three locations from grape arbours are summarised in Table 2. The table shows catches of two opposite yellow sticky traps (arbour as source; trap plant as sink). In all three locations the vector actively moved from the arbour to the cultivar Chardonnay trap plant. Depending on the trap, the rate of the catches of the migrating population was between 0 and 7.1%. *S. titanus* adults spread from the arbour to the old part of the vineyard at least 75 m in location 1.

Table 2. Results of the migration experiments: comparison of catches of *S. titanus* adults in two opposite yellow sticky traps placed in the source arbour, the opposite trap was placed in the sink (trap plant).

Site	Loc. 1	Loc. 2 N	Loc. 2 E	Loc. 2 S	Loc. 2 W	Loc. 3 -a	Loc. 3 -b	Loc. 3 -c
Distance (m) between the yellow sticky traps in the source (arbour) and the trap plants	75	4	6	13	6	4	3	5
Total catches of adults in the source	329	168	168	168	168	108	13	65
Total catches of adults in the trap plant	10 (2.9%)	4 (2.3%)	0	1 (0.6%)	0	6 (5.3%)	1 (7.1%)	4 (5.8%)

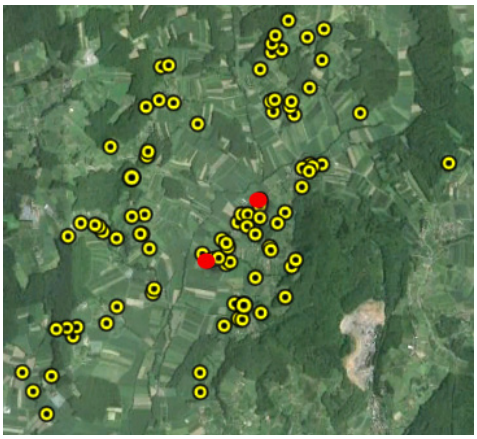

Figure 4. Survey of grape arbours within a focus zone in southeast Styria. Investigated arbours are marked as yellow circles, infected arbours are marked by red dots.

"Flavescence dorée" infection survey of grape arbours

The main variety of the investigated grape arbours was Isabella. In two out of 41 examined grape arbours the FD phytoplasma was detected (Figure 4). None of the arbours displayed grapevine yellows symptoms. Furthermore, the FD positive arbours were also only latent infected. Consideration shall be given to the possible risk as infection source, when these data are expressed in relative terms, which means 5% FD infected arbours in the fourth year after the first FD detection.

Conclusions

First *S. titanus* adults were recorded on the yellow sticky traps at the end of July with most individuals present in the middle of August. No catches were recorded when the weather was cold and wet. More *S. titanus* were recorded in the old (30 years) vineyard compared to the young vineyard (4 years), this can be explained by the vector preference for dense canopies and old wood for oviposition. However it can also be attributed to the spatial proximity of the old vineyard to the grape arbour where a higher numbers of *S. titanus* were recorded in the rows, close to the arbour than in the center and the far sides. It was assumed that the arbour acted as source for the migrating *S. titanus* adults. A further indication for this assumption may be the fact that in the rows close to the arbour all captured *S. titanus* adults were present on the yellow sticky trap side facing towards the arbour.
Spread dynamics of *S. titanus* was investigated in only few studies (Lessio and Alma, 2004). Lessio *et al.* (2007) found no significant differences in the captures of *S. titanus* adults on traps placed at the border and inside a vineyard and that this leafhopper is unlikely to perform considerable flights from American grapevine to close vineyards. In the study by Lessio and Alma (2004) hardly no catches were recorded in traps 24 m away from the vineyard. However, other authors report a distinct border effect with a decreasing gradient of *S. titanus* adult captures observed from contiguous untreated grapevines (Pavan *et al.*, 2012).

Results obtained from this research showed that more *S. titanus* were recorded in traps placed at the borders of the vineyard and at the edge close to the grape arbour than inside. These findings are in accordance with Maixner *et al.* (1993) who observed that adult *S. titanus* migrated into the peripheral parts of commercial vineyards at the end of the season. Based on the results of the migration experiments it is concluded that *S. titanus* adults spread from the grape arbour to the vineyard over a distance of at least 75 m and that an average 3.9% of a given *S. titanus* population migrated from an arbour with a high population density. Those individuals probably spread to the nearest attractive host plants available to lay eggs and form new populations in the following year. Obviously *S. titanus* adults were more attracted by the dense canopies of old grapevines than by the small groups of cultivar Chardonnay trap plants. The proximity of the old vineyard to the arbour had more influence on the number of *S. titanus* catches than the different cultivars in this study.

It is concluded that grapevine arbours may be an important factor in the epidemiology of FD if they are present in very high number in an area. In the municipality of Tieschen for example 505 arbours and hedges are registered on an area of 18.17 km^2 (2012). In such a case the disease might spread rapidly even when the vector occurs in only a small number of the grape arbours. Grape arbours pose another risk because *Vitis riparia* can be infected with FD but usually does not show symptoms (Maixner *et al.*, 1993).

From the results of this study it can be concluded that grape arbours act as a reservoir for *S. titanus* from which they spread to nearby vineyards and other arbours. Therefore grape arbours are an important factor with regard to epidemiology of FD and should be considered in the management strategy.

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Scaphoideus titanus and ''flavescence dorée" disease in Portugal

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Abstract

The grapevine "flavescence dorée" phytoplasma is included in the list of quarantine pathogens of the European Union and in the list A2 of EPPO. Official control and eradication measures are enforced. Following its recent detection in Portugal, additional emergency measures were established (Portuguese legislation 976/2008, 1 September). The program's procedures, which can be carried out into a national plan regarding the compulsory control of FD, include both the control of *S. titanus* and the removal of infected plants in infection *foci*. The control of *S. titanus*, which has been imposed to grapevine nurseries, is mandatory for all vineyards in the region, and is based on one or more seasonal spraying procedures, depending on epidemic risk. Removal of FD infected grapevines has been done by uprooting all symptomatic plants.

Key words: *Scaphoideus titanus*, ''flavescence dorée", disease, Portugal.

Introduction

"Flavescence dorée" (FD) is a grapevine disease that affects several production areas in Europe, namely in Portugal (Sousa *et al.*, 2003), it is associated with the presence of a phytoplasma belonging to 16SrV group and transmitted also in Portugal vineyards by the leafhopper *Scaphoideus titanus* Ball (Cicadellidae) (Quartau *et al.*, 2001; Sousa *et al.*, 2009).

From 2006 there was an increase in the presence of *S. titanus* in the northern regions of Portugal and in 2008 the insect has been detected for the first time in the central region, showing an increase of dispersion in the first two years. During 2011 it was however observed a containment in its spreading that can be attributed to the implementation of mandatory law measures. In 2010, its presence was detected for the first time on the island of Madeira. On the other hand the disease it is still restricted to north region of Portugal (Figure 1).

Despite the phytosanitary measures taken by the Regional Agricultural Services coordinated by the National Plant Protection Authority, the disease is not yet under control, so it becomes necessary to strengthen the ongoing actions and define additional measures involving the grapevine and wine producers.

It was also pointed out that there were several constraints to prevent the spread of the insect, namely the technical and logistical difficulty that regional services have in the control of application of the recommended insecticide treatments. Not collective performing of those treatments and poor dissemination of information to individual growers as well the predominance of small vineyards do not help the disease eradication. Another major constraint concerns the limited number of approved insecticides in Portugal.

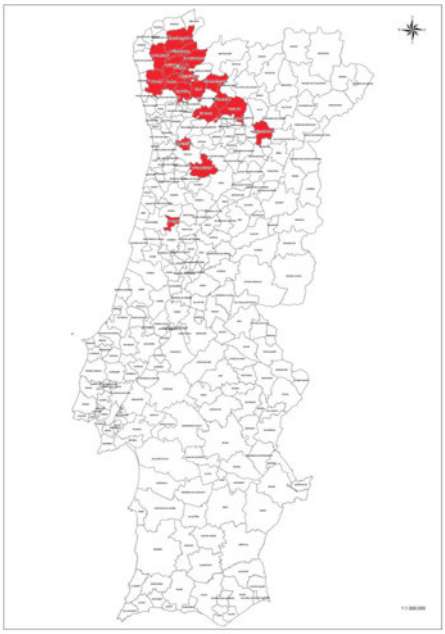

Figure 1. Presence of "flavescence dorée" in Portugal (2012).

PAN-FD's main objectives

Contain the disease within the wine-growing regions where it is declared and reducing to a minimum the impact on the grape production and wine industry in these regions is the major focus of this new research project.

The strategy of medium-term actions that can contribute to eradicating the disease, or, if it will be not possible, to reduce the presence of the disease to acceptable phytosanitary and economic levels was undertaken. Ensuring the health of grapevine propagation material and the confidence and sustainability of national nursery industry is also seeked. Some of these measures include grapevine sampling surveys, containment measures of insect vector and eradication measures.

The main factors that contribute to the technical difficulty in the implementation of legislative measures to eradicate the "flavescence dorée" disease in Portugal are summarized in Figure 2.

Several different kinds of new measures are included in this "FD-Plan of Action" that is an important tool to better assess the actual spreading of the disease and of the insect vector. To support the implementation of measures in the field, the adaptation of the "Information System of Vine and Wine - Sivv" managed by the "Portuguese Institute of Vine and Wine" and based on data obtained from plots geo-referenced will be

used (Figure 3). The adaptation of Sivv will enable the inclusion and processing of information already obtained and the data originate in future surveys of disease and insect vector presence, thus facilitating the definition of the "Priority Intervention Zones - ZIP" (Figure 4), for which particular phytosanitary measures are applied.
It is expected to include in Sivv the information already compiled of official surveys as well as the identification of vineyards in organic production, the fields of mother plants of rootstocks and varieties as well nurseries. Any other disease existing in the plots will also be registered.
The involvement and responsibility of various public and private bodies identified in the FD-Plan of Action when applying the various actions envisaged are critical to its application, not forgetting the key role of grapevine growers, winemakers, nurserymens. Several other aspects are included as the implementing measures defined for the vineyard organic farming, as well as the abandoned vineyards, reinforcement of training activities, information and awareness to farmers, official and private inspections.

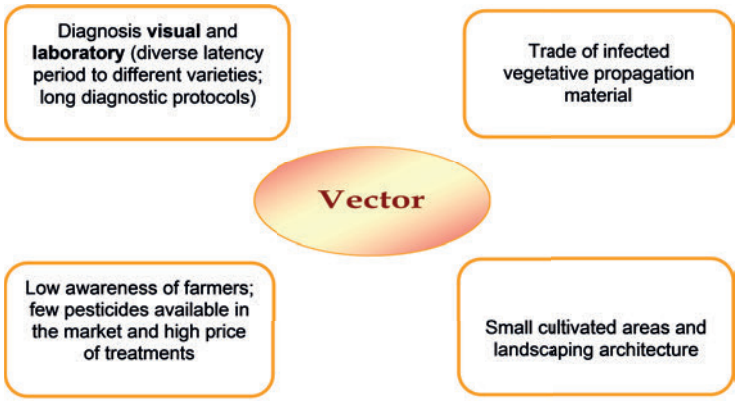

Figure 2. Factors that determined FD disease progression in Portugal.

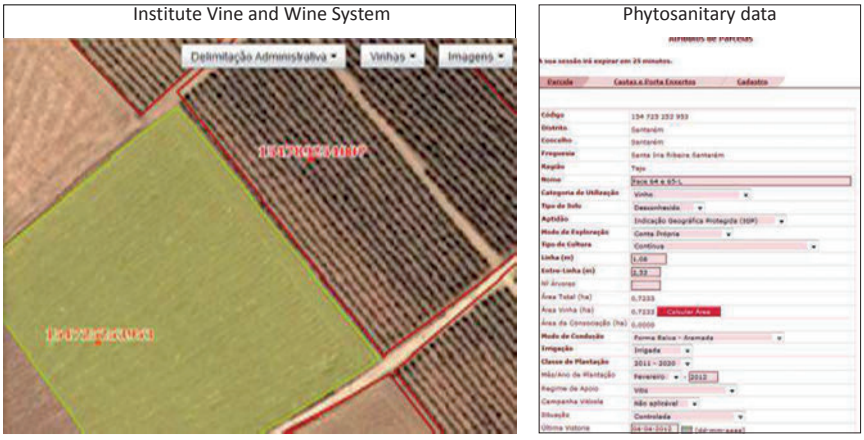

Figure 3. Example of the database of Sivv in which a column with phytosanitary data will be added.

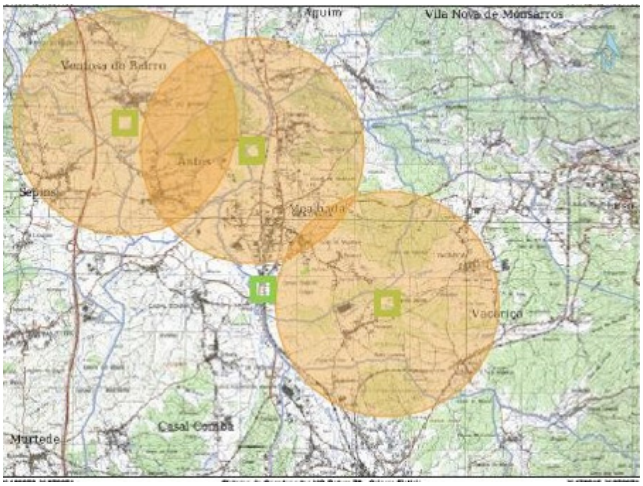

Figure 4. Example of SIvv utilization to define the 'ZIP' beginning from the localization of FD infection *foci*.

The definition of new measures based in FD-PAN will provide an effective control of the disease. The utilization of ZIPs together with the "National Service Avisos" will permit to apply only the number of chemical treatments required to control the *S. titanus* and the spread of the disease.

Preventive measures, such as the use of healthy certified propagation material and the respect of quarantine measures, are the most important approaches to reduce the risk of epidemic spreading of FD phytoplasma in areas where the disease is still not present. The control of the local vector populations and the elimination of infected plants are good agricultural practices that reduce the risk of contamination. Despite these measures, *S. titanus* is progressing along the Portuguese coast as FD disease. At the end of 2012 several stakeholders (government, researchers, farmers, nurserymen's representatives and wine organizations) have joined in order to prepare a National Action Plan to control the FD disease in the country called by PAN-FD (PAN-FD DGAV 2013), setting up new intervention measures for strengthening the existing ones.

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Current knowledge about recovery from phytoplasma diseases

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Abstract

Recovery is a spontaneous process that leads diseased plants back to the original asymptomatic condition. It occurs in perennial plants infected by different pathogens, and phytoplasmas in particular, however the precise causes of recovery are still not known. Biotic or abiotic factors are involved, but also agronomical management may play a role. Recovery can be permanent or transient; in the case of permanent recovery plants can also show resistance or tolerance to disease. In the literature examples are known of diseased plants that can recover from symptoms but not from the pathogen (e.g. apricot from European stone fruit yellows, ESFY), and plants that can recover from both symptoms and the agent (e.g. grapes from grapevine yellows, GY). It has been shown that an acquired tolerance to ESFY is transmissible to the progeny by grafting. Recovered plants can represent an opportunity in the management of plant diseases in areas where diseases are endemic. They can be used as a source of resistant or tolerant material to replace rogued plants. Morphological, physiological and genetic changes have been studied in the recent past in recovered plants. On the basis of this knowledge, recovery can be considered as a signal of inducible resistance.

Key words: phytoplasma diseases, tolerance, resistance, ESFY, GY.

Introduction

Since immunity to pathogens is not a common condition for many cultivated plant-species, disease is an expected event in plants exposed to natural inoculation pressure. In fact, plants - as well as animals - are normally exposed to a range of biotic and abiotic stresses. Animals have evolved various immune system mechanisms to combat pathogens. For animals, recovery is generally retained as a common phenomenon, which occurs after infection and disease. However, this situation has no correspondence in diseased plants, in which recovery is not a predictable event.

Nevertheless, in recent years recovery has become a phenomenon better understood and considered effective also for diseased plants. According to the most recent discoveries it is appropriate to say that plants, like animals, can recover and recovery is described as the ability to spontaneously return to a non-symptomatic state (Caudwell, 1961; Kunze, 1976). The real recovery is when the phenomenon is permanent and not transient. In particular, the current opinion regarding phytoplasma diseases is that real recovery is when symptoms have been absent for at least three consecutive years (Maixner *et al*., 2011).

Recovery from phytoplasma diseases is well known in perennial crops. The most relevant examples are: apple recovered from apple proliferation (AP) (Kunze, 1976; Seemüller *et al*., 1984; Osler *et al*., 2000; Carraro *et al*., 2004; Musetti *et al*., 2004),

pear from pear decline (PD) (Giunchedi *et al*., 1995), stone fruits from European stone fruit yellows (ESFY) (Morvan *et al*., 1986; Castelain *et al*., 1997; Osler *et al*., 2000; 2014; Kison and Seemüller, 2001; Musetti *et al*., 2005; Poggi Pollini *et al*., 2012), and grapevine from yellows (GY), "flavescence dorée" (FD) (Caudwell, 1961; 1966; Osler *et al*., 2000; 2002; Musetti *et al*., 2007), and "bois noir" (BN) (Osler *et al*., 1993; 2000; Garau *et al*., 2008; Romanazzi *et al*., 2007; Angelini *et al*., 2010; Maixner *et al*., 2011; Ermacora *et al*., 2011). This intriguing phenomenon has been also reported for virus diseases (Smith, 1999; Conti and Faoro, 2012), and in such cases the involvement of RNA silencing mechanisms has been hypothesized (Jovel *et al*., 2007).

Recovery and phytoplasma diseases

Several examples are reported below about recovery from phytoplasma diseases of grapevine and economically important fruit trees in Europe.

Grapevines and FD. The first observed and described case of recovery from phytoplasma diseases was a French work on grapevine recovery from FD (Caudwell, 1961). Later on, studies on the recovery from FD were also reported outside France (Osler *et al*., 2000; 2002; Carraro *et al*., 2009). The phenomenon is influenced by both scion and rootstock. A number of worldwide known grapevine varieties, like Chardonnay, Prosecco, Pinot, and Merlot, quite frequently recover from FD (Caudwell *et al*., 1987; Bianco *et al*., 2002; Osler *et al*., 2002; Morone *et al*., 2007) with an annual mean rate of about 20% (Osler *et al*., 2012). In contrast, the cultivar Perera does not recover, in all the conditions studied so far (Pavan *et al*., 1997). The Prosecco district in Veneto Region represented an impressive case where recovery was so efficient that it solved an FD epidemic: three million symptomatic grapevines recovered within four years from the first symptoms (Osler *et al*., 2002). Recently, it has been demonstrated that grapevines recover from both symptoms and the agent (Morone *et al*., 2007). Recovered plants can become infected and symptomatic again, but with a lower percentage when compared with grapevines that were never infected, indicating that recovery is linked to a form of inducible resistance (Ryals *et al*., 1996; Durrant and Dong, 2004; Vallad and Goodman, 2004; Musetti, 2010). Moreover, it has been demonstrated that uprooting can increase the annual recovery rate up to 70% (Osler *et al*., 2002; Carraro *et al*., 2008).

Grapevines and BN. The general behaviour of grapevine recovery from BN (Osler *et al*., 1993; Mutton *et al*., 2002; Bellomo *et al*., 2007; Maixner *et al*., 2011) is similar to the one of FD (Caudwell *et al*., 1987). In particular, recovery can be strongly influenced by pruning: in the case of pollarding, annual recovery rates can be increased up to 65-80% (Reggiani *et al*., 2008; Carraro *et al*., 2008; Riedle-Bauer *et al*., 2010; Romanazzi *et al*., 2008; Bianco *et al*., 2002; Garau *et al*., 2007; Bellomo *et al*., 2007). Moreover, recovery seems to be influenced by rootstock typology: its annual rate is considerably lower on rootstock 420A (Romanazzi *et al*. 2008; Ermacora *et al*., 2011). Similar to FD-infected grapevines, uprooting or partial uprooting can increase the recovery percentage (Osler *et al*., 1993; 2002; Romanazzi and Murolo, 2008). Recently, it has also been demonstrated

that the application of resistance inducers can promote the recovery of BN-infected grapevines with no adverse effects on the plants (Romanazzi *et al*., 2009; 2013).

Stone fruits and ESFY. Most of the work on recovery from ESFY has been carried out on apricots; the first experiments were reported in France by Morvan *et al*. (1986). The authors demonstrated that apricot plants are able to recover from severe symptoms, even though the proportions can be low. Interestingly, the recovered trees were shown to harbour avirulent or hypo-virulent strains of the pathogen (Morvan *et al*., 1986; 1991; Castelain *et al*., 1997). This suggests that the hypo-virulent strains might protect the plants from subsequent challenges by severe strains. In fact, plants grafted by using buds from recovered trees were shown to be resistant to the disease (Morvan *et al*., 1986).

Similar experiments were carried out in Italy confirming that symptomatic apricot plants do recover completely from symptoms, even if the percentage is low (Osler *et al*., 2000). The cause of recovery was recently interpreted as the result of an induced tolerance (Osler *et al*., 2014) instead of a cross-protection process (Morvan *et al*., 1986; Marcone *et al*., 2010). It was also confirmed that plants obtained from recovered mothers continued to exhibit tolerance, and that the recovery tendency is transmissible from recovered mothers to progeny by grafting. As proposed by Morvan and colleagues in 1986, it is of primary interest that a "transmissible factor" seems to exist in plants propagated from recovered mothers. As for grapevines, the recovery capacity in apricots is also highly influenced by the rootstock (Osler *et al*., 2014). Concerning other stone fruit species, Japanese plums are also reported to recover from ESFY. The cultivar Shiro recovers very efficiently but Ozark Premier quite rarely. European plum varieties are strongly tolerant to ESFY and therefore it is not possible to verify the recovery from symptoms (Carraro *et al*., 1998; Osler *et al*., 2000).

Apple and AP. Apples can recover from AP (Kunze, 1976; Seemüller *et al*., 1984; Osler *et al*., 2000; Carraro *et al*., 2004), but in the most specialised fruit growing areas rouging of symptomatic plants and insecticide vector-control are preferred instead of stimulating a recovery. Also, some scab resistant cultivars like Florina can recover from AP (Loi *et al*., 1995) with an annual rate of about 20% (Osler *et al*., 2012). In recent observations in northeastern Italy, apple cultivars grafted on EM 106 may recover efficiently and stably from AP. Recovery occurs from both the symptoms and the agent in the crown, but not in the roots where phytoplasmas survive (Seemüller *et al*., 1984). Plants that recover can eventually become symptomatic again, but to a minor extent (4.1 fold less) in comparison to newly infected plants (Osler *et al*., 2000). This confirms the hypothesis that recovery can be linked to a type of induced resistance. However, in the case of apple it is not yet clear whether this kind of inducible resistance is transmissible by grafting.

Direct primary causes of recovery in phytoplasma diseased plants

Recovery is a phenomenon that can be promoted and influenced by different factors and agronomic practices (Kunze, 1976). Although important physiological and genetic bases of recovery have been discovered, very little is known about its origin.

The hypothesized causes of recovery include: i) interaction of pathogenic strains with non-pathogenic microbial community; ii) host plant reaction to the pathogen; iii) environmental conditions and agronomical practices.
Concerning point i), one common hypothesis is based on the active presence in the plant of hypovirulent strains of the pathogen (Morvan *et al*., 1986; 1991; Castellain *et al*., 1997; Musetti *et al*., 2005; Loi *et al*., 2008; Ermacora *et al*., 2010). Avirulent strains have been proposed as responsible for cross-protection reactions that protect plants from attacks by virulent pathogens (Kison and Seemüller, 2001; Marcone *et al.*, 2010b). So far, a great genetic variability has been described for '*Candidatus* Phytoplasma prunorum' and '*Ca*. P. mali' (Martini *et al*., 2010; Danet *et al*., 2011), and a substantial effort has been made to differentiate hypo and hyper-virulent strains (Seemüller *et al.*, 2011). The involvement of endophytic fungal and bacterial microorganisms is also reported as a possible cause of recovery (Musetti *et al*., 2007; Martini *et al*., 2009; Bulgari *et al*., 2011; Bianco *et al*., 2012).
About point ii), a plant-mediated process is reported to govern inducible resistances/tolerances (Musetti *et al*., 2013; Osler *et al*., 2014). The factors involved in such plant responses (Durrant and Dong, 2004; Ahmad and Eveillard, 2011) are also assumed to be the possible causes of recovery (Musetti *et al*., 2013; Osler *et al*., 2014).
Regarding point iii), pollarding and strong pruning are reported to increase the probability of recovery (Reggiani *et al*., 2008; Carraro *et al*., 2008; Riedle-Bauer *et al*. 2010). Moreover, recovery was proved to be inducible by transplanting grapevines infected by FD or BN. For example, Chardonnay cultivar grapevines showing severe symptoms of yellows, were transplanted in April and six months later recovered at a rate of 66% (Osler *et al*., 1993). These results were confirmed in different regions of Italy (Zucchetto, 1998; Carraro *et al*., 2008). This intriguing reproducible recovery is possibly linked to an inducible resistance promoted by wounding/stress, which has already been proposed for fungus diseases (Francia *et al*., 2007). Further research is needed to confirm this fascinating and promising theory. As mentioned previously, also the application of resistance inducers is an agronomical practice for inducing recovery in BN-infected grapevines (Romanazzi *et al*., 2009; 2013).

Morphological, physiological and genetic changes in recovered plants

The physiological basis of recovery is not completely known, however, during recent years studies have been carried out with the aim of gaining new insights into the phenomenon. Cytochemical analyses have revealed that recovery is accompanied by biochemical changes in the phloem. It has been demonstrated that recovered plants are able to accumulate H_2O_2 in the sieve elements, this is a stable reactive oxygen species whose antimicrobial and signalling roles are well known. It has also been observed in these plants that the activities of two main enzymatic H_2O_2 scavengers (catalase and ascorbate peroxidases) are selectively and stably down regulated (Musetti *et al*., 2004; 2005; 2007). The variation of the sieve-element oxidative status leads to modifications of

phloem protein (P-protein) conformation and phloem occlusion expression patterns. An anomalous accumulation of callose and protein associated with the up-regulation of callose synthase- and P-protein- coding genes has been observed in the sieve elements of recovered apple trees (Musetti, 2010). This is supporting the hypothesis that recovered plants are able to develop resistance mechanisms dependent on Ca^{2+} signal activity (Musetti, 2010; Musetti *et al*., 2013). Recently, the activation of the jasmonate (JA)-related defence mechanism via JA gene up-regulation has also been demonstrated in apple trees recovered from apple proliferation disease (Patui *et al.*, 2013; Musetti *et al*., 2013).

Regarding grapevine and BN interaction, the possible involvement of callose, as well as callose synthase, sugar transporters, and cell wall invertase genes, has been investigated during infection and after recovery (Santi *et al*., 2013). Ultrastructural investigation of leaf tissue showed that callose accumulated in the sieve elements of diseased grapevine. Moreover, two genes encoding for callose synthase were up regulated in the infected leaves. Expression analysis showed that also sucrose transport and cleavage were severely affected by BN phytoplasma presence, that induced the establishment of a carbohydrate sink in the source leaf, and this is in agreement also with that obligate biotroph pathogens acquire most of their nutrients from the host plant. Interestingly, whereas the transcript level of sucrose synthase in recovered plants was similar to the one in healthy plants, sucrose transporters as well as cell wall invertase were expressed to a greater degree in recovered leaves than in the healthy ones. These results indicate that recovered plants seem to acquire structural and molecular changes leading to increases in sucrose transport ability and defence signalling (Santi *et al*., 2013).

Applications of recovery in phytoplasma-disease control and concluding remarks

The conventional attempts to control the phytoplasma epidemic diseases (rouging of the sources of inoculum, use of healthy plants to replace the infected ones and vector control) do not work in practice (Osler *et al*., 2003). Moreover, at present no resistant or tolerant cultivars are available against phytoplasma diseases. In such a situation, recovery may represent a chance to control phytoplasma diseases (Osler *et al*., 2012).

There are two ways to approach recovery: direct or indirect recovery, depending on the annual recovery rate and stability. For FD- and BN-infected grapevines, in which recovery is generally efficient, a direct recovery could be tried. This strategic plan was largely confirmed to be effective in the area of Prosecco (Italy) and it is more cost-effective than uprooting (Pavan *et al*., 2012). The spontaneously recovered grapevines revealed that they carry also a persistent resistance to FD. Similar observations were reported by Ermacora *et al*. (2011) regarding cultivar Chardonnay grapevines infected by BN in Friuli Venezia Giulia (Italy).

In contrast, apricot rarely recovers from ESFY. In this case direct recovery is not a feasible solution. However, a recent study (Osler *et al*., 2014) showed that stably recovered plants can become tolerant to ESFY and this character is transmissible to the

progeny by grafting. As a consequence, it is possible to obtain plants hosting inducible tolerance that can be grown in infected areas. The main advantage of this solution is the possibility of obtaining plant genotypes of interest, which have been selected in the field for tolerance/resistance to ESFY. The results gained from apricot and ESFY indicate that recovery is expected to be directly or indirectly effective in apple and AP, pear and PD and also grapevine and FD or BN. In the case of grapevine yellows, since recovery is also from the agent and not only from symptoms, the realistic hope is to obtain plants that are resistant to their phytoplasmas. In this way, the well-known limits of using tolerant plants that carry the pathogen in infected areas would be avoided.
Even if the understanding of recovery is much wider than in the past, it still remains an underestimated way to control plant phytoplasma diseases. The results obtained particularly with FD, BN and ESFY encourage future researches.

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Chapter 6

Genomes and their expression in COST FA0807


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From genomics to the characterization of virulence mechanisms of phytoplasmas

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Abstract

Phytoplasmas are insect-transmitted bacterial pathogens that induce dramatic changes in plant development, including proliferation of stems (witches' brooms) and reversion of flowers into leafy structures. Phytoplasma-infected plants are often more attractive and susceptible to insect vectors. It was hypothesized that phytoplasma secrete virulence proteins that act as effectors to interfere with plant pathways involved in the regulation of developmental processes and defense to insects: a summary of main achievement in this field is reported.

Key words: phytoplasmas, effectors, secreted proteins, gene regulation, disease.

Introduction

Phytoplasmas are insect-transmitted bacterial pathogens that induce dramatic changes in plant development, including proliferation of stems and reversion of flowers into leafy structures, often resulting in sterile plants that only serve to help phytoplasmas reproduce and propagate (zombie plants). As well, phytoplasma-infected plants are often more attractive and susceptible to insect vectors. It was hypothesized that phytoplasma secrete virulence proteins that act as effectors to interfere with plant pathways involved in the regulation of developmental processes and defense to insects (Bai *et al*., 2006; Hogenhout and Loria, 2008; Hogenhout *et al*., 2008; Sugio *et al*., 2011). In plants, phytoplasmas are restricted to the phloem and the virulence effectors may unload from the phloem and migrate to different plant tissues (Hogenhout and Loria, 2008; Sugio *et al*., 2011).

To test this hypothesis, the genome of aster yellows phytoplasma strain witches' broom (AY-WB) was sequenced to completion (Bai *et al*., 2006). Candidate virulence effectors were identified by finding genes potentially encoding secreted proteins that have a cleavable signal peptide at the N-terminus and absence of transmembrane domains in the mature protein (part of the protein without signal peptide) (Bai *et al*., 2009). To assess the functions of the AY-WB candidate virulence effectors in plants, the corresponding AY-WB genes were stably expressed in *Arabidopsis* plants under control of the *Cauliflower mosaic virus* (CaMV) *35S* promoter and transgenic plants were examined for changes in development, such as increased stem production, changes in leaf shape and structure, altered flower development and increased susceptibility of plants to insect vectors (MacLean *et al*., 2011; Sugio *et al*., 2011a). A yeast two-hybrid screen was conducted to find targets proteins in the plant host (Sugio *et al*., 2011a).

Phytoplasmas and virulence effectors

The approximately 700 kb AY-WB genome consists of one chromosome and four plasmids with about 700 predicted genes (Bai *et al*., 2006). Of these genes, 56 were predicted to encode secreted proteins; these were named secreted AY-WB protein (SAPs) and are candidate virulence effectors (Bai *et al*., 2009). SAP11 and three other SAPs contain a nuclear localization signal (NLS); SAP11 was detected in nuclei of cells beyond the phloem in AY-WB-infected plants suggesting that it is secreted by AY-WB and unloads from the phloem (Bai *et al*., 2009). Transgenic *Arabidopsis* plants for about 50 SAPs were generated and those for SAP05, SAP11 and SAP54 showed changes in development (MacLean *et al*., 2011; Sugio *et al*., 2011a). The *35S::SAP05* transgenic plants had changed leaf shapes, increased production of aerial rosettes and altered flowering time, the *35S::SAP11* plants had changed leaf shapes and increased stem production and the *35S:SAP54* plants produced leafy flowers with indeterminate growth (MacLean *et al*., 2011; Sugio *et al*., 2011a). A yeast two-hybrid screen of SAP11 against an *Arabidopsis* seedling library identified CINCINNATA (CIN)-related TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTORS (TCP) class II transcription factors as targets of SAP11 (Sugio *et al*., 2011a). SAP11 destabilizes these transcription factors resulting in changes in leaf shape, increased production of stems, downregulation of jasmonic acid production and greater susceptibility of plants to AY-WB leafhopper vectors (Sugio *et al*., 2011a). Thus, AY-WB phytoplasma produces virulence effectors that are secreted and unload from the phloem to perturb specific plant transcription factors leading to changes in plant development and increased susceptibility of plants to insect vectors that together will help phytoplasmas reproduce and propagate.

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Complete genomes and deduced metabolism of acholeplasmas in comparison to members of '*Candidatus* Phytoplasma'

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Abstract

Acholeplasmataceae comprises the genera *Acholeplasma* and the phytopathogenic bacteria of the provisory taxon '*Candidatus* Phytoplasma'. Several *Acholeplasma* species were reported as commensals of animals, insects and plants. As data from genomes provide valuable information for many subsequent studies, eight complete genome sequences from *Acholeplasmataceae* were determined comprising strains from '*Candidatus* P. asteris', '*Ca.* P. australiense', '*Ca.* P. mali', *A. laidlawii*, *A. palmae* and *A. brassicae*. Complete phytoplasma chromosomes vary in size between 602-960 kb (G + C content: 21-28%) and are thereby separated from acholeplasma chromosomes with a size of 1,497-1,878 kb (G + C content: 29-36%). Genome stability and rare duplication events in the acholeplasmas also distinguish them from phytoplasmas. Corresponding to genome sizes, the number of protein encoding genes ranges from 481-1,100 for and 1,380-1,690, respectively. The acholeplasma genomes encode a rich repertoire of transport systems, a F_0F_1 ATP synthase, components of the NADH oxidoreductase complex and an extensive carbohydrate metabolism. In contrast, phytoplasmas are separated by encoding effector proteins, in particular membrane proteins, a reduced number of transport systems and an adapted comparable poorly equipped metabolism characterizing the evolution of these parasites in their hosts.

Key words: *Acholeplasmataceae*, genome, transport, metabolism, pathogen-host interaction.

Introduction

Genome analysis, in particular the determination of complete genomes, is of high impact for phytoplasma research. Genome derived data were used in a wide range of studies including diagnosis, pathogen-host interactions, metabolism and phylogenetic

analyses. The provisory taxon '*Candidatus* Phytoplasma' (IRPCM, 2004) and the genus *Acholeplasma* are composing the family Acholeplasmataceae, which is part of the class *Mollicutes*. Initial phylogenetic analysis based on 16S rRNA suggested an early split of the Acholeplasmataceae from other *Mollicutes* (Weisburg *et al*., 1989). In contrast to many other *Mollicutes*, Acholeplasmataceae follow the usage of the bacterial genetic code using UGA as a termination signal in general. Despite the close relationship of the monophyletic taxon '*Ca*. Phytoplasma' (Lim and Sears, 1992; Toth *et al*., 1994) with the genus *Acholeplasma*, differences in lifestyle are obvious. Phytoplasmas are characterized as obligate parasites of the plant phloem and insects, and are associated as insect-borne phytopathogenic bacteria to several hundred of plant diseases including many important crops (Strauss, 2009). Acholeplasmas are generally characterized as saprophytes and not as primary pathogens. *Acholeplasma* species were identified in many different habitats. Some species, for instance *A. laidlawii,* are ubiquitary distributed indicating a manifold metabolic repertoire encoded by these commensals. Plant-derived isolates such as *A. brassicae* and *A. palmae* from *Brassica oleracea* var. *italica* and *Cocos nucifera* (Tully *et al*., 1994), respectively, highlight their origin by the scientific name, whereas other species were identified on plant surfaces (e.g. *A. laidlawii*, *A. axanthum* and *A. oculi*) (Brown, 2011). It is remarkable that species of this genus were also identified in insects and that some species even multiply in phytoplasma vectors (Eden-Green and Markham, 1987). However, interaction of acholeplasmas and insects remains unclear. Phytoplasmas depend on their natural spread by insect vectors. Therefore, phytoplasmas have to deal with different habitats such as the sieve tubes in the plant host and different environments in the insect vector (Oshima *et al*., 2011). The crossing of the gut barrier, colonization of the haemolymph and salivary glands of the phloem-sucking insect vector are essential steps in the infection cycle of phytoplasmas (Sugio *et al*., 2011; 2011a). In consequence, phytoplasmas have to deal with changes in the composition of available metabolic substrates but they are always surrounded as endocellular parasites by a nutrient-rich environment (Kube *et al*., 2012). The availability of essential metabolites is thus not a limiting factor compared to the situation present for many free-living bacteria.

Genome condensation is evident in the evolution of both genera. Acholeplasma genome sizes are ranging from about 1.2-2.1 Mb with a G + C content of 27-38% whereas the smaller phytoplasma chromosomes range from 0.5 to 1.4 Mb and show a decreased G + C content of 21-33% (Carle *et al*., 1995; Marcone *et al*., 1999; Neimark and Kirkpatrick, 1993). Beside these general genome benchmarks and some shared evolutionary processes, genomes of these genera are supposed to differ with respect to pathogenicity and host interaction at least.

General features of the *Acholeplasmataceae* genomes

The chromosome of '*Ca*. P. asteris' strain OY-M (onion yellows-mild) (subgroup 16SrI-B) was the first fully sequenced phytoplasma genome (Oshima *et al*., 2004).

Genome analysis highlighted the reductive evolution taking part in phytoplasmas encoding fewer metabolic functions compared to many other mycoplasmas such as the absent pentose phosphate cycle and ATP-synthase subunits. Apart from the completely encoded, ATP-providing glycolysis, Oshima and colleagues highlighted the high number of incomplete pathways. Incomplete pathways and poor transporter environment were considered to be compensated by the access to host-derived metabolites. Furthermore, a remarkably high number of duplicated genes in the chromosome was observed and interpreted to be responsible for an enlargement of the chromosome size compared to the non-redundant gene content (Bai *et al*., 2006; Oshima, *et al*., 2004; Tran-Nguyen *et al*., 2008). The determination of the genome sequence of the closely-related aster yellows witches' broom phytoplasma strain AY-WB (subgroup 16SrI-A) showed the largely conserved sequence synteny of both strains (Bai *et al*., 2006). This analysis also allowed the identification of complex transposons (so-called potential mobile units, PMUs) with a strong impact on duplication of genomic regions. In combination with the findings on the exchange of DNA between the encoded plasmids of strain AY-WB and the on-going loss of genetic elements led to the hypothesis of genome instability and rapid evolution of phytoplasmas. This hypothesis was underlined by the suggestion of large phage-derived parts in the genomes (Wei *et al*., 2008). Instability of phytoplasma chromosomes is also characteristic for the two sequenced '*Ca*. P. australiense' strains (Tran-Nguyen *et al*., 2008; Andersen *et al*., 2013). Beside the previously described elements, retrotransposons were identified. In summary, duplication and integration events result in instability of the phytoplasma chromosomes (Kube *et al*., 2012). In addition, an increased number of repeats also forces rearrangements. However, these strains that belong to the major phylogenetic branch of phytoplasmas, share a general genetic core of features, involved in housekeeping functions but also in transport and metabolism. This image of phytoplasma genomes was modified by the analysis of the genome sequence of '*Ca*. P. mali' strain AT (Kube *et al*., 2008). This genome, belonging to a different major phylogenetic branch in the phytoplasmas, is organized in a linear chromosome with long terminal inverted repeats. Duplication and integration events are of limited impact for this genome resulting in a low number of rearrangements and higher stability. Thereby, this chromosome sequence indicated that a genome reduction without a high number of sequence repeats in phytoplasmas resulted in the low genome size of 602 kb and a low G + C content of 21% (Table 1).

In contrast to the phytoplasma genomes, which are reconstructed from metagenomic data, acholeplasma genome projects derived from a DNA template obtained from an axenic culture. The genome of *A. laidlawii* strain PG-8A was published in 2011 (Lazarev *et al*., 2011). In 2013, the full sequences of the circular chromosomes of *A. brassicae* strain O502 and *A. palmae* strain J233 were published (Kube *et al*., 2014). *A. laidlawii* encodes the smallest acholeplasma chromosome with 1.5 Mb while *A. brassicae* has a size of 1.9 Mb. The fully sequenced circular chromsomes encode 1,380-1,690 proteins. The lowest G + C content was identified in the chromosome of *A. palmae* with 29%.

Table 1. Genome features of the complete Acholeplasmataceae chromosomes.

	Size (bp)	G + C	Number of *rRNA* genes[1]	tRNAs	CDS[2]	Accession number
'*Ca*. P. asteris' strain OY-M	853,092	28%	6 (3; 3)	32	752	AP006628.2
'*Ca*. P. asteris' strain AY-WB	706,569	27%	6 (3; 3)	31	671	CP000061.1
'*Ca*. P. australiense' strain Rp-A	879,959	27%	6 (3; 3)	35	684	AM422018.1
'*Ca*. P. australiense' strain NZSb11	959,779	27%	6 (3; 3)	35	1100	CP002548.1
'*Ca*. P. mali' strain AT	601,943	21%	6 (3; 3)	32	481	CU469464.1
A. laidlawii strain PG-8A	1,496,992	32%	6 (3; 3)	34	1,380	CP000896.1
A. palmae strain J233	1,554,229	29%	6 (3; 3)	35	1,439	FO681347.1
A. brassicae strain O502	1,877,792	36%	14 , (4 + 3; 3 + 4)	45	1,690	FO681348.1

[1]Number of each unit are given in brackets
[2]CDS (Coding DNA Sequence) assignment is limited to protein coding genes

No plasmids were determined for these strains. All three chromosomes show a switch of the coding strand preference in accordance to a bidirectional replication and a stable cumulative GC-skew in contrast to the phytoplasma genomes (Figure 1).
The most regular GC-skew for the phytoplasmas can be calculated from the condensed genome of '*Ca*. P. mali', which also harbours a low number of PMUs (Kube *et al*., 2008). Gene duplication is strongly limited in acholeplasmas, in contrast to the phytoplasmas. Exceptions include the unusual duplication of the rRNA operon in *A. brassicae* resulting in four *rRNA* gene clusters, two of them encoding *16S-23S-5S-5S-rRNA* genes. Duplicated genes include the single-stranded DNA binding protein (ssb), which is encoded at least two times in phytoplasmas and two times in acholeplasmas (Kube *et al*., 2014). Phylogenetic analysis of the deduced ssb protein highlighted a different phylogenetic origin of this gene copies with one shared by *Acholeplasma* and '*Ca*. Phytoplasma' (ssb1). Only the copy showing same phylogenetic origin (ssb1), could be confirmed as expressed in *A. palmae*, *A. laidlawii*, *A. brassicae* and '*Ca*. P. mali' strain AT all carrying two *ssb* genes. Besides having this independent shared evolutionary event in common, the number of shared protein coding genes of both genera is below 300 (Lazarev *et al*., 2011; Kube *et al*., 2014). Shared gene content is frequently organized in units on the chromosomes indicating a common origin of both taxa, but independent evolution (Kube *et al*., 2014). An indicator for this genomic feature is the low number

of paralogs in the acholeplasmas and the high number of paralogs in the phytoplamas after an early split.
Acholeplasmas posses a richer genetic repertoire of genes encoding proteins involved in replication and repair, which is shared in parts for the phytoplasmas. Proteins involved in cell division such as FtsZ and SepF are encoded on the acholeplasma genomes but absent in the completely sequenced phytoplasma genomes.

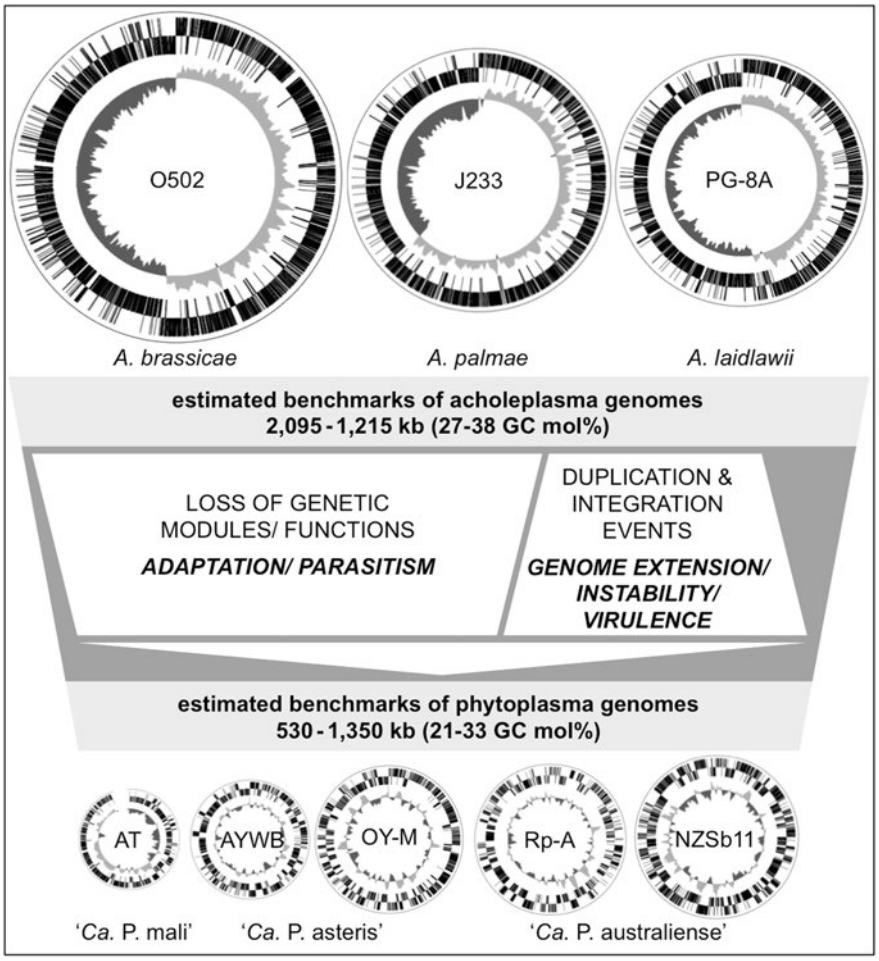

Figure 1. Evolutionary driving forces in Acholeplasmataceae highlight the reductive evolution and genome extension of phytoplasmas compared to the acholeplasmas. Genome circles of the complete chromosome sequence are drawn to scale and illustrate the switch in strand preference (black bars indicate protein coding genes on forward and reverse strand) and the regular cumulative GC skews (grey graph) in the *Acholeplasma* spp. versus the phytoplasma strains.

Secretion and transport

Phytoplasmas interact with the host tissue via membrane proteins (Fabre *et al*., 2011; Galetto *et al*., 2011; Neriya *et al*., 2011; Boonrod *et al*., 2012) and the secretion of effector proteins manipulating plant host and vector (Hoshi *et al*., 2009; MacLean *et al*., 2011; Sugio *et al*., 2011; 2011a). The effector protein SAP11 was identified in association with PMU-like element supporting an impact of PMUs in host adaptation (Toruno *et al*., 2010). However, genes encoding phytoplasma effectors were not identified in the genomes of acholeplasmas (Kube *et al*., 2014). Both taxa encode *Sec*-dependent secretion pathway, which is completed by SecG in the acholeplasmas. Phytoplasmas may encode, in addition a type IVB secretion system as indicated by the presence of the IcmE/DotG core component (Bai *et al*., 2006; Tran-Nguyen *et al*., 2008; Saccardo *et al*., 2012), which was not identified in the acholeplasmas (Kube *et al*., 2014).

Establishment of intracellular parasitism of the phytoplasmas resulted in a loss of transport systems to a minimal core compared to the acholeplasmas (Kube *et al*., 2014). This core comprises ABC-transporters for sugars, spermidine/putrescine, methionine, oligopeptides, amino acids, manganese/zinc and cobalt (Kube *et al*., 2012). This repertoire also comprises a P-type ATPase, multidrug efflux pumps, a mechanosensitive channel protein and a symporter for malate/citrate and sodium. Particularities of the phytoplasmas are the uptake of cofactors such as manganese and substrates such as malate. The limited amount of transport systems might be interpreted with respect to the specialised metabolism of phytoplasmas.

Carbohydrate metabolism

Genome analysis of '*Ca.* P. asteris' strain OY-M revealed the Embden-Meyerhof-Parnas pathway (glycolysis) as the central energy-yielding pathway in phytoplasmas (Oshima *et al*., 2004). The glycolysis is also part of a complex carbohydrate metabolism of acholeplasmas in addition to the pentose phosphate cycle and the metabolism of rhamnose, galacturonate, mannonate, galactose, glycogen and glycerophospholipids (Lazarev *et al*., 2011; Kube *et al*., 2014). Both taxa lack a conventional phosphotransferase system (Oshima *et al*., 2011; Kube *et al*., 2014). Acholeplasmas encode core components, which might be completed by not yet in detail characterized enzymes. There are no hints for such starting points in phytoplasmas. It was speculated that the ancestors of phytoplasmas encoded a sucrose phosphorylase enabling the usage of the major sugar component of the phloem sap. Truncated genes were identified in '*Ca*. P. asteris' strain OY-M and '*Ca*. P. solani' strains supporting such a scenario (Oshima *et al*., 2011; Mitrovic *et al*., 2013). The option of an uncharacterized phosphorylase or the uptake of a phosphorylated hexose from the environment was also discussed, taking into account the intracellular lifestyle of phytoplasmas (Kube *et al*., 2012).

As already pointed out, glycolysis was suggested to be the major energy-yielding pathway in phytoplasmas with respect to the poorly encoded carbohydrate metabolism.

Coding of this pathway was identified in the closely related '*Ca*. P. asteris' strains OY-M and AY-WB, the strains of '*Ca.* P. australiense' and the three acholeplasma genomes. In contrast, the genome of '*Ca*. P. mali' lacks the energy-yielding part of glycolysis (Kube *et al*., 2008). An additional ATP-providing pathway was suggested, taking into account particularities of the core genome of phytoplasmas (Kube *et al*., 2008; 2012). All sequenced phytoplasmas encode a symporter allowing the uptake of malate/citrate. Malate is suggested to be oxidatively decarboxylated by the malate dehydrogenase to pyruvate in phytoplasmas. The encoded pyruvate dehydrogenase multienzyme complex performs the oxidative decarboxylation to acetyl-CoA, which is converted by the phosphotransacetylase to acetyl phosphate, latter is converted by the acetate kinase to acetate and ATP. The key enzymes of this pathway are the symporter enabling the uptake of malate or a similar substrate from the environment and the malate dehydrogenase. Both genes were not identified in the acholeplasmas (Kube *et al*., 2014), highlighting a significant difference in the metabolism of both taxa.

The available genome information on Acholeplasmataceae and their encoded metabolism is still limited. Several genome studies are in progress and will provide new insights and a more complete picture of this bacterial family.

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In vitro expression of phytoplasma immunodominant membrane proteins

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Abstract

Strategies for *in vitro* expression in *Escherichia coli* of Imp immunodominant membrane proteins from '*Candidatus* Phytoplasma asteris' chrysanthemum yellows strain (CY) and "flavescence dorée" (FD) phytoplasmas are reported. The entire *imp* genes of CY and FD were cloned, sequenced and aligned with homologous sequences of phytoplasmas of different phylogenetic groups. Partial CY and FD *imp* gene sequences devoid of the transmembrane domains were cloned into the pQE32 and pRSetC vectors, but following IPTG induction, both fusion Imp proteins were expressed at low levels. To increase protein expression of the partial FD Imp, codon usage of the gene and accessibility of translation initiation were modified according to that of *E. coli*. The new construct showed 80% nucleotide similarity with the original FD *imp* gene, identical amino acid sequence and improved expression level. A second protein (about 28 kDa) was eluted under the same conditions as the putative partial His-tagged FD Imp, but the latter protein was the only specifically recognized by an anti-histidine antibody in Western blot. These results are the first indication that, at least for FD, even in the absence of the troublesome transmembrane domain, an optimized codon usage is useful to achieve *in vitro* expression of immunodominant protein.

Key words: CY, FD, Imp, pRSetC, pQE32.

Introduction

Three types of non homologous, highly abundant and immunodominant membrane proteins (IDPs) have been identified in phytoplasmas: Amp, IdpA, and Imp (Kakizawa *et al*., 2006). The three proteins are not all present in each phytoplasma genome: Imp is found in sweet potato witches' broom (Yu *et al*., 1998), apple proliferation (Berg *et al.*, 1999), European stone fruit yellows (Morton *et al*., 2003), pear decline (Morton *et al.*, 2003), peach yellow leaf roll (Morton *et al*., 2003) and lime witches' broom (Siampour *et al*., 2013) phytoplasmas; IdpA is present in western X-disease (WX) and poinsettia witches' broom phytoplasmas (Blomquist *et al*., 2001; Neriya *et al*., 2011), and Amp is found in aster yellows (Barbara *et al*., 2002), clover phyllody (Barbara *et al*., 2002), and onion yellows (Kakizawa *et al*., 2004) phytoplasmas. Moreover, in several cases, genes encoding two types of these proteins are present in phytoplasma genomes and one of them may be predominantly expressed (Kakizawa *et al*., 2009; Neriya *et al*., 2011).

Evolution under strong positive selection has been demonstrated for Amp and Imp (Kakizawa *et al*., 2006a; 2009; Siampour *et al*., 2013) supporting a possible role of both proteins in determining phytoplasma fitness. Recently, *in vitro* studies have supported the existence of interactions between phytoplasma IDPs and host proteins. In the case of two strains of the '*Candidatus* Phytoplasma asteris', specific *in vitro* interactions have been demonstrated between phytoplasma Amp and several leafhopper vector proteins (Suzuki *et al*., 2006; Galetto *et al*., 2011). Phytoplasma IDPs also interact with plant proteins, such as the case of '*Ca*. P. mali' Imp and plant actin (Boonrod *et al*., 2012). The expression of phytoplasma membrane genes in heterologous systems is a prerequisite for successive studies. Here we report on strategies for the *in vitro* expression of Imp from '*Ca*. P. asteris' chrysanthemum yellows strain and "flavescence dorée" phytoplasmas.

Materials and methods

CY and FD *imp* gene identification and cloning into expression vectors

Primer pairs to amplify *imp* gene of CY (CYimp) and FD (FDimp), and to directionally clone them into the expression vectors pRSetC (Invitrogen) and pQE32 (Qiagen) are listed in Table 1. These primers were designed with Primer3 software (http://primer3.sourceforge.net/) based on *dna*D and *pyr*G sequences of onion yellows phytoplasma (AB469007, OY) for CYimp, and on most conserved regions of the ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) alignment of *dna*D and *pyr*G sequences of different phytoplasmas obtained from Kakizawa and co-workers (2009) for FDimp. According to restriction analyses of obtained CYimp and FDimp sequences (Nebcutter: http://tools.neb.com/NEBcutter2/, and DNAMAN 4.02, Lynnon BioSoft), *Bam*HI (5' end) and *Eco*RI (3' end) were selected for the directional cloning into pRSetC and *Bam*HI (5' end) and *Hind*III (3' end) for pQE32. All PCRs were conducted in a standard master mix with 2 ng template DNA, 200 μM dNTPs, 2 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Polymed) in a S1000™ Thermal Cycler (BioRad). Amplicons (5 μl) were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized under UV light. PCR products were purified by GeneCleanTurbo kit (MP Biomedicals, LLC), ligated into pGEMT vector (Promega), transformed into *E. coli* DH5α competent cells (Invitrogen), and screened by PCR with universal vector primers M13F and M13R, following a routine protocol. Plasmid DNA was extracted from recombinant colonies with the FastPlasmid Mini Kit (5 PRIME) before sequencing (Bio-Fab Research s.r.l.).

Synthetic FD *imp* gene construction

A synthetic gene, including ribosome binding site, methionin starting codon, N-terminus His tag and partial FDImp sequence devoid of transmembrane domain, was designed starting from the obtained sequence of FDimp construct in pRSetC vector. The synthetic gene was synthesized (Life Technologies) and cloned into pMA-T

plasmid (pMATModFDImp), and the plasmid was transformed into DH5α *E. coli* competent cells.

Table 1. List of primers used in this study. CY: chrysanthemum yellows phytoplasma, FD: "flavescence dorée" phytoplasma. Restriction sites and added nucleotides are indicated in bold and italics, respectively.

Primer name	Sequence 5´-3´	Target gene	Source organism	Position from imp ATG
Imp 1045 Fw	ACCCGATGAAAAAGCAGACA	*dna*D	OY-W	-181
Imp 1893 Rv	AGCCATCCTTGGTTTATTGC	*pyr*G	OY-W	660
CYImpFwBamHI	*AAAA***GGATCC***TT*AACTGGCTTTCTCCTAAAAAAG	*imp*	CYP	105
CYImpFwBamHI2	*AAAA***GGATCC***AA*AAACAAGCAGCTCAAAATTTG	*imp*	CYP	135
CYImpRvEcoRI	*AAAAAA***GAATTC**TTATTTGGTTAAATTATTAAC	*imp*	CYP	-447
CYImpRvHindIII	*AAAAAA***AAGCTT**TTATTTGGTTAAATTATTAAC	*imp*	CYP	-447
DNAd Fw 1	ARACWATWGAANWDWTAGAA	*dna*D	OY-W, PWBK, TWB, RYD, AP	234
PyrG Rv 1	GTWCAATTTCATYCWGARTT	*pyr*G	OY-W, PWBK, TWB, RYD, AP	547
FDImpFwBamHI	*AAAA***GGATCC**CAACAATTACTGAGAAAG	*imp*	FDP	114
FDImpFwBamHI2	*AAAA***GGATCC***CT*TTAACAACATTTAAAACAG	*imp*	FDP	168
FDImpRVEcoRI	*AAAAAA***GAATTC**TTATTTTTCTTTAATTTCTTC	*imp*	FDP	-462
FDImpRvHindIII	*TGGC***AAGCTT**TTATTTTTCTTTAATTTCTTC	*imp*	FDP	-462

In vitro expression and Western blot

The recombinant plasmids (pRSetC_CYImp_1 and _16, pQE32_CYImp_9 and _23; pRSetC_FDImp_7 and _23, pQE32_FDImp_2 and _3, pMATModFDImp _1 and _2) were purified with Fast Plasmid Minikit (5 PRIME) and transformed into competent *E. coli* BL21 (DE3) pLysS (pRSetC constructs and pMA-T construct) or M15 (pQE32 constructs) with a standard protocol. pRSetC and pMA-T recombinant colonies were grown on LB agar plates with ampicillin (50 μg/ml, Sigma) and chloramphenicol (35 μg/ml, Sigma), and pQE32 constructs were grown with ampicillin (50 μg/ml) and kanamycin (25 μg/ml, Sigma). Single colonies were incubated over night into 1.5 ml LB (pQE32 constructs) or SOB (pRSetC and pMA-T constructs) media containing appropriate antibiotics at 27 or 37°C. Fresh dilutions (1:20, final 20 ml) of the over night cultures in the presence or in absence of the correct antibiotic combination were further incubated (at 27 or 37°C) to OD600 of 0.4-0.6 when 1 mM IPTG (Sigma) was added to start induction of expression. Samples (1 ml) were taken just before IPTG induction (T0) and at different times post inductions. Cells were harvested, lysed, and purified according to the NiNTA Spin Columns (Qiagen) protocol under denaturing conditions to isolate any tagged protein, irrespective of their solubility in different cellular compartements. For SDS-PAGE analyses, 5 μL of 3x SDS-PAGE sample buffer

(Laemmli, 1970) was added to each sample aliquot (10 µl) and heat denatured (5 min); proteins were loaded on 15% SDS-PAGE gel, electrophoresed and stained with colloidal Coomassie blue following a standard protocol. Protein molecular weight markers (Unstained Broad Range Protein Standards, Bio-Rad) were also loaded. Western blot experiments were conducted as previously described (Galetto *et al*., 2008) with A416 polyclonal antiserum (reacting against the histidine tag of the fusion antigen) and a secondary goat anti rabbit antibody HRP-conjugated (Sigma).

Results and discussion

Molecular cloning of CY and FD *imp* genes

Primer pair Imp 1045 Fw/Imp 1893 Rv amplified a CY-specific fragment (849 bp) that was cloned and sequenced (double coverage). It included 55 C-terminal bases of *dna*D gene, 126 bp intergenic region, the entire *imp* gene (468 bp), 79 bp intergenic region, and 120 C-terminal bases of *pyr*G gene of CY (KJ402358). CY *imp* gene showed about 98% sequence identity with OY and PvWB corresponding sequences (onion yellows, AB469007 and porcelain vine witches' broom AB469011, respectively), while sequence identities with other phytoplasmas were lower than 63% (Table 2). CY *imp* gene coded a 155 amino acid protein, with a predicted MW of 17.8 kDa, and pI 9.47. A signal peptide (C, Y, S, and D values of 0.394, 0.531, 0.993, and 0.672) and a predicted signal anchor (probability: 0.958) were predicted as well as one transmembrane domain (amino acid 13 to 32) with a prediction of outside location for the remaining portion of the protein (amino acids 33 to 155, Figures 1A, B, C). CY Imp protein showed more than 94% sequence homology with corresponding proteins of OY and PvWB phytoplasmas ('*Ca*. P. asteris' strains) and less than 17.5% with Imp protein of the remaining phytoplasma strains (Table 2).

Table 2. Similarity table for *imp* gene DNA (lower, left part) and amino acid (upper, right part) of chrysanthemum yellows (CY) and "flavescence dorée" phytoplasmas (FD), '*Ca.* P. asteris' strains (OY-W, PvWB), '*Ca*. P. mali' (AP), and other phytoplasmas belonging to 16SrIII (PWBK, TWB) and 16SrXI (RYD).

	CY	FD	OY-W	PvWB	PWBK	TWB	AP	RYD
CY		18.06	94.81	94.12	10.97	17.42	14.19	12.26
FD	58.55		18.83	20.26	24.2	18.75	19.38	25.0
OY-W	98.02	58.15		94.77	10.39	14.94	15.58	12.99
PvWB	98.05	56.49	96.48		11.76	16.34	15.03	11.11
PWBK	58.97	65.61	62.11	60.17		22.29	20.38	21.02
TWB	60.04	63.35	60.57	61.69	62.03		13.94	12.35
AP	62.39	63.77	64.54	61.69	59.92	58.03		14.81
RYD	56.41	60.66	55.07	59.74	61.39	60.94	59.71	

FD-specific amplicons of the expected sizes, obtained with degenerate primer pair DNAd Fw1/PyrG Rv1 (predicted amplicon size: 1027 bp), were cloned; sequencing (double coverage) of three of the obtained plasmids confirmed that they were identical. The cloned sequence (1,038 bp) included 254 C-terminal bases of *dna*D gene, 124 bp intergenic region, the entire *imp* gene (483 bp), 75 bp intergenic region, and 102 C-terminal bases of *pyr*G gene of FD (KJ402359). FD *imp* gene showed less than 66% sequence identity with the corresponding gene of all other phytoplasma tested (Table 2) and coded a 160 amino acid protein, with a predicted MW of 18.1 kDa, and pI 9.60. A predicted signal anchor (Signal anchor probability: 0.996) was detected together with one transmembrane domain (amino acid positions 21 to 43) with a prediction of outside location for the remaining portion of the protein (amino acids 44 to 160, Figures 1D, E, F). FD Imp protein showed about 25% sequence homology with corresponding proteins of Korean potato witches' broom phytoplasma and '*Ca*. P. oryzae' (RYD strain), respectively and less than 20% with Imp protein of the remaining phytoplasma strains (Table 2).

Full length CY *imp* gene was cloned with non degenerate primers designed on the closely related OY operon, while degenerated primers on most conserved regions from the alignment of different phytoplasma species were required to obtain *imp* gene of FD. Indeed, sequence similarity at the gene level showed that FD imp was poorly conserved among other phytoplasmas, especially compared with homologous genes of other '*Ca*. P asteris' strains, and it clustered with group III phytoplasmas (16SrIII) according to Kakizawa and co-workers (2009). On the contrary, CY imp was confirmed to be closely related to that of '*Ca*. P asteris' group and showed low identity with *imp* genes of 16SrXI group members. Similarity of Imp proteins within the '*Ca*. P. asteris' was higher than 94%, while it decreased to less than 20% with the homologous amino acid sequences of other '*Ca*. Phytoplasma' species, thus confirming the high variability of this protein among phytoplasmas and the evolutionary pressure to which it is subjected (Kakizawa *et al*., 2009; Siampour *et al*., 2013), a clue of its role in interactions with hosts, as already shown for Amp of OY (Kakizawa *et al*., 2006a).

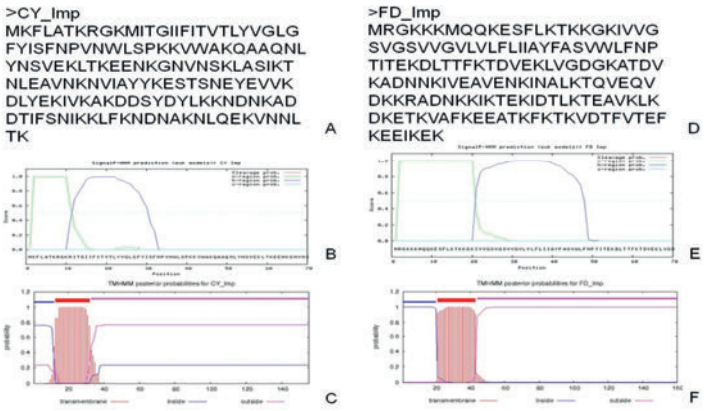

Figure 1. Chrysanthemum yellows (CY) and "flavescence dorée" phytoplasmas (FD) Imp protein sequences (A, D), results of SignalP prediction (B, E), and of transmembrane domain prediction by TMHMM software (C, F).

Figure 2. DNA (Panel A) amino acid (Panel B) alignments of pRSetCFDImp and pMATModFDImp.

Heterologous expression of CY and FD Imp proteins

Primers were designed to clone partial CY and FD *imp* gene sequences devoid of the transmembrane domains and few of the following amino acids into the pQE32 and pRSetC vectors. Sequencing of two transformed plasmids for each target showed identical sequences and *in silico* translation indicated expected MWs of 17.9 and 14.2 kDa (156 and 123 amino acids, for pRSetC_CYImp and pQE32_CYImp, respectively) and 16.7 and 13.4 kDa (146 and 117 amino acids, pRSetC_FDImp and pQE32_FDImp, respectively) for the four partial fusion Imp proteins. The pRSetC encoded fusion proteins contained the 120 (CYP) and 110 (FDP) C-terminal Imp aminoacids of each phytoplasma, while the pQE32 constructs expressed the C-terminal 111 amino acids for CYP Imp and 103 amino acids for FDP Imp.

The transformed pRSetC and pQE32 plasmids with partial CY and FD *imp* genes were transformed into BL21 and M15 *E. coli* host cell strains, respectively. Following IPTG induction, both fusion Imp proteins were expressed, although at low levels, irrespective of the expression vector used (data not shown).

To increase protein expression of the partial FD Imp in *E. coli*, the codon usage of the gene and accessibility of translation initiation were modified and the pMATModFDImp construct was produced. Alignment of both gene and protein sequences of pRSetC_FDImp and pMATModFDImp showed that, despite the low (80%) identity at the nucleotide level, the predicted primary sequences of the FD Imp partial fusion proteins were identical (Figure 2). Following IPTG induction of the pMATModFDImp transformed clone, a partial FD Imp fusion protein (MW: 16.7 kDa, 146 amino acids) was produced 1 h after IPTG induction and the expression increased up to 4 h post induction. The partial FD fusion Imp was then purified by binding to NiNTA beads.

Gel electrophoresis of the different elution phases, showed that a second protein (about 28 kDa) was eluted under the same conditions as the putative partial FD fusion Imp (about 17 kDa) (Figure 3A), although only the latter protein reacted specifically with the anti histidine (His) antibody in Western blot, therefore confirming the expected presence of the His tag, while the 28 kDa protein did not (Figure 3B). *In vitro* expression of full length Imp from several phytoplasmas has been reported (Yu *et al*., 1998; Berg *et al*., 1999; Neriya *et al*., 2011) and, in some cases removal of the transmembrane domain from the coding sequence was the key to obtain enough fusion antigen for the successive production of Imp-specific antibodies (Kakizawa *et al*., 2009). It is known that LWB (lime witches' broom) and AlfWB (alfalfa witches' broom) (16SrII group) partial Imp proteins can be expressed to high concentration in *E. coli* (Siampour *et a*l., 2013), and these genes have low sequence identity/similarity with corresponding genes/proteins of CY and FD (this study). Moreover, expression of a recombinant phage library of the sweet potato witches' broom phytoplasma (SPWB, 16SrII group) indicated that SPWB *imp* gene expression was independent of the *E. coli* promoter (Yu *et al*., 1998). On the other hand, pMATModFDImp, which was designed to improve the codon usage and accessibility of translation initiation for expression in *E. coli*, but showed identical amino acid sequence as FD Imp, was expressed at a much higher level compared to the original FD *imp* gene present in pRSetCFDImp. Optimization of the codon usage for expression in *E. coli* seems a most promising strategy to improve yield of recalcitrant recombinant phytoplasma membrane proteins.

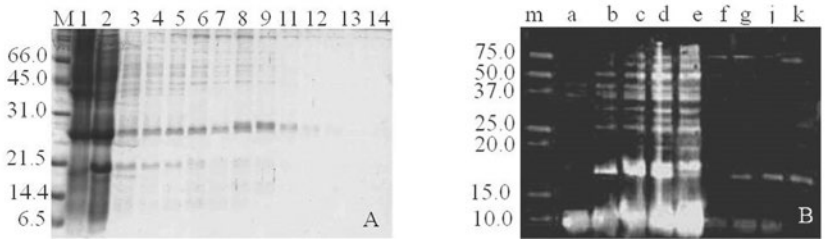

Figure 3. Coomassie stained SDS-PAGE (A) and Western blot with A416 antibody (B) of different phases of the NiNTA beads (Qiagen) purification of partial fusion "flavescence dorée" phytoplasmas (FD) Imp (16.7kDa) from pMATModFDImp transformed BL21 (DE3) pLysS cells at 4 h post IPTG induction. 1: lysate; 2: flow through; 3: washing; 4 to 14: serial elutions with different pH buffers. M: unstained SDS-PAGE standard Broad Range (BioRad). a: before IPTG induction control; b to e: total proteins at 1, 2, 4 and 24 h after IPTG induction; f to k: purified protein by NiNTA resin and eluted with low pH buffer; m: prestained SDS-PAGE standard Broad Range (BioRad).

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Identification and molecular features of suppressive strains of '*Candidatus* Phytoplasma mali' and their effect on disease development

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Abstract

To study antagonistic interactions of '*Candidatus* Phytoplasma mali' strains, *Catharanthus roseus* and *Nicotiana occidentalis* were inoculated with mild strains 1/93Vin and 1/93Tab respectively as suppressors and with two aggressive strains as challengers. Inoculation of the suppressors was carried out in either the cross protection modus prior to grafting of the challengers or by co-inoculating suppressors and challengers. In the cross protection trials with *C. roseus*, suppressor 1/93Vin was present in all root and randomly collected stem samples over the entire observation period. In contrast, the challengers were never detected in such stem samples and rarely in the roots. Following simultaneous inoculation, the suppressor colonized all stem and root regions whereas detection of challenger AT steadily decreased. However, this strain remained detectable in up to 13% and 27% of stem and root samples, respectively. The cross protection trials with *N. occidentalis* yielded results similar to that with *C. roseus*. Examination of differently infected apple trees indicated that suppression of severe strains also occurs in apple. Phylogenetic analysis using a variable fragment of AAA+ ATPase gene AP460 of '*Ca.* P. mali' revealed that 1/93Vin and 1/93Tab together with several mild strains from apple, cluster distantly from obviously nonsuppressive strains that were predominantly highly virulent.

Key words: '*Candidatus* Phytoplasma mali', apple proliferation, suppression, disease development, phylogenetic clustering.

Introduction

The wall-less bacterium '*Candidatus* Phytoplasma mali' is the agent of apple proliferation (AP), one of the most important phytoplasma diseases in Europe. This psyllid-transmitted pathogen induces a range of specific and nonspecific symptoms, associated with reduced vigor and size and value of the crop. Previous work showed that symptom expression depends on the virulence of the infecting strain(s). Long-term observations showed that virulence of '*Ca.* P. mali' accessions ranged from being avirulent or mildly virulent to strongly virulent (Seemüller and Schneider, 2007). However, symptom expression is often subject to fluctuation. In particular, the formation of the most characteristic witches' broom symptom is often typical for newly diseased trees and can be observed in the first few years of disease. Then, trees may recover and show no or only mild symptoms for shorter or longer periods, after which severe symptoms may reappear

(Carraro *et al*., 2004; Seemüller *et al*., 1984; 2010). From recent work, there is evidence that such fluctuations can be attributed to multiple infections of apple trees by distinct strains of '*Ca.* P. mali'. Such infections are common and are usually composed of strains that greatly differ in virulence. Obviously due to antagonistic interactions between mild and severe strains, shifts in the population may occur that result in increased or, more often, reduced virulence of the infecting phytoplasmas (Seemüller *et al*., 2010; 2011).
The multiple-strain accession 1/93 is an example for such variations. Trees infected by this source were largely symptomless for many years. Following transmission to periwinkle (*Catharanthus roseus*) and *Nicotiana occidentalis*, two distinctly different strains, referred to as 1/93Vin and 1/93Tab, respectively, were identified by single-strand conformation polymorphism (SSCP) and sequence analysis of a variable fragment of AAA+ *hflB* protease gene ATP464. The same strains were identified in the largely symptomless donor tree of accession 1/93, jointly or separately. Each of the two strains identified in apple, and the experimental hosts showed distinct SSCP profiles and clustered with other mild strains in phylogenetic analysis. However, following graft transmission of accession 1/93 from a field-grown tree to a clonal rootstock and growing the recipient trees in an unheated greenhouse, one of several inoculated trees developed witches' broom symptoms. Using SSCP and sequence typing, a third strain was identified in the diseased tree that clustered with severe strains of other accessions in phylogenetic analysis. Further investigations revealed that this strain also occurred in low numbers in the roots of the symptomless donor tree, together with the two predominant mild strains (Seemüller *et al*., 2010; 2011).
In this contribution, further indication of antagonistic interaction between AP phytoplasma strains, mainly on the suppressive action of the largely avirulent periwinkle- and tobacco-maintained strains 1/93Vin and 1/93Tab on aggressive strains is reported. The occurrence of suppressive actions of AP phytoplasma strains in infected apple trees was inferred by linking SSCP and nucleotide sequence data of the AAA+ ATPase gene AP460 with the disease history of the trees and the symptoms shown at sampling. The ATPase AP460 gene sequence proved to be suitable to distinguish established and supposed suppressive and nonsuppressive strains. In addition, the nucleotide and deduced amino acid sequences of this gene and those of hflB protease ATP464 enable distinction of largely avirulent and aggressive AP phytoplasma strains (Seemüller *et al*., 2011a; 2013).

Suppressive action in periwinkle

Six to eight month-old cutting-propagated periwinkle plants were graft-inoculated at three evenly distributed sites with the mild strain 1/93Vin used as suppressor and, at three other evenly distributed sites, with either aggressive strains AT or AP15 used as challengers. In the three trials carried out, inoculation of suppressor 1/93Vin was carried out 2.5 to 4 month prior to the inoculation of challengers ("cross protection

modus") or strains 1/93Vin and AT were co-inoculated at the time challenger AT was grafted to pre-inoculated plants. In each trial, three healthy controls and three singly inoculated plants were included. Following the second inoculation term, the plants were observed for symptom development over 33 to 43 month. The presence of suppressors and challengers was monitored employing multiplex real-time PCR with strain-specific TaqMan probes as described (Schneider *et al*., 2014). Approximately 600 samples from 45 plants were examined.

The symptoms of the inoculated control plants differed considerably. Suppressor 1/93Vin induced only mild symptoms. Four months postinoculation (p.i.), infected plants showed mild foliar yellowing, nearly normal-sized and only weakly undulated leaves, and flowers slightly smaller than normal. At termination of the trials, the plants did not differ from healthy controls. In contrast, strains AT and AP15 caused much more pronounced symptoms by inducing earlier and more severe yellowing symptoms, strongly undulated and distinctly smaller leaves, and much smaller flowers than 1/93Vin. Flowering of AT- and AP15-inoculated plants ceased 8 to 10 months p.i. At the end of the observation periods, all AT- and AP15-inoculated plants were either dead or declining.

Periwinkle plants pre-inoculated with strain 1/93Vin and challenged with strains AT or AP15 developed predominantly the mild symptoms of strain 1/93Vin alone. Only the growth developed from the challenger scions, and leaves of the grafted shoot below the grafting site showed the typical symptoms induced by strains AT and AP15. These symptoms persisted for about 10 months and then slowly disappeared. Sixteen months after of the secondary inoculation, no AT- or AP15-specific symptoms could be observed. Flowering of the cross protected plants remained until the end of observation periods as described for the plants inoculated with 1/93Vin alone. In addition, the overall symptomatology was similar. The symptoms of the plants co-inoculated with the suppressor and the challengers differed from that of cross-protected plants: foliar AT symptoms, mainly on the growth of the challenger scions persisted for up to two years. At the end of the observation periods, no differences between healthy controls, inoculated 1/93Vin controls, cross-protected and co-inoculated plants were observed. There was no decline of cross-protected or co-inoculated plants.

In the three trials carried out, all randomly collected shoot samples taken from pre-inoculated plants tested positive for suppressor 1/93Vin over the entire monitoring period, independently of the first sampling date ranging from 3.5 to 8 months after the second inoculation. In contrast, challenger strains AT and AP15 were never detected in shoot samples from such plants. Similarly, strain 1/93Vin was from the beginning of sampling detected in all root samples of cross-protected plants, whereas strain AT was not or rarely detected in root samples. The figures obtained following co-inoculation differed significantly from those of cross-protected plants. At the first sampling dates, detection frequency in stem samples was similar for suppressor and challenger. Then, detection rate of 1/93Vin steadily increased, reaching 100% 12 to 27 months p.i. of the

challenger. In contrast, detection of the challenger successively decreased. However, up to 13% of the samples remained strain AT-positive at the end of the trials. In the roots of co-inoculated plants, suppressor and challenger were at first detected in all samples. This was true for the suppressor until the end of the observation period whereas detection of the challengers decreased to 20-27%. An example of the results obtained in one of the trials is shown in Figure 1.

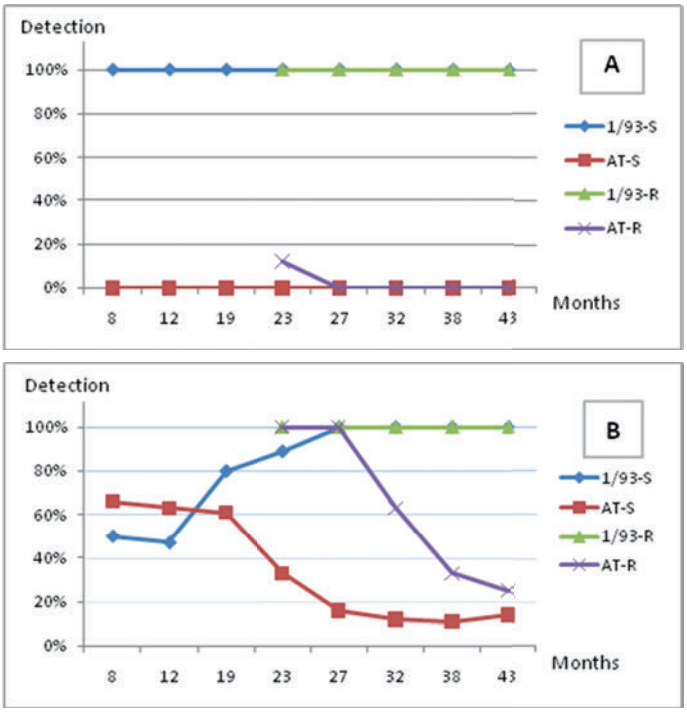

Figure 1. Detection of suppressive strain 1/93Vin and challenger strain AT of '*Ca*. P. mali' in shoots (S) and roots (R) of periwinkle following inoculation of suppressor and challenger in the cross protection modus (A) or simultaneously (B). Monitoring 8 to 43 months after inoculation of the challenger and co-inoculation.

Suppressive action in *Nicotiana occidentalis*

Two trials were carried out in which plants grown from seeds were inoculated in the cross protection modus. In each trial, seven eight-week-old plants were inoculated with suppresser 1/93Tab and 3 or 6 weeks later with challenger strain AT. Sampling started 4 or 6 weeks after challenging, and was repeated three times in the following three months. The presence of suppressor and challenger was monitored employing multiplex real-time PCR with strain-specific TaqMan probes as described (Schneider *et al*., 2014). Suppressor 1/93Tab, which proved to be largely avirulent in apple trees (Seemüller *et al*., 2013), induced distinct symptoms consisting of foliar yellowing associated with enlargement and browning of veins. With the development of foliar symptoms,

flower size decreased and flowering ceased 2 to 3 month p.i. The plants declined 4 to 5 months p.i. of the challenger. Symptomatology was similar on plants pre-inoculated with 1/93Tab and challenged with strain AT. However, plants inoculated with strain AT alone showed more pronounced symptoms, leading to decline 1 month earlier than plants cross-protected with 1/93Tab.

In both cross protection experiments phytoplasma detection was similar to that of the cross protection trials with periwinkle. In both *N. occidentalis* trials, suppressor 1/93Tab was detected in all randomly collected stem samples whereas challenger AT was never detected. In addition, the suppressor was detected in all root samples collected at the end of the experiment whereas the tests were negative for the challenger. In contrast, the challenger was detected in the new growth of the AT inoculated scion, and in lateral shoots below the AT grafting side. However, there were distinct differences between the two trials, due to the different incubation period of the suppressor of 3 versus 6 weeks prior to challenge inoculation. These differences are evident by a much higher detection rate of the suppressor, and a much lower detection rate of the challenger after the longer incubation period of suppressor. However, despite these differences, the suppressor was detected in all samples by the end of the experiments, whereas detection of the challenger decreased to zero in both trials.

Molecular features of suppression and evidence of suppression in apple

To identify molecular features related to suppression, SSCP analysis of a variable cloned fragment of gene AP460 of approximately 455 bp in length was used as described (Schneider *et al*., 2014). This approach revealed that suppressive strains 1/93Vin and 1/93Tab show similar profiles consisting in one group of bands. This profile is different from those of aggressive, periwinkle- and *N. occidentalis*-maintained challenger strains AT and AP15 that are composed of two groups of bands. Both types of profiles were identified in cloned PCR fragments of gene AP460 in root samples from multiple-strain accession 1/93 maintained in apple consisting of mild and severe strains (Figure 2).

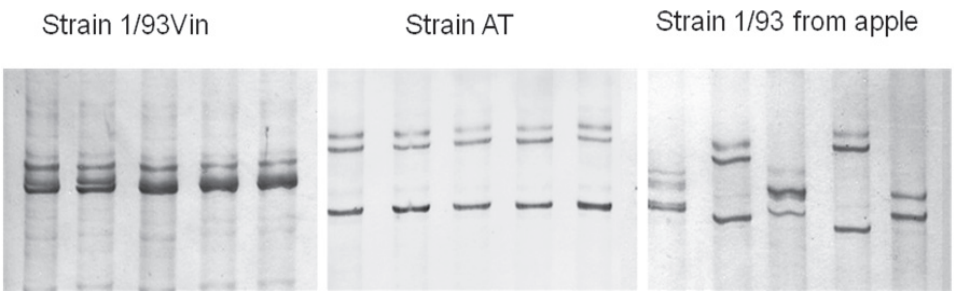

Figure 2. SSCP profiles of suppressive strain 1/93Vin and aggressive, nonsuppressive strain AT. Multiple-strain accession 1/93 from apple consisting of suppressive and nonsuppressive strains shows both types of profiles.

In order to relate the presence of these markers to the symptomatology of infected apple trees, 12 other trees with known disease history were examined. This analysis revealed a close correlation between the gene AP460 markers, and the symptoms shown at sampling. In accessions inducing severe symptoms, only cloned fragments showing the profile of nonsuppressive strains were identified, whereas in most symptomless trees only suppression-associated profiles were observed. Both types of fragments were detected in trees exhibiting moderate symptoms. An exception of these patterns was shown by the phytoplasma type present in a nonsymptomatic tree that exhibited the profile of severe strains. This observation seems to indicate that not all mild strains are suppressive, and that the suppression-associated profiles are not markers for virulence like sequences in other regions of gene AP460 and other AAA+ protein genes. It thus appears that both strain virulence and suppressive action of strains determine disease development.
Based on the SSCP profiles, gene AP460 inserts of recombinant plasmids were sequenced and the variable, suppression-relevant 319- to 322-bp 5' fragments used in phylogenetic analysis. Construction of the phylogram resulted in two homogenous major branches represented by fragments of suppressive strains 1/93Vin and 1/93Tab and by the putatively nonsuppressive strains AT and AP15, respectively. The suppression-associated sequences of mild strains including those of accession 1/93 from apple, periwinkle and *N. occidentalis*, and several other accessions from apple were identical or nearly identical and clustered in one major branch (Schneider *et al*., 2014).

Discussion

In previous work, we were able to distinguish and specifically amplify both mild and severe strains of the AP phytoplasma based on sequences of several AAA+ ATPase and *hflB* protein genes. Furthermore, we could show that multiple strain infections are common and that they usually consist of mild and severe strains. Under still poorly understood circumstances, shifts in the composition of the populations may occur. Because symptom expression depends on the prevailing strain(s), such changes determine disease appearance and severity, and may result in recovery or in reappearance of severe symptoms. As observed in this work with accession 1/93, avirulent and mild strains predominate in the roots of recovered trees whereas severe strains persist latently only in low numbers (Seemüller *et al*., 2010; 2011). Due to the observations on accession 1/93, a suppressive action of the mild strains was taken into consideration. The inoculation experiments with the experimental hosts periwinkle and *N. occidentalis* reported here confirmed this assumption. The antagonistic action of suppressors 1/93Vin and 1/93Tab was most pronounced in the trials with periwinkle and *N. occidentalis* carried out in the cross protection modus. In these experiments, the suppressor was identified in all randomly collected stems and in all root samples over the entire observation period. In contrast, the challengers were not detected in such stems and rarely in the roots. Following simultaneous inoculation of periwinkle, the suppressor successively colonized

all stem and root regions whereas detection of challenger AT decreased. However, this strain usually remained detectable in up to 13% of the stem samples and 27% of the root samples. This data indicate that strains 1/93Vin and 1/93Tab are powerful suppressors that either eliminates the challengers or reduces their population below the detection level and to a concentration that did not markedly affect the plants. These results with the experimental host periwinkle and *N. occidentalis* together with the data obtained with apple suggest that suppressive strains are involved in the widespread recovery phenomenon.

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