COST ACTION FA0807 FINAL MEETING

LISBON, PORTUGAL

Carcavelos

September 30th - October 1st 2013

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Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Abstract Book

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

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Background and objectives
All these countries located in the Northern part of the EU are believed to be not seriously affected by phytoplasmas, however, during the project a few reports have emerged on the presence of phytoplasmas in Norway, Finland and Lithuania.

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. Mixed infection of both phytoplasma and '*Ca*. L. solanacearum' also was detected in 20% of symptomatic plants.
Results of a survey confirmed the presence of both psyllid vector species of '*Ca*. P. 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 much 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. To establish 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 and were shown to be apple proliferation subtypes AT-1 (Lemmetty *et al*., 2013).

Lithuania
Studies on phytoplasma diseases in this country has at least a fifteen year history allowing identification of several phytoplasmas in diverse 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 phytoplasma group), new subgroup 16SrI-Q were detected. 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.
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 both 16SrI-C and a phytoplasma classified in subgroup 16SrIII-B. These findings extend the known geographical ranges of subgroup I-C and

subgroup III-B taxa. Phytoplasma strains belonging to subgroups 16SrIII-P and 16SrI-C were identified in leafhoppers collected from a meadow and an orchard. Phytoplasma strains belonging to subgroup 16SrI-C were found in *Euscelis incisus* and *Macrosteles sexnotatus*. Phytoplasma strains belonging to subgroup 16SrIII-P were detected in *E. incisus*. 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 sub-group containing phytoplasma strains that have to date been found only in Lithuania.

Norway

Apple production is an important disease in specific areas in both the western and the eastern parts of the country. AP is listed as a 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. There was not found any infected trees in the nursery production in any of the nurseries due to a strict use of healthy propagation material and good control of potential vector populations (Blystad *et al*., 2012).

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Phytoplasma diseases and vectors in United Kingdom, The Netherlands and Belgium

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Here we give an overview of the current status of phytoplasma diseases, their vectors and recent research efforts in Belgium, UK and The Netherlands.

Belgium

In 1981, apple proliferation (AP, associated with '*Candidatus* Phytoplasma mali', EPPO A2 list) was discovered for the first time in Belgium. Until recently symptomatic trees had sporadically been reported and found infected but they were subsequently destroyed. In 2009 two samples collected in a nursery in the south-west of Belgium tested positive by PCR. 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 '*Candidatus* Phytoplasma pyri' (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 (apple and pear) and revealed the presence of both quarantine organisms in root (AP) and leaf samples (PD). Psyllids were collected as well and identified. Following *Cacopsylla* sp. were found: *C. mali, C. melanoneura* and *C. picta* in apple, *C. melanoneura, C. peregrina, C. affinis* and *C. crataegi* in adjacent hawthorn hedges, *C. pyri* and *C. pyricola* in pear. Further research and official inspection of orchards and nurseries is currently on going and preliminary results will be presented.

The Netherlands

Both diseases are also present in the Netherlands - AP at low incidence, PD at moderate rate - as well as the vector species *C. melanoneura, C. pyri* and *C. pyricola*. Another phytoplasma was detected in gladiolus and hyacinth – '*Candidatus* Phytoplasma asteris' – as well as its vector *Macrosteles sexnotatus.*

United Kingdom

In the UK, phytoplasmas are detected only sporadically in minor crops. However imported poinsettias contain phytoplasma but this does not constitute a disease.

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

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Czech Republic (CR)

'*Candidiatus* Phytoplasma prunorum' is a long-standing problem in the CR. Survey was done in apricot, peach and cherry orchards. Different methods of DNA extraction and PCR detection were evaluated, including comparison of symptom variability after inoculation in different *Prunus* spp.. Occurrence of '*Candidatus* Phytoplasma pyri' (PD), '*Ca*. P. mali' (AP) and '*Ca.* P. asteris' (16SrI-B and 16SrI-C subgroups) was confirmed in orchards as well as wild growing trees. Extensive study of AP revealed prevalence of P-I 16S-23S profile, rpX-A subgroup, subtypes AP-15 and AT-2.

'*Ca.* P. solani' (stolbur) was detected in many plant species and its epidemics have been recorded in tomato, pepper and celery in South Moravia. Study of genetic variability of stolbur also by use of polymorphic gene *vmp* 1 in annual crops and wild plants revealed presence of 4 genetic variants. First mass occurrence of *Hyalesthes obsoletus* (vector of stolbur) in South Moravia since 1950´ was recorded in 2010. Alarming information were obtained about the infectivity of overwintering *Cacopsylla pruni* and *C. picta* that in which phytoplasmas were detected in up to 23% of the samples and *Anaceratagallia ribauti* with 50% of stolbur positive individuals. *Trifolium pratense* and *Chenopodium album* were found to be new hosts for stolbur phytoplasma that was also found to be associated with "bois noir" (BN) disease of grapevine.

Asparagus officinalis and *Plantago lanceolata* were found to be hosts for '*Ca.* P. asteris' (AY, 16SrI-B). Phytoplasmas belonging to 16SrI-C ribosomal subgroup were firstly reported in *Echinacea purpurea*, *Rhododendron hybridum*, *Ribes rubrum* and *Apium graveolens*; phytoplasma of subgroup 16SrIII-B in *E. purpurea* and *Chenopodium album* and phytoplasmas of 16SrVI-A subgroup in *Rhododendron hybridum*. The report about *Ulmus minor* affected by elm yellows (EY) in Moravia represents the northernmost occurrence of this phytoplasma on elm trees within Europe.

AP and PD were sporadically monitored 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. Examination of hemipterans in Czech vineyards revealed 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.

Hungary

National survey of grapevine yellows disease allow to determine the distribution of BN in all the grape-growing regions of Hungary. Molecular characterisation of '*Ca.* P. solani' proved the presence of different genotypes all resulted to belong to the tuf-type b. Most of the isolates clustered in tuf-type b, STAMP cluster II, while one isolate from tomato resulted to belong to STAMP cluster III.

Grapevine isolates from north-west Hungary clustered together in a monophyletic branch of the STAMP tuf genotype.
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 average of three-years 83.8% and 0.5% of the tubers and daughter plants, respectively resulted positive to the presence of 16SrXII-A subgroup phytoplasmas.
The 2010 survey conducted in the maize production areas for the occurrence of maize redness (MR) disease and of its known vector *Reptalus panzeri* allow to detect the presence of '*Ca*. P. solani' in MR symptomatic maize plants and *R. panzeri* at one locality.
Although *Scaphoideus titanus*, the vector of "flavescence dorée" (FD) was found in the southern counties since 2006 and its spreading to north is continuous, FD has not been detected in grapevine. 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 for the presence of 16SrV-C and -D phytoplasmas. Phytoplasmas belonging to group 16SrV were detected by nested PCR/RFLP analyses in alders (86%) and in clematis (71%). Characterisation of the *map* gene revealed that both strains have the same *map* gene sequence as the one reported in FD epidemic strains. This survey has also identified the presence of *Oncopsis alni* and *Dictyophara europaea* reported to be phytoplasma vectors.
'*Ca*. P. pyri' the causal agent of PD was detected in *C. pyri* and *C. pyricola* and '*Ca*. P. prunorum' from stone fruits as well collected in central region of Hungary.

Poland
Epidemiological study on stone fruit phytoplasmas in several regions in Poland revealed predominantly '*Ca*. P. prunorum' presence. Moreover, in single peach and sweet cherry trees '*Ca*. P. mali' and '*Ca*. P. pyri' were identified, respectively. Survey of the apple orchards and several home gardens and study on identification of phytoplasmas revealed the presence of AP and in several cases of AY (16SrI-B). Most of the AP isolates were identified as belonging to subgroup rpX-A, subtype AP-15. Only two of them were classified to rpX-B, subtype AT-1. AP was identified also in *C. melanoneura* and *C. picta*, while AY in a single leafhoppers batch.
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*. X-disease (16SrIII), was detected in *Picea abies* and *P. glauca* while '*Ca*. P. asteris' (16SrI) was identified in *Picea pungens* tree. The presence of phytoplasmas was found in about 42% of the tested plants which originated from the witches' brooms or showed pronounced shoot proliferation symptoms as well as in some hybrid trees without disease symptoms.
Pisum sativum with stunting, shoot proliferation and leaf chlorosis was found to be a new phytoplasma host infected by stolbur. AY (16SrI-B) was firstly identified in *Brassica* interspecific hybrids with green foliage and complete flower bud failure, in Brussels sprout with stunted growth, severe leaf malformation and flower bud failure, in Chinese cabbage with abnormal flowers, virescence and phyllody, in oilseed rape and European hazel and in *Fraxinus excelsior* showing ash yellows symptoms. Oriental lily hybrids were found to be infected by AY (16SrI-B) and AP. A phytoplasma closely related to members of 16SrI-C subgroup was detected in tomato plants in the western region of the country. The natural occurrence of EY, AY and X diseases in *Rubus* spp. with stunting, short and thin shoots was firstly reported in this Country.

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

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Background and objectives
Phytoplasmas are quarantine organisms causing several economically important diseases on different crops all over Europe. For this reason, updated knowledge on their distribution as well as on the presence of their vectors is very important for pest risk assessment and phytosanitary decisions. Within WG2 (Epidemiology and Vector Ecology) of the COST Action FA0807, a questionnaire about distribution of phytoplasma diseases and their putative vectors throughout European regions has been drafted and distributed among all members of the action. The objective of this survey was to compile current data about the spread of different phytoplasma diseases and the presence of their proven or putative vectors. This abstracts describes the particular situation in Germany and Switzerland.

Materials and methods
Phytoplasma detection in plants and insects has been carried out by molecular analysis via PCR as cited in references. 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 in Germany for *Hyalesthes obsoletus*, *Cacopsylla picta* and *C. pruni* as cited in references.

Results and discussion
GERMANY: the most important grapevine-related phytoplasma disease is “bois noir” associated with ‘*Candidatus* Phytoplasma solani’ (16SrXII-A-group) present in 11 of 13 viticultural regions of Germany (Darimont and Maixner, 2001). Two other locally spread diseases on grapevine are: the Palatinate grapevine yellows associated with phytoplasmas of the elm yellows group (16SrV) found in viticultural areas of Palatinate, Mosel and Franken (Maixner *et al*., 2000) and another grapevine yellows associated with a phytoplasma belonging to the aster yellows group (16SrI) occasionally found in Palatinate and Mosel areas, respectively (Ipach *et al*., 2010). The cixiid *Hyalesthes obsoletus*, the proven vector for ‘*Ca*. P. solani’ to grapevine (Maixner *et al*., 1994) has been found in all areas where the disease is present while the agent of Palatinate grapevine yellows has only found being erratically transmitted from *Alnus glutinosa* to grapevine by *Oncopsis alni* (Maixner *et al*., 2000). The vector(s) for the aster yellows-related phytoplasmas in grapevine are still unknown. So far, “flavescence dorée” has not been found in Germany. The most important and most widespread phytoplasma diseases on fruit crops in Germany are: apple proliferation (‘*Ca*. P. mali’), European stone fruit yellows (‘*Ca*. P. prunorum’) and pear decline (‘*Ca*. P. pyri’) with all agents belonging to the apple proliferation group (16SrX group). Proven vectors are the psyllid species *Cacopsylla picta* for ‘*Ca*. P. mali’ (Jarausch *et al*. 2003: 2007; 2011) and *C. pruni* for ‘*Ca*. P. prunorum’ (Jarausch *et al*., 2007). All pear psyllids, *C. pyri*, *C. pyricola* and *C. pyrisuga* are present in Germany, but only *C. pyri* and *C. pyricola* have been found infected with ‘Ca. P. pyri’ (Monien *et al*., 2012). Detailed information about the spread of ‘*Ca*. P. mali’ and the infection status of its vector *C. picta* is described by Jarausch *et al*. (2011) and online at www.apfeltriebsucht.de. The distribution of ‘*Ca*. P. prunorum’ and its vector

C. pruni is recorded in detail for Southwestern Germany (Jarausch *et al*., 2007; 2008) while for pear decline no comprehensive study is published. Further phytoplasma diseases of minor incidence in Germany are: rubus stunt associated with '*Candidatus* Phytoplasma rubi' (16SrV, elm yellows group) and potato "stolbur" (16SrXII-A group) found in Hesse and Rhineland-Palatinate (Ulrich *et al*., 2010).
SWITZERLAND: both important diseases on grapevine, "bois noir" and "flavescence dorée" are present in Switzerland. While '*Ca*. P. solani' and its vector *H. obsoletus* are widespread all over the country (Kehrli *et al*., 2010; 2011), the agent of 'flavescence dorée' (16SrV-group) so far has only been found in the Tessin region (Linder *et al*., 2007).; its proven vector, the leafhopper *Scaphoideus titanus*, is found not only in canton Ticino, but also in cantons Vaud and Geneva. The fruit crop diseases apple proliferation and pear decline are widespread in Switzerland (Bünter and Schaerer, 2012) while European stone fruit yellows (ESFY) is only reported from some local stone fruit growing regions, mostly in canton Valais, where its proven vector, *C. pruni*, is also present (Genini and Ramel, 2004; Ramel and Gugerli, 2004). *C. picta* was present in the cantons Aargau and Solothurn, but infected with '*Ca*. P. mali' only 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 been episodically reported from local blackberry and raspberry cultivations. Potato stolbur is also found, but rarely.

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Recent insight on phytoplasma diseases and vectors in France

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Phytoplasma diseases mainly affect the French grapevine, stone fruit and lavender production. These diseases are mainly spreading by sap feeding hemipteran insect vectors.

Two strains of "flavescence dorée" (FD) phytoplasmas are present in French FD outbreaks and surrounding wild *Vitis* regrowth and infected alders and may constitute potential epidemic reservoirs.

In France, 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 once 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 "bois noir" (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 different genetic clusters associated with FD outbreaks. In France, the genetic cluster 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). This two strains have been isolated and transmitted to broad bean. Experiments in controlled conditions showed both strains multiply at the same kinetics in plant, so they should have the same epidemiological properties. The prevalence of the strain mapFD2 may have resulted from 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 isolates 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.

BN is endemic in French vineyards and '*Candidatus* Phytoplasma solani' strains detected belong to the s*tamp* cluster IA and IV. Specific strains are responsible for severe outbreaks of lavender decline.

The "bois noir" disease associated with '*Ca.* P. solani' presence is endemic in France and is transmitted by *Hyalesthes obsoletus,* a planthopper residing in weeds such as bindweeds *(Convolvulus arvensis)* and stinging nettles *(Urtica dioica)* which also is endemic and acts as plant reservoirs.

'Ca. P. solani' is also affecting solanaceous crop such as tomato, tobacco, eggplant, pepper but also sugar beet and small fruit production such as strawberry. 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) but one specific strains is prevalent the *sec*Y strain S17 which was representing about 75% of the disease cases in 2010.

A gene encoding a variable surface protein of 'Ca. P. solani' was recently isolated and used for strain genotyping (Fabre *et al.*, 2011a; 2011b). *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 isolates corresponded to genetic clusters tuf-type b and stamp IA corresponding to strains from bindweed in Western Europe and tuf-type a stamp IV corresponding to strain detected in *U. dioica*.

European stone fruit yellows and its psyllid vectors

'*Ca.* P. prunorum' induces economic damages essentially to apricot and Japanese plum production and two cryptic species corresponding to *Cacopsylla pruni* are present in France as vector of European stone fruit yellows; they have overwintering and latency stage on conifers (Thebaud *et al.,* 2009; Peccoud *et al.,* 2013). Eleven different genotypes of '*Ca.* P. prunorum' have been detected so far in France (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 since 2009

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Background and objectives

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 problems, the importance of "bois noir" phytoplasma disease (BN, '*Candidatus* Phytoplasma solani') and on the constant improvements in diagnostic approaches. This overview summarizes the main achievements in phytoplasma and vector research from 2009 onwards entailing the readers' familiarity with the previous publications. The data were gathered from the published papers and conference contributions available to the author for the years 2009-2013. For brevity, only the most relevant contributions not recorded in the COST Action FA0807 yearly publication lists (www.costphytoplasma.eu) are cited here.

Austria

Significant increase of BN infected vineyards has been noted in the recent years in Austria. Systematic field investigations of the diseased vineyards as pathosystems consisting of infected vines, insect vectors and weed phytoplasma enabled both detecting new members of the pathosystem and experimenting with measures like pruning and pollarding to manage BN. Agalliinae leafhopper *Anaceratagallia ribauti* captured on grapevine, and reared in the laboratory, was proven to transmit BN to *Vicia faba* in the experimental conditions. The BN transmission by *A. ribauti* was also demonstrated to occur from *Convolvulus arvensis* to *Catharanthus roseus* (Riedle-Bauer *et al*., 2013) introducing potentially a new member in the BN epidemiological cycle. In this recent grapevine epidemiology research, the evidence of nettle and bindweed infections with '*Ca*. P. convolvuli' was found. The outbreaks of FD in Austrian vineyards were not recorded until 2009. The *foc*i in Southeast Styria and later in South Styria regions included indigenous varieties and an American hybrid, as well as *Clematis* plants. Mixed infections of BN and "Flavescence dorée" phytoplasmas (FD) were also recorded. Apricot, pear and apple orchards were surveyed for psyllid vectors and the insects were investigated for phytoplasma presence. The expected *Cacopsylla* species were found in the corresponding orchards but apparently *C. pruni* as an ESFY agent ('*Ca*. P. prunorum') vector has become a major concern in the Austrian apricot production. Expectedly, *C. pruni* was found positive for ESFY in molecular tests whilst the pear decline phytoplasma (PD; '*Ca*. P. pyri') was detected in *C. pyricola*, *C. pyri* and *C. pyrisuga*. In apples, both *C. melanoneura* and *C. picta* were present but without AP detected, as yet.

Croatia

The BN impact on the Croatian vine growing is still the largest. Correspondingly, BN molecular epidemiology and diversity is the most investigated resulting in finding its vast molecular diversity by multigene sequence analysis (about 20 different genotypes). Closed *Hyalestes obsoletus*-bindweed-grapevine pathosystems have been found but also indications of different pathosystems at some locations (Šeruga Musić *et al*., 2012; 2013). The first FD foci were recorded in 2009-10 both in commercially well-known and indigenous grapevine varieties. FD presence has been confirmed in *Clematis* and *Scaphoideus titanus* (Šeruga Musić *et al.,* 2012). FD1 and FD3 (16SrV-C) strains were found at different locations. '*Ca*. P. asteris' (AY) was sporadically detected in grapevines, *S. titanus*, fruit trees and *C. pyri*. Even though symptoms of PD and AP were reported in the country long ago,

the molecular characterization of pathogens and the vector survey are more recent. '*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 which was later confirmed by multigene sequence analysis of some local strains within an international consortium. 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 for about 5 years (Šeruga Musić *et al*., 2013). A phytoplasma known to infect conifer species '*Ca*. P. pini' was detected in Croatia for the first time in 2011 in *Pinus mugo* and *P. halepensis* (Ježić *et al*., 2012).

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, genomics and transcriptomics approaches and to introduce new diagnostic procedures like real-time PCR and loop-mediated isothermal amplification (Prezelj *et al*., 2013; Rotter *et al*., 2013). Besides the main BN vector *H. obsoletus*, *Euscelis incisus*, *S. titanus*, *Reptalus cuspidatus* and *R. panzeri* also harbour BN apparently without major epidemiological significance. Besides in symptomatic *Echinacea purpurea* plants, the aster yellows phytoplasma (AY) has not been detected in any other host in the country. The FD in Slovenia has been present in grapevine and *Clematis vitalba* since 2005, however, the comparison of *Clematis* and neighbouring grapevine FD isolates was undertaken in 2010 and yielded interesting results regarding this phytoplasma molecular diversity and epidemiology. Besides *S. titanus*, harbouring FD2 (16SrV-D) phytoplasmas, the mosaic leafhopper *Orientus ishidae* was found infected with FD adding it to the complexity of the FD pathosystems. Alders (*Alnus glutinosa* and *A. incana*) and *Oncopsis alni* were found to host phytoplasmas with sequence similarities to all three FD clusters, as well as those similar to other alder yellows strains. The presence of AP, PD and ESFY in Slovenia is long 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*.

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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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Background and objectives
The examined countries showed increasing interest in phytoplasma research trough the Action that was shown by increasing number of publication mainly focused on phytoplasmas and insect vectors identification as detailed below.

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 mainly related with "bois noir" strain characterization, however considering the presence in southern districts of *Scaphoideus titanus* (Delić *et al*., 2007) extensive monitoring were 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 belongin 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 of these species (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*., 2011a). 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*., 2011b). 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 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*); the same phytoplasma was also detected in weeds, particularly *Convolvulus 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", "flavescence dorée" in grapevine, European stone fruit yellows, apple proliferation, pear decline, aster yellows, 16SrII-E, 16SrIII-B, bermudagrass white leaf, '*Ca.* P. ulmi', '*Ca.* P. rhamni'. The new '*Ca.* P. convolvuli' was also reported in bindweed together with "stolbur" phytoplasmas. Epidemiological studies on stolbur and aster yellows phytoplasmas insect vectors have been conducted (Jović *et al.*, 2009; Drobnjaković *et al.*, 2010). A new marker, groEL gene, has been used for analyses of some of the examined phytoplasmas (Mitrović *et al*., 2011).

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Review of occurrence of phytoplasmas in Spain, Portugal and Malta

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Phytoplasmas and 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 the BN, it is present in different vine growing regions such as "La Rioja", Alava, Navarre, Catalonia and Aragoón. The first outbreak of FD in Spain was detected in 1996 in the French boarder in Northeast of Catalonia and by now the disease is eradicated. FD is the most dangerous disease caused by phytoplasmas in Portugal where it was detected since 2002 (Sousa *et al*., 2003:2009) after the first identification of its vector, the ampelophagous leafhopper *Schapoideus titanus* Ball in 1998 (Quartau *et al*, 2001). The phytoplasma associated with FD in Spain and Portugal belongs to the ribosomal group 16SrV-D. This strain shows also epidemic activity in other countries as France and Italy. '*Candidatus* Phytoplasma solani' is the phytoplasma associated with BN. The study of the geographical 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, Aragon and Navarre. In *Hyalesthes obsoletus* signoret only tuf-type b was identified until now (Batlle *et al.,* 2009).

In Spain *S. titanus* was identified in the north of the country: Catalonia (Northeast) and Galicia (Nothwest). 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 BN is present but in low 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 (Sabaté *et al.,* 2007).

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

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 (Laviña *et al*., 2004).

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 (Garcia-Chapa et al. 2003). 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.
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* present in the orchards. Sampling carried out in apple plots of different geographical areas of Spain indicated the presence of the two species of *Cacopsylla* reported as vectors of the apple proliferation disease. The population evolution of *C. picta* and *C. melanoneura* showed two peaks, one for adults re-immigrants in early April and the other for new generations between June and July. The populations of *C. picta* are higher than those of *C. melanoneura* (Laviña *et al.,* 2011)

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 Valencia Province (Eastern Spain), in *Dianthus caryophyllus,* in solanaceous and in other horticultural crops. Phytoplasmas clustering in the 16SrIII group, the type member of which is X-disease phytoplasma, were detected in faba bean (*Vicia faba* L.) plants showing symptoms of shoe stringed leaves, phyllody and flower abortion in Málaga (Southern Spain). *'Ca*. P. pini' has been detected in *Pinus halepensis* in the Mediterranean coast of Spain. Phytoplasmas related to phytoplasmas of group 16SrVI, of which the type member is clover proliferation phytoplasma, where detected in *Capsicum annuum* plants showing short internodes and green flowers buds. '*Ca*. P. asteris' has been detected in ornamental and horticultural plants.

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

Region/s	Phytoplasma/s	Strain/s	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 /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/s		Infection rate		Vector host\s
	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; Lavandula; Solanum nigrum* Portugal: grapevine
S. titanus	medium	high	FD	FD	n.d	high	Grapevine

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

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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 also for the EU as well. The information concerns new plant hosts of phytoplasmas, new vectors and 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 species 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 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 this technique made 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 group phytoplasmas and of phytoplasmas belonging to group 16SrIX (Ertunc *et al.*, 2013), the latter phytoplasma group is known to contain phytoplasmas associated with a tremendous diseases of almond and other stone fruit trees in EU geographically contiguous Countries such as Lebanon.
As for FD, 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 the vector *Hyalesthes obsoletus*, survival in field. 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 outbreak of the disease like in Italy and Greece (Rumbou *et al.,* 2011). The studies on 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 accompanied with the phytoplasma strain information based 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.

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

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Background and objectives
A national survey was conducted in Lebanon in 2009/2010 and updated in 2012 in order to detect the almond witches' broom disease (AlmWB) spread in the country, as well as preliminary researches about the disease epidemiology and phytoplasma characterization. Over four years of work, Lebanese and Italian efforts were joined to define focus and endemic areas, to discover the phytoplasma vector(s) and to elaborate preliminary management plans.

Materials and methods
Lebanon is divided in 26 districts, where local offices of the "Ministry of Agriculture extension services" are located. Training sessions for sixty technicians have been developed in order to train the extension services personnel about the disease symptoms and the sample collection. According to the national census and the field condition, 561 stone fruit cultivated regions have been visited, in order to collect leaf samples for the detection of the phytoplasma presence in the stone fruit orchards. A common protocol of detection has been established and shared among three laboratory units at AUB, LARI and USEK departments, by using the specific primer pair AlWF2/AlWR2. 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 partially achieved results and the strategies to be implemented among all the involved partners and subjects. Four meetings have been organised so far and a final event will resume the four year works in December 2013. Since the research about the phytoplasma vector(s) is still on going, in order to reduce the presence of *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 and discussion
Almond, peach and nectarine were severely affected and the number of infected trees was increasing. The disease presence, detected in 16 districts out of 26 in 2010, was also confirmed in 2 different districts in 2012 (Bent Jbeil and Bcharre); 216 out of 561 monitored villages have been recorded with positive samples collected in stone fruit orchards.
About the nursery sector, 282 samples were collected and analyzed from registered and non-registered nurseries. Five out of 136 visited nurseries were found selling positive seedlings; a prompt decision from the Lebanese Ministry of Agriculture was adopted to destroy all the seedlings. Extension services have been developed for farmer and nurseryman awareness. The pilot area for

tree elimination and replacement has been implemented in 14 villages, with the elimination of 182 infected trees. Within the last year, a plan of 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 2,666 infected trees have been eliminated until the end of July by the field team in 61 villages. The farmers will receive from the Ministry of Agriculture new crops, adapted to the different regions, in support of their economic losses.

The cooperation for the complex research for '*Candidatus* Phytoplasma phoenicium' epidemiology and control is the necessary tool to achieve the expected and necessary results. Regulation and control measures must urgently adopted to contain the diffusion of almond witches' broom in Lebanon but also to avoid its spread in the Middle East and in Europe.

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

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Background and objectives

The presence and rapid spread of almond witches' broom disease (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 we focused the survey on the cixiid-fauna present in almond and nectarine orchards of Lebanon with particular attention on their natural infection by phytoplasmas.

Materials and methods

Deepened samplings were carried out through the years 2010-2012 by means of Malaise and yellow sticky traps in two AlmWB infected orchards planted respectively with almond and nectarine trees. Additional direct samplings with a hand-held D-vac were performed in the same orchards and surroundings. 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, using respectively the semi-specific 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 to identify 16Sr group/subgroup.

Results and discussion

The following cixiid genera were collected during the different insect field collections: *Cixius*, *Tachycixius*, *Eumecurus*, *Oliarus*, *Pentastira*, *Pentastiridius* and *Hyalesthes*. The molecular analyses pointed out *Cixius*, *Tachycixius*, *Eumecurus* and *Hyalesthes* as carriers of '*Ca*. P. phoenicium'. These results highlighted the role of planthoppers in the epidemiology of AlmWB disease. Nevertheless further studies about their vector activity as well as their specific identification are needed. Concerning this latter a deep systematic revision is

desirable to solve the taxonomic critical situation of this family. Moreover, since almost no information is known about their biology, new surveys will be important to better understand the disease spread and define suitable control strategies.

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

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Background and objectives
"Bois noir" (BN) is a disease of the grapevine yellows (GY) complex associated with '*Candidatus* Phytoplasma solani' phytoplasmas (subgroup 16SrXII-A) mainly transmitted by the polyphagous Cixiidae *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, in South Africa, in Iran and in China (Botti and Bertaccini, 2006; Gajardo *et al*., 2009; Karimi *et al*., 2009; Duduk *et al*., 2010). In the present work, we communicate the first report of BN in Jordan.

Materials and methods
A survey was carried out in Jordan vineyards in August and October 2012, grapevine plants showing typical grapevine yellows (GY) 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 carried out using universal primer pairs P1/P7 followed by R16F2n/R16R2 (F2n/R2) (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. F2n/R2 PCR products amplified from grapevines and bindweeds 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 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 of phytoplasmas identified in grapevine and bindweed in Jordan 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). 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 Cixiidae responsible for the BN phytoplasma transmission in Europe, and other possible insect vector(s) in the BN spread in Jordan.

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Identification and molecular characterization of '*Candidatus* Phytoplasma' strains from maize in four Countries

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Background and objectives
Maize is affected by phytoplasma diseases in some of its cultivation areas where losses of production can be higher than 50%. However diverse phytoplasmas can be associated with similar disease symptoms such as stunting, reddening of leaves, early and abnormal ripening, precocious death, poor and shriveled grains in kernels that are in some cases also malformed. Phytoplasmas infecting corn in Americas are associated with maize bushy stunt disease and belong to 16SrI-B ribosomal subgroup (aster yellows or '*Candidatus* Phytoplasma asteris'), while in Europe the same symptoms were mainly associated with corn reddeness and belong to ribosomal subgroup 16SrXII-A ("stolbur") (Bedendo *et al*., 2000; Duduk and Bertaccini, 2006). Besides the identification of "stolbur" phytoplasmas in Serbia, Hungary and Bosnia and Herzegovina, in Italy preliminary identification of phytoplasmas belonging to 16SrI (aster yellows), 16SrIII (X disease) and 16SrXII ("stolbur") groups, in same cases in mixed infection was reported (Calari *et al*., 2010). Molecular analyses and characterization were carried out on aster yellows and "stolbur" strains from different geographic areas.

Materials and methods
Plants showing typical disease symptoms were collected and aster yellows and "stolbur" strains from Colombia, Serbia, Cuba and Italy were selected after 16S rDNA identification, and characterized by MLT (multi locus typing) assays. Nucleic acid was extracted from 0.4 – 1.0 g of leaf tissues, stem and young cobs (Prince *et al*., 1993). Direct PCR analyses with P1A/P7A (Lee *et al*., 2004) followed by nested PCR by F1/B6 (Duduk *et al*., 2004), R16F2n/R2, R16(I)F1/R1 (Gundersen and Lee, 1996; Lee *et al*., 1994) were carried out. RFLP analyses with *Tru1*I were carried out for aster yellows and "stolbur" identification and with *Hha*I and *Mbo*II respectively, for strains characterization. Aster yellows strains from Cuba and Colombia were further studied by direct sequencing with F1, B6, F2n and R2 of P1A/P7A amplicons and by RFLP analyses with *Tru1*I, *Hpy8*I, *Taa*I, *Alu*I e *Tsp509*I and direct sequencing of *groEL*, *tuf, amp*, pseudo helicase and *rp* genes (Schneider *et al*., 1997; Duduk and Bertaccini, 2006; Kakizawa *et al*., 2004; Martini *et al*., 2007; Mitrović *et al*., 2011a).

Results and discussion
From all countries phytoplasmas were identified only in samples from symptomatic materials and aster yellows phytoplasmas (16SrI-B) were identified in Colombia, Cuba and Italy, while "stolbur"(16SrXII-A) phytoplasmas were only detected in Serbia and Italy. While "stolbur" strains collected in either in Serbia or in Italy show polymorphism in 16Sr DNA, aster yellows strains from corn collected in Colombia, Cuba and Italy show no polymorphisms in this gene.
Further molecular characterization of aster yellows strains from Colombia and Cuba on g*roEL* gene allow distinguishing the aster yellows strains detected in these countries in maize from all other aster yellows phytoplasmas used as reference (Mitrović *et al*., 2011a; 2011b). RFLP analyses with *Tru1*I and Tsp*509*I on *amp* gene amplicons and with *Tru1*I, *Hpy8*I, *Taa*I, *Alu*I on *rpL22*/*rpS3* gene showed differential profiles between strains infecting maize in Colombia and in Cuba and reference

phytoplasma strains. Moreover the *Hpy8*I restriction on the latter gene show a SNP distinguishing Colombian strain from the strain from Cuba and Mexico (Accession number AY265208). The results of RFLP analyses were confirmed and consistent with those obtained by sequencing for the aster yellows phytoplasmas. Predicted translation products for all genes evaluated, confirmed the differentiation of phytoplasmas in corn from Cuba and Colombia from all other strains used for the analyses.

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Advances in knowledge about phytoplasma diseases in Argentina

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Background and objectives

Two well defined situations can be seen in Argentina as regards the presence of phytoplasmas, similar to what is reported in several other Countries. On one hand, plant species that develop symptoms of infection sporadically, and with low frequency. In such situation only the pathogen characterization can be performed and eventually maintain them under controlled conditions on experimental plant hosts. On the other hand, there are other situations in which systematically affected plants are found in the crop, and it is possible to study the pathogen involved, identify insect vectors, establish the disease incidence, the losses and the cycle of the disease. Currently the work team is focused on detection and molecular characterization of new phytoplasmas; identification of different species of *Hemiptera* vectors and the components involved in the transmission; epidemiological studies of phytoplasmas diseases in different crops; study of proteins potentially involved in host-pathogen interaction.

Materials and methods

IDENTIFICATION AND MOLECULAR CHARACTERIZATION. DNA from symptomatic plants is purified and amplified by PCR using universal primers (Lee *et al*., 1993; 1998). Usually amplicons produced by PCR are analyzed by RFLP, cloned, sequenced and aligned to database sequences. When possible transmission of the pathogen into periwinkle (*Catharanthus roseus*) by grafting or by *Cuscuta subinclusa* is also carried out.

EPIDEMIOLOGICAL STUDIES. In crops systematically affected by phytoplasma diseases, the epidemiological studies were carried out. In production areas of strawberry (*Fragaria x annanassa*), alfalfa (*Medicago sativa*) and garlic (*Allium sativa*) crops where sampled. The incidence and prevalence were determined. When possible, *Hemiptera* fauna in each crop has been studied, and losses caused by the disease have been established.

VECTORS. The transmission of the agent associated with "escoba de bruja de la alfalfa" (ArAWB) was studied in an experimental way. Hemiptera insects collected from alfalfa fields were identified and the presence of phytoplasma determined. The insects are being reared under controlled conditions in order to study each species transmission parameters.

PHYTOPLASMA HOST INTERACTIONS. In order to characterize and immunolocalize pathogen proteins in infected tissue, we are producing antisera against effector, immunodominant membrane and SecA proteins of selected phytoplasmas from Argentina.

Results and discussion

IDENTIFICATION AND MOLECULAR CHARACTERIZATION. We have identified four different 16Sr pytoplasma groups in more than 20 plant species. The highest number and widest distribution of strains was found in 16SrIII group (X-disease) with subgroups -J, -W and -X (Galdeano *et al*., 2004; Fernandez *et al*., 2013b). Phytoplasmas of 16SrI group (aster yellows) subgroup -S were described (Torres *et al*., 2011) and of group 16SrVII (Ash yellows) with subgroups -B and -C (Conci *et al*., 2005; Meneguzzi *et al*., 2008; Fernandez *et al*., 2013a). 16SrXIII group (Mexican periwinkle virescence phytoplasmas) was

detected in China tree and in strawberry (Arneodo *et al*., 2005, Fernández *et al*., 2008). Several of these pathogens have been identified exclusively in South America.

EPIDEMIOLOGICAL STUDIES. Phytoplasmas present in strawberry, garlic and alfalfa are present with high prevalence and low incidence. We have also demonstrated that the symptoms in strawberries are not always related to phytoplasma presence suggesting the presence of other pathogens or abiotic stresses involved in the symptom development. In alfalfa, the incidence increased with the crop age and lead to premature death of the affected plants. The severity of the disease is extremely high in garlic and leads to death of the plant in early infections. "Morado" genotype is significantly more susceptible to garlic decline (16SrIII-J group phytoplasmas) than "nco" and "Colorado" types. However, the absence of an efficient vector reduces the disease incidence and symptom remission during sprouting has also been observed.

VECTORS. *Ceresa nigripectus* (Membracidae, treehoppers) was reared under experimental conditions and its biological cycle was determined. Under the hypothesis that it is a terminal host, we are now rearing the Hemiptera vector of ArAWB with the aim of studying protein interactions and to establish the transmission capacity.

PHYTOPLASMA-HOST INTERACTIONS. Effector proteins: the recombinant protein of 11kDa expressed from AY-ACLL was designed as SAP11ar and was confirmed by MALDI-TOF analysis. We are adjusting purification protocols for SAP11ar from bacterial culture to rabbit sensibilization to produce a specific antiserum. SecA protein: the SecA protein of ChTYXIII was expressed in bacterial culture and an antiserum was developed for its detection and immunolocalization in infected tissues.

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An update on the status of grapevine yellows disease in vineyards of the Olifants River region of South Africa

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Background and objectives

Grapevine yellows is a widespread phytoplasma disease in Europe where it causes serious damage ranging from lower yields to the death of vines (Magarey, 1986). Symptoms of grapevine yellows disease 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). Symptoms of the disease initially occurred on grapevines in two production regions, Olifants River and Wabooms River, but recently they were also found 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). Observations of grapevine yellows disease spread necessitated the monitoring of its incidence and spread.

Materials and methods

Thirteen trial sites in the Olifants River region, planted with vines of seven different cultivars (Chardonnay, Chenin blanc, Sauvignon blanc, Colombar, Shiraz, Cabernet Franc and Pinotage), with ages ranging from 6 months to 18 years at the time of the initial disease evaluation (January 2010), and all displaying symptoms of varying severity, were selected for the study. Annual vine-to-vine mapping of vineyards was conducted during the past four seasons (January/February, 2010-2013). Each grapevine plant was marked as healthy, AY-affected or missing/dead. Grapevines were considered AY-affected if any one of the visual symptoms of the disease were present: (1) aborted fruit clusters, (2) downward rolling and yellowing/reddening of leaves, (3) green, immature canes and/or (4) die back of shoot tips and shoots. The Patchy Programme (Maixner, 1993) was used to determine incidence of the disease. In order to confirm visual symptom evaluation of grapevines, five symptomatic and five asymptomatic grapevines were sampled per vineyard and subjected to PCR analysis. Total nucleic acid was extracted from leaf veins according to Angelini *et al*. (2001). The presence of AY phytoplasma was determined by using PCR-RFLP, as described by Lee *et al*. (1998), using the restriction enzymes *Alu*I, *Hha*I, *Hpa*I and *Rsa*I. Nested PCR was performed using two sets of universal primers (P1/P7, followed by R16F2n/R16R2).

Results and discussion

Previously reported results of detailed annual mapping of 13 selected vineyards in the Vredendal region (Carstens *et al*., 2011) showed that disease incidences varied between the different cultivars and vineyards. The cumulative incidence of AY in all 13 vineyards increased from year to year over the survey period, but varied significantly between vineyards. The cumulative incidence of AY increased by 2.1%, 4.6%, 5.1%, 5.2% and 32.7%, 62.2%, 63%, 63.5% in two different Chardonnay vineyards; and by 0.9%, 2.3%, 4.9%, 5.6%; and 7.9%, 8.5%, 11.3, 14.6% in two different Pinotage vineyards. For the verification of visual symptom evaluation of grapevines, leaf samples (symptomatic and asymptomatic) were collected every year for PCR analysis. A correlation of 83% was found between PCR positive results and visual symptom analysis during the first two seasons and a 95% correlation the last season. This could be ascribed to the technical team becoming more experienced in recognising AY symptoms in the different cultivars. During the survey two vineyards (Pinotage and Shiraz) where symptoms were previously observed showed some reddening of leaves,

but no typical AY symptoms. This observation may suggest an example of "recovery phenotype", but needs to be further investigated by PCR diagnostics. This study reports the current status of AY disease incidence in South African vineyards. Results showed that AY disease is spreading in vineyards located in a region of high disease incidence. Moreover, the disease has emerged in two new production areas in the Western Cape. Detailed annual mapping showed varied disease incidences in different cultivars and vineyards of different ages. A clearer picture of the spreading *tempo* should emerge after data of the past season has been analysed.

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'*Candidatus* Phytoplasma mali' - a new phytoplasma species molecularly characterized in Croatia

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Background and objectives

A survey on presence and diversity of phytoplasmas infecting fruit trees and their vectors in Croatia has been conducted since 2002. Although the presence of '*Candidatus* Phytoplasma pyri' and '*Ca*. P. solani' was sporadically associated with symptomatic apples (Križanac *et al*., 2010), there was no direct evidence of the presence of '*Ca*. P. mali', the main agent of apple proliferation disease (AP) in neither plant or insect vector samples.

Materials and methods

Throughout 2011 and 2012, orchards from all fruit growing regions of Croatia were surveyed for AP symptoms and nearly 70 samples of different apple cultivars/varieties were collected. Psyllid samples were collected from two selected orchards in the north-western part of the country only in the year 2012. Total nucleic acids were extracted following CTAB procedure. PCR/RFLP analyses of phytoplasma 16S rDNA were performed by using P1/P7 primers (Deng and Hiruki, 1991; Schneider *et al.*, 1995) in a direct PCR assays followed by nested PCR using R16(X)F1/R1 (Lee *et al*., 1995) primers. Amplicons from nested PCR were digested with *Rsa*I and *Ssp*I in order to determine the affiliation to the '*Ca*. P. mali' species. To confirm the results, real-time PCR experiments amplifying 16S rDNA were performed as well (Baric and Dalla-Via, 2004). Furthermore, *pnp* and *aceF* genes encoding polynucleotide phosphorylase and pyruvate dehydrogenase (dihydrolipoamide acetyltransferase component) were amplified, sequenced and analyzed to assess the genetic variability among '*Ca*. P. mali' isolates.

Results and discussion

For the first time, in the year 2011, the presence of '*Ca*. P. mali' was detected in samples collected from apple orchards at seven locations from continental as well as Adriatic Croatian regions while the survey in the following year one additional location with samples positive for the '*Ca*. P. mali' presence was obtained. The analysis of psyllid vectors revealed one positive finding of '*Ca*. P. mali' in *Cacopsylla picta* (Foerster) sample from one orchard in the north-western Croatia. PCR/RFLP results were consistent with the results obtained by real-time PCR analyses. Phylogenetic analysis of *pnp* and *aceF* gene sequences have shown very close relatedness to the reference '*Ca*. P. mali' genes (NC_011047) with 99% of sequence identity for most of the obtained sequences. This is the first finding and molecular characterization of '*Ca*. P. mali' in Croatia, from both apple and insect vector species.

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Molecular characterization of `*Candidatus* Phytoplasma mali` and `*Candidatus* Phytoplasma pyri` isolates from Romania

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Background and objectives

Apple proliferation (AP) and pear decline (PD) are quarantine diseases associated with the presence of phytoplasmas '*Candidatus* Phytoplasma mali' (16SrX-A) and *'Ca.*P. pyri' (16Sr16X-C), respectively and they are considered of major importance for apple and pear orchards in Europe (Seemüller and Schneider, 2004). This paper aims to detect and molecular characterize the 16S rDNA and the 16S-23S rDNA spacer region of phytoplasma isolates infecting apple and pear trees in Romania.

Materials and methods

Samples of symptomatic apple (35) and pear (11) of different varieties, collected in autumn 2012 from four pomicultural regions of Romania as well as 10 insect of *Cacopsylla melanoneura* (6), *Fieberiella florii* (2) and *Metcalfa pruinosa* (2) were tested for phytoplasma presence. Total DNA extracted using the CTAB-based protocol, was employed for PCR-RFLP analyses in the Virology Section of the Research Institute of Horticulture in Skierniewice, Poland. Direct PCR was conducted with P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) or fU5/rU3 (Lorenz *et al*., 1995) and AP group specific primers fAT/rAS (Smart *et al*., 1996). Nested PCR with primers F1/B6 (Davis and Lee, 1993; Padovan *et al*., 1995) or R16F2n/R16R2 (Gundersen and Lee, 1996) was applied on P1/P7 amplicons diluted 1:29 in water. Healthy periwinkle and apple infected with AP-15 subtype strain of `*Ca*. P. mali' were used as negative and positive controls, respectively. RFLP analyses were conducted on F1/B6 amplicons digested with *Mse*I, *Hpa*II, and *Ssp*I enzymes. The restriction patterns were compared to those for the reference strains (Lee *et al*., 1998; Paltrinieri *et al*., 2010).

Results and discussion

PCR with primers fAT/rAS showed that eight out of 35 apple and four out of 11 pear samples were infected with phytoplasmas from AP group. Negative results were obtain for insect samples. The RFLP analyses on F1/B6 amplicons digested with *Hpa*II showed three restriction profiles: P-I, P-II and P-III (Fig. 1, table 1). The P-I profile was detected in majority of *'Ca*. P. mali', PII was also detected in symptomatic apple while a different profile was detected in pear (P-III).

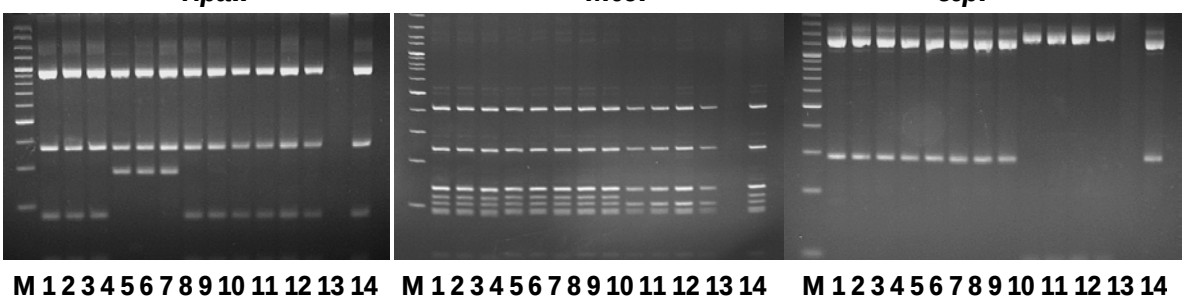

Fig. 1. RFLP patterns on F1/B6 amplicons of 16 rDNA fragments digested with *Hpa*II, *Mse*I and *Ssp*I. M-molecular marker 100 bp. Samples: apple 1-8; pear 9-12; 13- healthy plant, 14- positive control `*Ca*. P. mali'.

Table 1. Results of RFLP analyses of 16S rDNA and the 16S-23S rDNA spacer region of phytoplasmas infecting apple and pear trees.

No.	species/cultivar	Isolate	restriction pattern			Profile
			*Mse*I	*Hpa*II	*Ssp*I	
1	apple/Golden Delicious	GD1.DB	A	A	A	P-I
2	apple/rootstok	Root1.DB	A	A	A	P-I
3	apple/Golden Delicious	GD2.DB	A	A	A	P-I
4	apple/Golden Delicious	GD1.Bu	A	B	A	P-II
5	apple/Golden Delicious	GD2.Bu	A	B	A	P-II
6	apple/Generos	G1.BN	A	B	A	P-II
7	apple/Generos	G2.BN	A	A	A	P-I
8	apple/Iris	Is1.AG	A	A	A	P-I
9	pear/Williams	W1.Bu	B	A	B	P-III
10	pear/Conference	C1.Bu	B	A	B	P-III
11	pear/Williams	W2.Bu	B	A	B	P-III
12	pear/unknown	Un1.Bu	B	A	B	P-III

This is the first report on molecular characterization of '*Ca*. P. mali' and '*Ca*. P. pyri' isolates from Romania. The presence of these phytoplasmas represents a serious threat for national fruit production, thus further investigations are needed on their distribution and incidence.

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Fruit tree phytoplasmas in Turkey

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Background and objectives

Although fruit tree phytoplasmas have been studied since 1999 in Turkey, most reports were on symptomological, microscopic and molecular characterization of European stone fruit yellows, pear decline and apple proliferation associated diseases and their epidemiology (Çağlayan *et al*., 2011). Recently phytoplasma-like symptoms were observed on cherries, pomegranate, persimmon (kaki) and also olives. The main symptoms on apricots were off season flowering in winter and leaf rolling whereas decline was the most obvious symptom on plums. Cherries exhibited proliferation of branches, off-season flowering and declining whereas yellowing was the main symptom on pomegranate, kaki and olives.

Materials and methods

More than 500 cultivated and wild *Prunus* plants in or nearby germplasm nurseries and commercial orchards and 116 pear samples during 2002-2009 were tested by using universal primers P1/P7 and fU5/rU3 for direct and nested PCR, respectively. Amplification products were digested with the *Rsa*I and *Ssp*I enzymes. Some symptomatic pomegranate, persimmon (kaki) and olive samples were also tested by the same method.

Results and discussion

The average incidence of '*Candidatus* Phytoplasma prunorum' was detected as 10.19%. The most infected stone fruit species were apricot and plum followed by almond and peach, but cherries were always negative for this phytoplasma. When 116 pear trees were tested for '*Candidatus* Phytoplasma pyri' presence 61 samples were found infected by this phytoplasma (52.58%). Despite '*Ca*. P. mali' was also detected in different regions of Turkey, its presence was much lower comparing to other 16SrX group phytoplasmas. Although other symptomatic fruit tree samples (kaki, pomegranate and olive) gave positive results by using universal phytoplasma primers P1/P7 followed by R16F2n/R2, characterization studies are still under investigation.

Vector psyllids of 16SrX group phytoplasmas were found in limited locations in Turkey. Many overwintered individuals of *Cacopsylla pruni* were collected from *Abies nordmanniana* subsp. *bornmulleriana* and *Pinus* spp.. DNAs of individual psyllid species of *C. pruni* and *C. pyri* were analysed by RFLP and the presence of '*Ca.* P. prunorum' and '*Ca.* P. pyri' was confirmed, respectively. As a potential vectors of '*Ca*. P. mali' ; *C. picta*, *C. affinis* and *C. melanoneura* were also reported. Healthy pear and plum plants were able to be inoculated with '*Ca.* P. pyri' and '*Ca*. P. prunorum' by using new generation of *C. pyri* and *C. pruni* respectively. Experimental transmission trials of '*Ca.* P. mali' is still in process.

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

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Background and objectives

Phytoplasma-associated diseases affect wild and cultivated raspberry, blackberry, and crosses of these species throughout Europe, north-eastern USA and Turkey. The associated disease agents are phytoplasmas belonging to several 16Sr groups: 16SrV, elm yellows (Marani *et al*., 1977; Bertaccini *et al*., 1995;), 16SrIII, X disease (Davies, 2000), 16SrI, aster yellows (Fahmeed *et al*., 2009) and 16SrXII, "stolbur" (Borroto Fernández *et al*., 2007). The infected plants show stunting, shoot and flower proliferation, and fruit malformations. Detection and molecular characterization of the 16S rRNA gene of the phytoplasmas infected *Rubus* sp. Plants in Poland are reported.

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 their natural environments in Poland. Shoot samples were collected from selected red raspberry, blackberry (*Rubus fruticosus*), as well as from hybrids loganberry (R. *loganobaccus*) and tayberry (*R. loganobaccus* x *R. idaeus*). Nested PCR of DNA extracted from diseased plants and from healthy raspberry were conducted using phytoplasma universal and group-specific primer pairs. The RFLP and sequence analyses of 16S rDNA fragment amplified with universal primers R16F2n/R16R2 were performed to identify the phytoplasmas.

Results and discussion

On the basis of PCR/RFLP with *Hpa*II, *Rsa*I, and *Bfa*I (Fig. 1) 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 groups. Most of *Rubus* sp. plants were infected by '*Candidatus* Phytoplasma rubi' belonging to subgroup E of the Elm yellows phytoplasma group, 16SrV (Davis *et al*., 2001).

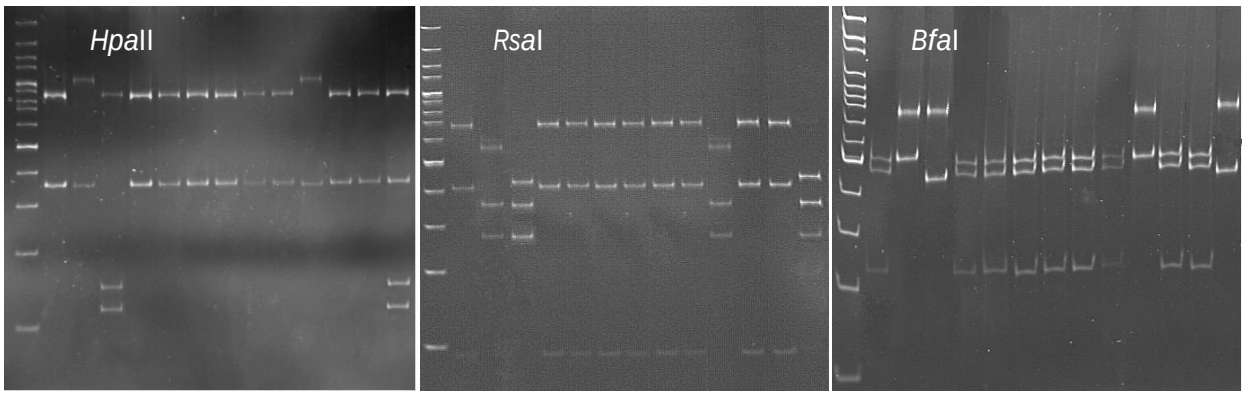

Fig. 1. RFLP profiles of 16 rDNA fragments amplified from samples of *Rubus* spp. with primers R16F2n/R16R2 and digested with *Hpa*II, *Rsa*I and *Bfa*I restriction enzymes. M - 100 bp DNA Ladder (Fermentas, Lithuania). Lines: 1 – EY-1; 2 – CX; 3 – AY-1; 4-7, 11, raspberry; 8, 9, 13 – blackberry; 10 – loganberry; 12 – tayberry.

Phylogenetic analysis showed that they form a monophyletic cluster with the reference strain RuS from *Rubus fruticosus* from Italy (GenBank accession no. Y16395) and that they are genetically related to the strain FD isolated from *Vitis vinifera* from France (GenBank accession no. X76560) (data not shown).

It was possible to identify phytoplasma belonging to subgroup 16SrIII of the X diseases group in loganberry plant. Based on the results of phylogenetic analysis this isolate was assigned to one cluster with a reference strain CYE of the clover yellow edge phytoplasma isolated from clover in Canada (GenBank accession no. L33766). X disease phytoplasma was previously identified in loganberry in the UK (Davies, 2000). Supposedly, the infected loganberry plant growing in germplasm collection was imported from the UK.

One of the blackberry plants (*Rubus fruticosus*) from forest was infected by a phytoplasma from subgroup B of the Aster yellows phytoplasma group ('*Candidatus* P. asteris', 16SrI-B). The analysis revealed high similarity of the sequence of this isolate with the sequences of reference strain of *'Ca.* P. asteris' OAY from periwinkle from Michigan, USA (GenBank accession no. M30790) and SAY isolated from celery in California, USA (GenBank accession no. M86340).

The partial sequences of the 16S rDNA region of the five isolates of phytoplasmas detected in *Rubus* spp. plants were deposited in the GenBank database under accession numbers: GU125723-27.

Although the phytoplasmas are transmitted mainly with infected plant material, *Rubus* spp. plants growing in a natural environment can become a reservoir of these pathogens. The phytoplasmas belonging to group 16SrV were reported in wild and cultivated *Rubus* spp. in Germany, France, Italy (Mäurer and Seemüller, 1994), the UK (Davies, 2000), and Poland (Cieślińska, 2011). X disease phytoplasma was identified in loganberry in the UK (Davies, 2000) and Poland (Cieślińska, 2011), as well as in black raspberry (*Rubus occidentalis*) with witches' broom symptoms in Oregon (Davis *et al*., 2001). It was shown that aster yellows phytoplasma (16SrI-B) infected wild raspberry and blackberry growing in Austrian forests (Borroto Fernández *et al*., 2007), and blackberry found in Pakistan (Fahmeed *et al*., 2009) and in the UK (Reeder *et al*., 2010).

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Phytoplasmas associated with *Rubus idaeus* and *Rubus fruticosus*

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Background and objectives

Phytoplasmas infecting *Rubus* fruit species are considered to be a major challenge in their production. Common 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 and Duduk, 2009; Cieslinska, 2011). So far, *Rubus* spp. has 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; Vindimian *et al*., 2004; Borroto Fernández *et al*., 2007; Valiunas *et al*., 2007; Cieslinska, 2011). Due to its distinct biological niche and genomic differentiation, the Rubus stunt phytoplasma of the elm yellows group (16SrV) was proposed as novel distinct candidate taxon: `*Candidatus* Phytoplasma rubi´ (Malembic-Maher *et al*., 2011). An early detection of phytoplasma is of prime importance to minimize spread of disease to larger areas.

Materials and methods

For this reason PCR, restriction fragment length polymorphism (RFLP) and cloning were employed to detect and identify phytoplasmas infecting this plant species. PCR using the universal and specific phytoplasma primers listed in table 1 was conducted on total DNA extracted from symptomatic and asymptomatic plants.

Table 1. Primers employed for the *Rubus* phytoplasma detection

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	(Deng and Hiruki, 1991)
RubF	GTGGTGCATGGTTGTCGTCAG	this study
RubR	CTAACATCTCACGACACGAACTGA	this study
PA2F	GCCCCGGCTAACTATGTGC	(Heinrich *et al*., 2001)
PA2R	TTGGTGGGCCTAAATGGACTC	(Heinrich *et al*., 2001)

Results and discussion

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 revealed the presence of a phytoplasma having 98% to 99% similarity to phytoplasmas belonging to group 16SrV in *R. idaeus*. For RFLP analyses, a nested PCR was performed with the primer pairs R16mF2/R16mR1 followed by R16F2nM/R16R2. Products were digested with *Mse*I and the results confirmed that the assayed *Rubus* plants were infected by phytoplasma belonging to the group 16SrV.

Positive samples produced the expected 1,250 bp fragment of phytoplasma 16S rDNA after nested PCR using primers R16F2nM/R16R2. From a total of 39 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 and 4 out 7 flowers (57.1%) and 1 out of 3 (33%) fruits. Furthermore, nested PCR with primer pair R16(V)F1/R16(V)R1 specific for group 16SrV phytoplasma gave positive results in 21 out of 34 (61.8%) samples.

Explants of positive plants were established as *in vitro* cultures and maintained in the Vienna collection of Fruit Pathogens. Sanitation experiments by *in vitro* thermotherapy and meristem preparation are currently underway.

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Phytoplasma infections in *Rhododendron hybridum*

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Background and objectives

Some reports of phytoplasma diseases on *Rhododendron* sp. were described in the past. Symptoms of yellowing, leaf malformation and variegation, shortened axillary shoots, reduced leaves, little leaf disease and witches´ broom always caused devastation of shrubs. "Stolbur" phytoplasmas (Mertelík *et al*., 2006), ´*Candidatus* Phytoplasma trifolii´ (Přibylová *et al*., 2009) and aster yellows (AY) 16SrI-T (Wei *et al*., 2011) phytoplasma subgroup have been identified in some plants.

Materials and methods

A rhododendron plant with symptoms of irregular shape of small malformed leaves, mosaic, leaf tip necrosis and multiple axillary shoots from Bohemia was examined by transmission electron microscopy and tested by PCR. Some phytoplasma-like bodies were found in phloem cells, and RFLP and sequence analyses enabled classification of the detected phytoplasma into 16SrI subgroup -C.

Results and discussion

A PCR product of 1.1 kbp obtained with primer pair R16(I)F1/R1 was digested with *Mse*I. The restriction profile corresponded to the profile of clover phyllody (16SrI-C). The resulting sequence of 1,724 bp, containing sequences of the 16S rRNA gene, 16S-23S rRNA spacer region and the start of the 23S rRNA gene confirmed the closest relationship of this phytoplasma with members of ribosomal subgroup 16SrI-C, however some differences were found between them and the sequence from the symptomatic rhododendron plant.

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Maize redness disease: current situation in Bosnia and Herzegovina

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Background and objectives

"Maize redness" (MR) disease symptoms were observed for the first time in 1957 in Banat district of Serbia. According to historical data during the sixties MR were also rerecorded in corn fields in Bulgaria and Romania (Bekavac *et al*., 2007). For long time the causal agent of the disease was unknown and many abiotic and biotic agents were suspected to be cause of MR. Nevertheless, Duduk and Bertaccini (2006) found that "stolbur" phytoplasma (*'Candidatus* Phytoplasma solani', subgroup 16SrXII-A) associated with MR symptoms was present in symptomatic corn plants. Ciixid planthopper *Reptalus panzeri* (Low) was proved to transmit "stolbur" phytoplasma to corn (Jović *et al*., 2007). Jović *et al*. (2009) found that johnsongrass, *Sorghum halepense,* and wheat, *Triticum aestivum,* play important role in disease epidemiology since roots of these plants serve as a bridge of overwintering populations (nymphs) of *R. panzeri*. Molecular analyses of infected corn plants showed also stolbur phytoplasma presence in Italy and Hungary (Calari *et al*., 2010; Acs *et al*., 2011).
During the past five years MR symptoms occurred in corn fields in Semberija region of Bosnia and Herzegovina causing significant yield losses. Consequently, in 2012 first survey was conducted in corn fields of Semberija in order to check the presence of phytoplasma and ciixid vectors in the region. Results of laboratory analyses showed the presence of "stolbur" phytoplasma in infected corn and johnsongrass plants as well as in *R. panzeri* (Kovačević *et al*., 2013). Since corn is an important crop in Bosnia and Herzegovina extended survey for phytoplasma presence and distribution was also conducted in 2013.

Materials and methods

During August 2013 selected corn fields in production areas of Bosnia and Herzegovina were surveyed for the occurrence of reddening symptoms on corn. Plants expressing symptoms such as midrib, leaf and stalk reddening were collected from ten sampling sites in Semberija, Brčko District and Posavina regions. On all surveyed localities, the symptomatic maize plants were in range from 10 to 50%. Maize (*Zea mays* L.), field bindweed (*Convolvulus arvensis* L.), johnsongrass [*Sorghum halepense* (L.) Pers.] and foxtail [*Setaria viridis* (L.) Beauv.] were sampled for laboratory analyses.
DNA was extracted from leaf midribs and roots of MR symptomatic maize plants and weeds collected randomly in areas adjacent to MR symptomatic fields following a CTAB protocol (Angelini *et al*., 2001). Phytoplasma identification was conducted using nested PCR assays on the 16S rRNA gene with primer pairs P1/P7 and R16F2n/R16R2 (Lee *et al*., 1998). The obtained nested-PCR products were digested with *Tru1*I restriction enzyme. Subsequently, all phytoplasma positive samples were retested in nested PCR employing tuf-gene and Stol11 "stolbur"-specific protocols (Langer and Maixner 2004; Clair *et al*., 2003). Molecular characterization of identified phytoplasma was performed by RFLP analyses of the *tuf* gene using *Hpa*II endonuclease.

Results and discussion

Corn fields with intensive wheat-maize crop rotation were selected for the survey. Wheat-maize crop rotation is a key factor for MR occurrence and persistence and found to favour the disease development because winter wheat roots can constitute a bridge (for overwintering stages of the

vector and the phytoplasma) between two maize crops (Jović *et al*., 2009). "Stolbur" phytoplasma (16SrXII-A) was identified in symptomatic maize plants collected from all three surveyed regions in Bosnia and Herzegovina. In that point of view, spreading of MR in new Bosnia and Herzegovina regions is in dynamic progress. Study of MR in Bosnia and Herzegovina showed that the disease play important role leading to significant losses in corn production in areas where wheat-maize crop rotation is practice. Presence of MR and possible correlation with longer periods of high temperatures during last few years and droughts can cause significant economic damage in maize production, since the presence of MR in new regions and the most severe damage were recorded during the warmest summer decade in 2012 and 2013.

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Fruit tree phytoplasmas and their vectors in pome fruit growing in Belgium: overview of current status and recent research efforts

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Background and objectives

Apple proliferation (AP, '*Candidatus* Phytoplasma mali') and pear decline (PD, '*Candidatus* Phytoplasma pyri') can cause severe damage and economic losses to the production of top fruit. Both pathogens are member of the 16SrX, apple proliferation group and are listed as quarantine organisms (EPPO A2).

Materials and methods

In a recent research project a survey was carried out to obtain data on the presence of AP and PD in Belgium. Root samples of symptomatic and apparently healthy apple trees were collected in 8 commercial and 3 non-commercial orchards and leaf samples of pear trees with premature yellow or red discoloration were collected in 14 commercial orchards, all samples were analysed by real-time PCR assays.

As some *Cacopsylla* spp. are able to transmit '*Ca*. P. mali' from infected trees to healthy trees within and between orchards, the dynamic of the psyllid population was studied in 4 apple orchards and in 5 hawthorn hedges from March till August.

Results and discussion

Results confirmed the presence of AP in both symptomatic and asymptomatic apple trees and in 8 of the pear inspected orchards an infection with PD was detected.

The study of the dynamic of the psyllid population studied in 4 apple orchards and in 5 hawthorn hedges from March till August allow the identification of several species were: *Cacopsylla mali, C. melanoneura* and *C. picta* in apple trees*, C. melanoneura, C. peregrina, C. affinis* and *C. crataegi* in hawthorn. PCR- analysis of individuals of *C. mali* and *C. peregrina* revealed their infectivity but in transmission trials performed with *C. mali* under lab conditions no transmission of AP to an artificial feeding source could be obtained. In a previous study *C. pyri* and *C. pyricola* were found to be present in pear trees.

These research efforts represent the first systematic survey on presence of fruit tree phytoplasmas in Belgium and the first confirmed detection of PD in our fruit growing region. The study also revealed insights into the diversity, infectivity and dynamics of the psyllid population complexes in and around pome fruit orchards.

Use of *groEL* gene in characterization of strains belonging to some '*Candidatus* phytoplasma' species

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Background and objectives
The highly conserved double copy 16S rDNA has been used for more than a decade as the primary marker for classification of phytoplasmas. However, many closely related strains, but ecologically or epidemiologically different, usually could not be differentiated by this marker while an accurate molecular distinction is necessary for phytoplasma strain characterisation and epidemiological studies. To overcome the high conservancy and the interoperon heterogeneity, new additional markers are needed (Lee *et al*., 2010).
The heat shock protein and molecular chaperone GroEL (also known as Hsp60, cpn60, MopA or Group I chaperonin), of about 60-kDa, is present in almost all bacteria but it is absent in many *Mollicutes* (Wong and Houry, 2004), while his homologues are present in plastids, chloroplasts, and mitochondria. This ubiquitous and highly conserved single-copy gene has been used for identification of many bacteria because of his species-specific sequence variations (Goh *et al*., 1996; Teng *et al.*, 2001). A new PCR system for amplification of *groEL* gene was developed and used for strain differentiation of several '*Candidatus* Phytoplasma' species (Mitrović *et al*., 2011a; 2012).

Materials and methods
Available phytoplasma and *Acholeplasma groEL* genes were acquired from the GenBank, aligned and primers were designed according to species specific sequences (Mitrović *et al*., 2011a; 2012). Phytoplasma positive samples identified on 16S ribosomal gene were subjected to PCR amplification on groEL. Successfully amplified products of representative strains were sequenced and obtained sequences were aligned with those of other representative phytoplasmas. Phylogenetic tree was constructed in MEGA 5 by Maximum Parsimony (MP) method employing *Acholeplasma laidlawii* as the outgroup; 1,000 bootstrapping was performed to estimate the stability and support for the inferred clades and nucleotide and amino acid sequence identity values were calculated.

Results and discussion
Sixty four AY strains belonging to seven 16SrI- ribosomal subgroups (-A, -B, -C, -M, -L, -F and -P) were divided into 11 groELI- subgroups (I-XI) according to their *Tru1*I and *Alu*I RFLP profiles (Mitrović *et al.*, 2011a; b).
Around 100 stolbur phytoplasma strains belonging to tuf-types a and b from six countries (Serbia, Italy, Croatia, Bulgaria and Germany) were tested. After examination of *groEL* gene sequences of the tested strains, six nucleotide differences were observed.
The majority of the strains showed no RFLP polymorphisms and were very similar also at the sequence level. However after examination of *groEL* gene sequences of the tested strains six nucleotide differences were observed. One of them was present only in some of the Serbian strains (*Tru1*I restriction site), three were differentiating the German strains belonging to tuf-type b, but one

of the showed only one SNP. One SNP was differentiating Croatian strains and the last one was specific for on of the Italian strains.
Four strains (two from Italy and one from Albania and Serbia) of '*Ca.* P. cynodontis' from *Cynodon dactylon* were successfully amplified. After examination of the *groEL* gene sequences, 39 nucleotide differences were observed - one was differentiating one of the Italian strains from the other three while the remaining 38 SNPs were differentiating the Serbian strain from the others.
The *groEL* gene was successfully amplified from three Serbian strains of '*Ca.* P. convolvuli', the obtained amplicon was shorter than those from the other ribosomal groups tested (around 1,000 bp compared to 1,400 to 2,200 bp) and the obtained sequences were identical.
Since the designed primers for 16SrI, 16SrXII ribosomal groups are specific, a multiplex PCR system for differentiating AY from stolbur phytoplasmas can be developed, which would be suitable for detecting mixed infection of these two phytoplasmas.
The phylogenetic tree based on *groEL* gene sequence analysis was highly congruent with the 16S rDNA based tree and confirmed delineation of distinct phylogenetic lineages for ribosomal groups among the phytoplasma strains analyzed. *GroEL* gene showed to have lower sequence similarity than 16S rDNA and more resolution power in separating closely related strains.

Acknowledgements
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Multiplex and real-time PCR assays for the specific detection of chicory phyllody phytoplasma (16SrIX-C)

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Background and objectives
In 2011 a severe outbreak of chicory phyllody has been reported in the Carlino area (Udine province) in Friuli Venezia Giulia region (FVG, North-eastern Italy). Plants of *Cichorium intybus* L., (chicory, family Asteraceae) showed symptoms of phyllody, virescence and proliferation of axillary buds. Molecular characterization of chicory phyllody (ChiP) phytoplasma strains (Martini *et al.*, 2012; Ermacora *et al.*, 2013) based on the three genes 16S rDNA, ribosomal protein (rp, *rpl22* and *rps3*) and *sec*Y showed that all the strains were nearly identical and were closely related to strains PEY (*Picris echioides* yellows) and NaxY (Naxos periwinkle virescence), belonging to 16SrIX-C, rp(IX)-C1 and secY(IX)C1 subgroups (Lee *et al.*, 2012). The aim of the present work was to develop new molecular tools (multiplex-PCR and real-time PCR) for specific, sensitive and robust detection of chicory phyllody and very closely related phytoplasma strains (16SrIX-C) on the basis of the two variable genes rp and *sec*Y.

Materials and methods
Chicory phyllody phytoplasma (16SrIX-C) specific primers have been designed based on rp (*rpl22-rps3*) and *sec*Y gene sequence alignments of 16SrIX phytoplasma strains including chicory phyllody phytoplasma strains and all strains used in the work by Lee *et al.* (2012).
A total of 11 primers, 5 on rp and 6 on *sec*Y gene sequences, have been designed for the specific detection and identification of chicory phyllody and very closely related phytoplasma strains (16SrIX-C). PCRs assays were performed on dilutions (20-60 ng/µl) of extracted DNA samples that were positive in previous nested-PCRs on 16S rDNA, and on 16SrIX phytoplasma group reference strains: PEY (16SrIX-C), NaxY (16SrIX-C), PPWB (16SrIX-A), KAP (16SrIX-C), AlmWB-A112 (16SrIX-B) (Lee *et al.*, 2012).
Multiplex-PCRs (duplex-PCR, with simultaneous amplification of ChiP phytoplasma rp and *sec*Y gene fragments) with four different primer combinations have been tested on 5 chicory phyllody phytoplasma strains, on 5 positive insect DNA samples and 16SrIX reference strains. Combinations of specific rp and *sec*Y primers were also used to develop a multiplex nested-PCR assay for a more sensitive detection and identification of ChiP phytoplasma.
Furthermore, one of the specific primer pair designed on *sec*Y gene (amplifying a fragment of 142 bp) has been validated in an EvaGreen® Real-time PCR protocol for sensitive and specific quantification of ChiP phytoplasma strains in plants and insects.

Results and discussion
All the four duplex-PCRs tested (with four different primer combinations), worked very well when used on plant DNA samples; however two of them resulted more sensitive allowing the simultaneous amplification of the two targets in the majority of the plant and insect samples analysed. All samples tested in multiplex nested-PCR resulted positive including the few negatives and weak positives obtained with the duplex-PCRs. Therefore the multiplex nested-PCR allowed a more sensitive detection and identification of ChiP phytoplasma in samples with a low target concentration. In the EvaGreen® Real-time PCR protocol a unique melting peak was observed with all positive samples and slopes of real-time PCR for quantification of ChiP phytoplasma were very similar, indicating a PCR

efficiency close to 100%. In all PCR assays the reference strains PEY and NaxY (16SrIX-C) yielded very good reactions, whereas all the others yielded weak or no PCR products.
In the present study, we have demonstrated that multiplex conventional PCR assays, using a combination of a rp-based and a *sec*Y-based primer pairs, effectively detected the target pathogen and gave dual signals that confirmed the pathogen identity unequivocally in a single PCR assay. Therefore, the specific multiplex-PCR and multiplex nested-PCR assays developed, represent valuable and robust tools to be used especially in epidemiological studies. Furthermore, the specific real-time PCR protocol established in the present work will allow the quantification of the target phytoplasma in both plant and insect hosts.

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Searching for new markers of the phytoplasma diseases of grapevine

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Background and objectives

Phytoplasma associated diseases cause big losses in yield and additional economic losses due to lost investment in application of pesticides to control the insect vectors, laboratory analysis for detection, follow-up costs for monitoring and eradication campaigns. Because of difficulties associated with research of phytoplasmas their early detection is hard and great efforts are put in search for new approaches and strategies for its improvement. In order to understand the determining factors of plant diseases to obtain sustainable practices for their strategic management several different data analysis tools have been employed in epidemiology, but similar approaches have not been used for phytoplasma diseases. In this work the results of data analysis based on the previous transcriptome study and carbohydrate analysis of grapevine plants infected with '*Candidatus* Phytoplasma solani', which is associated with the "bois noir" disease, were used for identifying new potential markers with high efficiency for disease forecasting.

Materials and methods

A production vineyard of grapevine (*Vitis vinifera* L.) cv. 'Chardonnay' in the southwestern part of Slovenia in the Brda region was selected for the study carried out from 2004 to 2010. 22 selected genes were analyzed as described (Hren *et al*., 2009). From the fully developed leaves glucose, fructose and sucrose were isolated and determined by the enzyme based spectrophotometric sugar determination SUFRG kit. Classification trees were calculated using Weka software using the J48 classifier.

Results and discussion

Construction of two classification trees based on the expression data from selected genes revealed two trees constructed of two genes each. Validation showed the accuracy of 92% for the first tree and 86% for the second one; the precision of 66% and 100%, respectively; and the sensitivity of 100% and 60%, respectively.

Sugar determination revealed healthy grapevine plants having either very low or high concentration of fructose. Only the low-fructose plants showed a significant change in fructose level upon the phytoplasma infection and symptom development. Up to a certain fructose concentration, each unit of sugar increase increases the odds of having a symptomatic plant in summer season by roughly 3-fold ($p<0.01$). In addition, statistical analysis revealed a significant connection between the fructose level and a gene involved in starch biosynthesis.

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Multilocus sequence typing of phytoplasma strains associated with "bois noir" in Italian vineyards

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Background and objectives

"Bois noir" (BN) is an important grapevine yellows (GY) that is widely spread in Europe and in the Mediterranean basin. BN is attributed to infection of *Vitis vinifera* by '*Candidatus* Phytoplasma solani' strains that are transmitted plant-to-plant mainly by the cixiid *Hyalesthes obsoletus* Signoret (Quaglino *et al*., 2013). Complex disease epidemiology related to the numerous weed plants hosting BN phytoplasmas within and around vineyards, and to the possible presence of additional vector(s) has increased the necessity to study the genetic diversity among BN populations in diverse ecological niches. Recent studies, based on molecular characterization of more (*16S rRNA*, *tuf*, *secY*) or less (*vmp*, *stamp*) conserved gene nucleotide sequences, evidenced an unexpectedly high genetic diversity among BN strains from European Countries (Foissac *et al*., 2013). In the present work, multilocus sequence typing (MLST) analyses including *16S rRNA*, *tuf*, *hlyC*, *cbiQ-glyA*, *trxA-truB*, *and rplS-tyrS-csdB* were carried out for investigating the genetic diversity among BN populations from distinct regions of Italy.

Materials and methods

BN strains identified in 108 grapevine plants collected from 2008 to 2012 in vineyards located in Lombardia (north-western Italy), Veneto (north-eastern Italy), Marche and Abruzzi (central Italy), and Sicily (southern Italy) regions were characterized by polymerase chain reaction (PCR)-based amplifications of the genes *16S rRNA*, *tuf*, *hlyC*, *cbiQ-glyA*, *trxA-truB*, and *rplS-tyrS-csdB*, followed by Restriction Fragment Length Polymorphism (RFLP) analyses. Reaction conditions were as previously described (Quaglino *et al*., 2009). PCR products and RFLP patterns were visualized on 1% and 3% agarose gels, respectively, under a UV transilluminator.

Results and discussion

RFLP analyses performed on 16S rDNA amplicons revealed that all BN strains analyzed in the present work belong to subgroup 16SrXII-A. Based on *tuf* gene characterization, BN strains were divided in two types, BN-type I (formerly called VK-I or tuf-type a) and BN-type II (formerly called VK-II or tuf-type b). BN-type I was prevalent (65 strains) in the examined Italian vineyards, but prevalence of the two BN-types differed depending on the region studied. Thus, BN-type I was prevalent in Veneto; type II in Marche/Abruzzi and in Sicily. In Lombardia, BN-types I and II were almost identical in regional prevalence. Based on *hlyC* RFLP analyses, two distinct restriction patterns were found, distinguishing the BN strains into two groups consistent with BN-types. Based on *cbiQ-glyA*, *trxA-truB*, and *rplS-tyrS-csdB* RFLP analyses, it was possible to identify two distinct restriction profiles for each of those genomic segments, distinguishing the BN strains into groups that were not consistent with those defined by *tuf*/*hlyC* gene RFLP patterns. Considering the *cbiQ-glyA*, *trxA-truB*, and *rplS-*

tyrS-csdB RFLP profiles of each BN strain as unique collective RFLP pattern, it was possible to evidence the presence of eight collective patterns: four associated with BN-type I and II; three unique for BN-type II; and one unique for BN-type I. In detail, five and seven collective patterns were identified among BN-type I and II strains, respectively, showing a possibly higher genetic variability among BN-type II. Such evidence reinforces previous results obtained by *vmp1*, *stamp,* and *groEL* gene sequence analyses (Murolo *et al*., 2010; Foissac *et al.,* 2013*;* Mitrović *et al*., 2013).

On the basis of overall RFLP collective patterns, MLST analyses including all the analyzed genes evidenced the presence of 12 BN lineages (BN1 to BN12) among BN phytoplasma populations analyzed in the present work. Prevalent lineages were BN1 (24 strains), BN4 (20 strains) and BN12 (15 strains), but in each examined region the lineage prevalence was found different. In detail, BN1 and BN4 were prevalent in northern Italy, BN12 in central Italy, and BN6 in southern Italy. Interestingly, no BN lineage was found in every region studied, suggesting a possible influence of region-specific weed host plants and/or insect vector(s) in selecting BN strain lineages and determining regional BN population composition.

Molecular markers identified in the present study will be employed for investigating more accurately the presence of BN lineages in grapevine, weed host plants and insect vector(s) in diverse ecological niches in order to clarify the "bois noir" epidemiology.

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DNA barcoding of phytoplasmas: a tool for their fast identification

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Background and objectives
Phytoplasmas infect a high number of plant species. Based on the 16S ribosomal gene sequence, over 30 RFLP groups have been established within the '*Candidatus* Phytoplasma' taxon, some of which are of quarantine status. 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 a universal DNA barcoding based tool for phytoplasma identification was developed and the system was validated for relevant quarantine phytoplasmas. The phytoplasma *secY* and *tuf* genes were employed as tools to provide reliable information in combination with 16SrDNA for phytoplasma strains differentiation (Makarova *et al*., 2012; 2013).

Materials and methods
Two sets of primers amplifying a fragment of the *Tuf* 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.

Results and discussion
The set of *Tuf* specific primers designed amplified a product (420-444 bp) from all phytoplasma strains tested and belonging to a number of 16S ribosomal groups including both EU quarantine and non-quarantine representatives. Similar results were obtained with primers amplifying a fragment of about 600 bp at the 3' of the 16S ribosomal gene. 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 sub-group level suggest that these barcodes are efficient phytoplasma identification tools. *Tuf* barcode sequence alignments and NJ tree construction showed that the *Tuf* tree was highly congruent with 16S rDNA derived trees. The *Tuf* amd 16S ribosomal barcodes could separate main ribosomal groups and most of their sub-groups. 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 of quarantine status. The obtained sequences can thus be used by plant health services and researchers for online phytoplasma identification following an amplification scheme reported in the same web page.

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To the problem of reliable detection and sanitation of European stone fruit yellows phytoplasma in apricots

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Background and objectives

The presence of European stone fruit yellows (ESFY) phytoplasma, '*Candidatus* Phytoplasma prunorum', (Bertaccini, 2005) was confirmed in the Czech Republic in 1990s (Navrátil *et al.*, 1998). The phytoplasma infected apricot trees died within two years after appearance of symptoms and infection was verified only in symptomatic apricot trees (Navrátil *et al.*, 2001). In 2002 ESFY phytoplasma was detected in symptomless apricot trees and they were not dying. The results of nested PCR were completely different from the biological test (Polák *et al.*, 2006). Therefore another experiment was established in apricot orchard where typical symptoms of ESFY were observed.

Materials and methods

Collection of samples from thirty eight trees of four apricot cultivars 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. All trees were tested by direct and nested PCR assays in every year. Specific primers fAT/r PRUS were used in direct PCR assays (Lorenz *et al.,* 1995). The first step of nested PCR was carried out with the primers R16F1/R16R0 (Lee *et al.,* 1995), the second step with two different pairs of primers, R16F2/R16R2 (Lee *et al.,* 1995) and fU5/rU31 (Lorenz *et al.,* 1995).
The hot water (temperature 45°C) was used for the elimination of ESFY in shoots of several apricot cultivars. *In vitro* cultures of apricot cultivars ´Leskora´ and ´Bergeron´ were prepared in trials to eliminate ESFY by combination of *in vitro* culture with chemotherapy. Six different media and two concentrations of tetracyklin (10mg/l and 20 mg/l) were applied.

Results and discussion

Eighteen trees showed no ESFY symptoms during the whole eight years period of evaluation. Six trees with ESFY symptoms in the first year of evaluation died by the end of the first vegetation period. Another twelve trees died during the next seven years, from that three trees died of apoplexy. The number of died trees during the eight years: 1. – 6, 2. – 0, 3. – 5, 4. – 1, 5. – 1, 6. – 3, 7. – 1, 8. – 2. Apricot trees showing ESFY symptoms died in the year when ESFY symptoms appeared, or in the next year. The biological indexing on GF-305 was not reliable because a lot of negative results were obtained from tested trees with ESFY symptoms. Some false positive reactions was recorded by nested PCR in several trees with no ESFY symptoms during the eight years of evaluation. The only reliable detection of ESFY resulted to be visual evaluation of specific symptoms in leaves and shoots. Thirty nine percent of apricot trees infected with ESFY died during the eight years of evaluation.
The attempts to eliminate ESFY by hot water and by *in vitro* cultures of apricots in combination with chemotherapy were not successful. Some positive results with hot water were obtained without confirmation by molecular tests. Cultures of apricots died in two months, therefore the thermotherapy was not suitable to use.

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Identification and characterization of European stone fruit yellows and pear decline phytoplasma strains in Austria

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Background and objectives

European stone fruit yellows (ESFY) and pear decline (PD) are widely spread in Austria and cause severe damage in affected cultures. So far little is known about present phytoplasma strains. Previous work showed a high variability in virulence of different ESFY strains in other European countries and cross protection has been reported as a possible tool for disease control (Kison and Seemüller, 2001; Ermacora *et al*., 2010). Identification and characterization of ESFY and PD strains in Austria aims to create a basis for further analyses and control strategies.

Materials and methods

Various apricot and pear orchards with different origin of planting material were sampled for phytoplasma identification and strain characterization. Symptom expression of trees in the orchards has been recorded. Samples were first screened for phytoplasma infections on 16S rDNA gene. Genes previously shown as being less conserved such as *imp*, *hflB* and *aceF* were chosen for strain characterization (Danet *et al*., 2011). Amplification products were sequenced and aligned in ClustalW. Sequences were further analysed by maximum parsimony (phylip) method and visualized in T-Rex.

Results and discussion

Analyses of the *imp* gene revealed polymorphisms in ESFY and PD sequences. Comparison with NCBI database showed homologies with published imp strains but also different polymorphisms could be observed. Interestingly different ESFY strains were present not only within Austria but even within orchards. Analyses of *aceF* gene displayed less dissimilarities. Amplification of *hflB* gene could only be obtained for a few pear decline samples but not for European stone fruit yellows.

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

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Background and objectives
"Bois noir" (BN) is widespread in several winegrowing regions in Austria. Investigations between 2003 and 2008 revealed an absence or low population densities of the confirmed vector *Hyalesthes obsoletus* in most viticultural areas of Austria. The phytoplasma was frequently ascertained in bindweed and grapevines whereas infections of nettles were very unusual. Analysis of tuf-types showed sole presence of tuf type b (Riedle-Bauer *et al*., 2006; 2008). The aim of the present study was to collect update data on the epidemiology of BN in Austria and to characterize previous and present "stolbur" strains by the molecular markers secY, stamp, tuf and vmp1.

Materials and methods
The investigations were carried out in more than 30 vineyards and their surroundings all over Austria. *H. obsoletus* and *Anacaratagallia ribauti* were collected by vacuum sampling directly from *Urtica dioica* and *Convolvulus arvensis* using a modified garden blower-vac (Stihl, Dieburg, Germany). Morevoer samples were 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 (Langer and Maixner 2004; Riedle-Bauer *et al*., 2008). Phytoplasma DNA was amplified directly as previously described with the primer pairs stampfw-0/rv-0 (stamp, Fabre *et al*., 2011), fTUFAY/rTUFAY (tuf, Schneider *et al*., 1997), POSecF1/POSecR1, TYPH10F/TYPH10R (secY and vmp1, Fialová *et al*., 2009), and for 16S with 5-CTAATACATGCAAGTCGAACG-3 (R16mF2m) and 5- TGACGGGCGGTGTGTACAAACC-3 (R16R2m) for 40 cyles, 30 sec 94°C, 45 sec 58°C and 90 sec at 72°C with 10 min final extension. The PCR products were sequenced with stamp fw-0, TYPH10R, rTUFAY and R16R2m, respectively. Field collected *H. obsoletus* and *A. ribauti* were employed transmission trials on *Catharanthus roseus* seedlings using 10 to 50 insects per experiment.

Results and discussion
Transmission experiments with *H. obsoletus* collected from stinging nettle and with *A. ribauti* collected from bindweed resulted in several infections of *C. roseus* and in successful transmission of three different strains based on stamp and vmp sequences. In contrast to earlier works (Maixner, 2011) we have not found the previously described association (Langer and Maixner, 2004) between tuf type and herbaceous host plant in the epidemic cycle. Positive *H. obsoletus* individuals trapped from stinging nettles were infected with tuf-type b stolbur phytoplasmas and transmitted this stolbur type to *C. roseus*. The same tuf type was identified in grapevines and in stinging nettles.
Sequence analysis shows, however, that only the minority of these tuf sequences are "pure" tuf-type b type as compared with reference strains, but the majority has a G instead of an A on position 568. For a better analysis of epidemiological cycles we characterized also secY, stamp and vmp1 sequences and identified at least 7 stolbur subtypes in Austria. The finding raises the question whether in the investigated vineyards tuf-type b stolbur phytoplasmas are transmitted in an epidemiological cycle including *H. obsoletus* and nettles. Moreover our data indicate 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 we now face high population densities of *H. obsoletus*

and frequent infections of nettles. BN is a disease characterized by sudden outbreaks and different epidemiological cycles (Maixner, 2011). It seems likely that a transmission cycle including nettles and *H. obsoletus* will account for future BN outbreaks in Austria.
Interestingly, we also found in three nettle and several bindweed samples 16S sequences corresponding to bindweed nettle phytoplasma '*Candidatus* Phytoplasma convolvuli', a recently described taxon (Martini *et al*., 2012) and could also describe strains within this phytoplasma based on vmp1 and tuf sequences.

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

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Background and objectives

"Bois noir" (BN) is one of the most important grapevine yellows in Europe and it is associated with the stolbur phytoplasma named *'Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013). BN 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 vectors is an important tool to find out more about the vectors and the host plants involved in the dissemination of BN. In Spain, BN disease was first identified in 1994 (Laviña *et al.,* 1995), but in recent years its incidence seems to have increased in different wine regions (Sabaté *et al*., 2007). According to the results obtained in previous studies, the tuf-type b was the prevalent in the regions studied in Spain. 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 arvensis* while the strain tuf-type a is associated with *Urtica dioica*. With the aim to determine the cause of the BN incidence increase, a new study about Bn strains 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 in *C. arvensis* and *U. dioica*. The insects were captured weekly with a D-Vac aspirator and 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 is being evaluated using PCR-RFLP analyses with primers for *tuf* gene encoding the elongation factor TU and primers stol-1H 10, for a gene *vmp* 1 encoding a membrane protein (Langer and Maixner, 2004, Cimerman *et al.,* 2009).

Results and discussion

In the surveys conducted in Spain until now, 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 the surveys conducted 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 obtained on *C. arvensis*. The maximum population in *U. dioica* was obtained in Navarre in August 6th, where 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. The individuals captured in *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 tyf-type b continues being the only one identified.

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Advances in molecular detection of the sesame phytoplasmas and resistance to sesame phyllody in Turkey

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Background and objectives
Sesame plants with symptoms typical for those associated with phytoplasma infections were observed in Antalya province of Turkey. Symptoms includes of proliferation of branches with smaller leaflets, yellowing, decline and witches' broom. Identification and molecular characterization of phytoplasmas in sesame have been reported in several countries. Restriction fragments length polymorphism (RFLP) analysis together with the sequencing of 16Sr phytoplasma genes and PCR amplification of rDNA gene are predominantly used for sesame phytoplasma detection and characterization. However, further molecular analyses combined with field observations would be necessary for better phytoplasma characterization and selection of resistant and/or tolerant plants. The objectives of the present study were detection and quantification of sesame phytoplasmas with real-time PCR and field selection of resistant genotypes in a world-wide sesame collection.

Materials and methods
A wide collection of sesame varieties was grown in experimental field of Antalya, Turkey for screening to phytoplasma infection. Genotypes were observed under both field and greenhouse conditions. In the field, genotypes were scored using 1-5 (resistant-susceptible) scale according to degree of phyllody symptoms. The possible resistant/tolerant varieties identified in the field were further analysed under greenhouse condition. The plants in the greenhouse were observed for symptom development or tested by molecular analysis. Molecular assays were conducted with real-time PCR assay. Total DNAs were extracted using a modified CTAB extraction method and real time PCR assays were used for detection and quantification of sesame phytoplasmas. This technique with relative quantification method allowed to verify the phytoplasma quantity for each variety.

Results and discussion
About 28% of the sesame collection scored 1 which means that they had no phyllody symptom. These genotypes were further analysed under greenhouse conditions and the disease transmission were made by the vector insect, *Orosius orientalis.* Up to date, 24 genotypes of possible resistant varieties identified under field conditions were tested under greenhouse conditions and the rest is currently under investigation. Three of those genotypes were identified as highly tolerant to the disease because either symptom development was no recorded or symptoms were observed one month later comparing to control plants. These accessions were also analysed by real time-PCR assays and as a preliminary result, their phytoplasma loads were at negligible level compared to control plants. In addition, these accessions were able to produce capsules and seeds. The study also showed that the real time PCR technique was very powerful for qualitatively and quantitatively detecting the pathogen in sesame.

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Monitoring programs for quarantine phytoplasmas on grapevine and fruit trees and problems for the phytosanitary control in Bulgaria

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Background and objectives

Apple proliferation (AP), pear decline (PD), apricot chlorotic leaf roll (ESFY) and "flavescence dorée" (FD) phytoplasmas are quarantine diseases described in Annex 1, Part A Chapter II of Ordinance № 1 of 27 May 1998 for phytosanitary control (Corresponding to Dir 2000/29/EC). They are the most economically important diseases in many grapevine and fruit-growing areas of Europe and fruit growing regions in Bulgaria (Topchiiska and Sakalieva, 2001; 2002; EPPO, 2006; Sakalieva *et al*., 2007, Avramov *et al.,* 2011a; 2011b). To verify their distribution in the country to implement adequate control measures, the phytosanitary inspectors from Bulgarian Food Safety Agency annually perform surveys in the country as part of the official monitoring program for quarantine pests on grapevine and fruit trees.

Materials and methods

SAMPLE COLLECTION. Since 2003 the control has been provided in Border Inspector Points on imported planting materials, in fruit plantations created by such material and mainly in nurseries, producing fruit seedlings, mother trees and growing materials (Table 1). Vector Insects for phytoplasma are usually collected from June until September (Avramov *et al*., 2011a; 2011b; Etropolska *et al.,* 2011).
DNA's were extracted from symptomatic and asymptomatic grapevine plants and fruit trees using a CTAB method (Doyle and Doyle, 1990), and from single insect vector by the method of Maixner *et al* (1995). Final products are suspended in 200 µl of sterile double distilled water or TA buffer [pH 8].
PCR ASSAYS. The extracted DNA was tested by nested PCR (Lee *et al*., 1995), using two pairs of generic primers (P1/P7, R16F2/R16R2) and specific primers for AP group (fO1/rO1) on fruit trees (Lorenz *et al.,* 1995) followed by RFLP analyses using restriction enzymes *Rsa*I *(Afa*I*), Alu*I and *Ssp*I (Thermo Scientific, Lithuania). PCR and RFLP products were analysed by electrophoresis on 1.5% agarose gel followed by staining with ethidium bromide and visualization with a UV transilluminator.

Results and discussion

Apple proliferation phytoplasma was detected in Blagoevgrad, Kjustendil, Pazardjik, Plovdiv and Veliko Turnovo regions (Table 1.) Pear decline phytoplasma was detected on the territory of 10 Regional Food Safety Services (RFSS) - Blagoevgrad, Kjustendil, Pazardjik, Plovdiv, Veliko Turnovo, Sliven, Silistra, Bourgas, Yambol and Haskovo. Apricot chlorotic leaf roll phytoplasma was detected in Pazardjik, Lovech, Sliven, Dobrich, Bourgas and Yambol.
"Flavescence dorée" phytoplasma was not detected in Bulgaria. During the monitoring program "bois noir" (BN) phytoplasma was detected (Avramov *et al*., 2008). Phytoplasma presence was confirmed in 181 samples in all Bulgarian vineyards. Infection on vectors from Psyllidae was detected in 13 out of 28 Regional food safety services and *Scaphoideus titanus* was identified but not found to be infected with FD. On the bases of the results to eliminate possibility to spread the infection, Phytosanitary department of BFSA took measures to eradicate 200 mother trees, 4,600 units of plants for planting – 1st year, 22,000 units 2nd year and up to 2 ha of garden plots. Second step was the optimization of the monitoring program and development of additional measures including - increasing the number of plant samples and samples from insects for analysis in high-risk areas, - development of preventive

measures in mother trees and nurseries and - focus on the monitoring of the vectors across the country, to get a comprehensive picture of their distribution in the region and of their population density. Works of optimization were performed also on the development of an effective scheme against vectors. Extremely small number of registered insecticides against vectors of phytoplasma and officially controlled stock production plots appears a major problem at the moment.

Table 1. Results of molecular tests on plant samples collected during the period of monitoring programs.

Period	Regions	Samples tested		Results		Regions with infection
		Grapevine	Fruit trees	Grapevine	Fruit trees	
2003-2004	Bourgas, Sliven, Pleven, Varna, Plovdiv, St. Sagora, Rousse, Blagoevgrad	83	-	0	-	None
2005	Pleven, Veliko Turnovo, Dobrich, Haskovo	352	-	10 BN	-	Sliven (7), Lovech (2), Veliko Turnovo
2006-2007	All 24 vine growing	962	-	23 BN	-	14
2008	All	578	326	34 BN	13 PD 12 AP	BN: all 24 regions PD: Rousse, Plovdiv, Kjustendil AP: Plovdiv, Pazardjik, Veliko Turnovo
2009	All	632	276	43 BN	5 PD; 5 AP 3 ESFY	BN: all regions; PD and AP: Plovdiv ESFY: Yambol, Dobrich
2010	All	274	226	19 BN	13 PD 3 ESFY 1 AP	BN: all regions PD: Bourgas, Rousse, Kustendil AP and ESFY: Yambol, Pazardjik
2011	All	305	348	1 BN	6 PD 4 ESFY	BN: Bourgas ; PD: Rousse, Pazardjik ESFY: Pazardjik, Kjustendil, Bourgas
2012	All	392	437	44 BN	31 PD 10 ESFY 5 AP	BN: all regions; PD: Rousse, Haskovo, Plovdiv, Silistra, Kjustendil ESFY: Sliven, Lovech, Bourgas AP: Plovdiv, Pazardjik

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Modeling epidemiological and economic consequences of "flavescence dorée" to Austrian viticulture as a novel decision tool for control strategies

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Background and objectives

The knowledge and understanding of the biology and the behaviour of *Scaphoideus titanus* and "flavescence dorée" (FD) is essential when planning effective control measures against the FD disease. Stochastic spread simulation is a very useful tool, providing insight into the spread dynamics of plant diseases and enabling the identification of critical control points and the prediction of high risk areas. The aim of the economic impact analysis is to evaluate different intervention and abatement strategies regarding the spread of FD. As FD is a new invasive disease in Austria and control experience is limited, the provision of novel decision tools for decision makers and stakeholders is important. Key objectives of the research project VitisCLIM include the development of a stochastic spread model to simulate the temporal and spatial spread dynamics of FD and the assessment of the potential economic impact of the disease on Austrian viticulture as a function of different pest management options.

Materials and methods

Two Austrian model regions (the communities Tieschen and Glanz in two different vine growing areas of Styria) were selected. These domains have differing situations in disease incidence, in the abundance of wild arbours, in the average acreage of vineyards and in the presence of organic vineyards.

Using an individual-based Monte-Carlo simulation model, geographic and topographical information could be incorporated into the spread model. Each vineyard or arbour was represented by a set of static and dynamic data. Dynamic data were created for each plot and each day in the season reflecting the spread of the disease and its vector. The model was initialized by using different realistic scenarios. The results of the model runs were compared with data and spread patterns of the real outbreaks in these communities and thereby the model was calibrated.

For a macroeconomic impact analysis the most appropriate method is input-output analysis (IOA). In the context of this topic a multi-regional IOA was used to determine the economic impact of FD based on a multiregional input-output table. For each model region (Glanz and Tieschen) the spread model evaluated different initial conditions. These considered the intensity of the initial disease outbreak (severe/limited) and the size of the initial leafhopper population (large/small). Based on the existing data eight scenarios of potential economic impact were calculated depending on the selected intervention scenarios as reaction to given outbreak scenarios. Different current control strategies depending on the type of municipality were tested in the economic impact model.

Results and discussion

For both model regions (Glanz and Tieschen), the spread simulation illustrated the importance of early detection. Furthermore, the result revealed that robust varieties in arbours favour the spread of the disease, as – apart from being the favourite host plant of *S. titanus* – they typically display an

unclear disease pattern or do not exhibit symptoms at all and, therefore, act as a reservoir for the disease.
The results of the economic impact model revealed that the potential losses calculated for eight scenarios vary from zero to 5-6 Mio Euro. The scenarios demonstrate that in situations of limited outbreaks potential economic losses are not likely to occur. The two situations that result in high potential losses are both related to severe outbreaks. A severe outbreak in a community of type Tieschen (many wild *Vitis* plants in hedges and arbours) demands a control strategy that includes both arbours and other wild *Vitis* sp.. In cases where outbreaks are limited, controls of the arbours are not effective from an economic point of view. In communities of type Glanz (limited number of wild plants) high intensity control strategies are necessary only in cases of severe outbreaks.
A scientific basis was developed to better understand the different factors involved in the local spread of FD and *S. titanus* in a grapevine growing area. The biology of the vector and the pathogen, as well as the topography and the agricultural structure of the domain were considered. The results can be used as a decision making tool in an outbreak-case, identifying the best specific risk reduction option with respect to its efficacy on the spread of FD and on its cost-effectiveness.

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Scaphoideus titanus Ball and ''Flavescence dorée'' disease in Portugal

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Background and objectives
"Flavescence dorée" (FD) is a grapevine disease that affects several wine production areas in Europe, namely in Portugal (Sousa *et al*., 2003). FD is associated with a phytoplasma belonging to ribosomal subgroups 16SrV-C/D which is spread, in Portugal as in the other infected areas of Europe, throughout the vineyards by the leafhopper *Scaphoideus titanus* Ball (Cicadellidae) (Quartau *et al*., 2001; Sousa *et al*., 2009).

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

Results and discussion
Since it is impossible to distinguish FD from other grapevine yellows (GY) just through observation of the symptoms, nested-PCR is used to confirm the symptoms and removal of FD infected plants has been done by uprooting all symptomatic grapevines in infected FD parcels. 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 diffusion of FD 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. However, despite these measures both, *S. titanus* and FD are progressing along the Portuguese northwest region.

At the end of 2012 several stakeholders (government, researchers, farmers, nurserymen's representatives and wine organizations) have joined in order to prepare a particular national action plan to control the FD disease in the country called by PAN-FD, setting up new intervention measures for strengthening of existing ones.
Towards this aim a new project was developed named PAN-FD DGAV-2013, "Plano de Ação Nacional para o Controlo da Flavescência Dourada (PAN-FD)" (www.dgav.pt). The PAN-FD's main objectives are to contain the disease within the wine-growing regions where it is declared, reducing to a minimum the impact on the grape production and wine industry in these regions. Starting with a strategy of medium-term actions can contribute to eradicate the disease, or, if it will be not possible, at least to reduce the presence of the disease to acceptable phytosanitary and economic levels.

Another important goal of the project is ensuring the health status of grapevine propagation material and ensure confidence and sustainability of national nursery industry. Some of this new measures were included in 'grapevine sampling surveys', 'containment measures of insect vector' and 'eradication measures'. The particular factors that determined disease progression in northeast region of Portugal in last years and the definition of new measures that will provide an effective control of the disease will be also undertaken.

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

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Background and objectives

The "flavescence dorée" (FD) is a grapevine yellows disease causing severe damage in European vineyards. The phytoplasma associated with the disease, is transmitted 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 vineyards untreated with insecticides (cultivated or abandoned) high population levels of *S. titanus* were found (Pavan *et al*., 2005a, 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 al*., 2005a, 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 in Italy only in grape-growing areas where FD is present (Barba, 2005; Pavan *et al*., 2005b). These areas are annually identified by the local governments, but 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 without *S. titanus* control to the 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 vineyards are present.
YEAR 2011. Breganze location (45°42.43' N - 11°34.50' E, 117 m a.s.l.), Merlot and Cabernet Sauvignon cultivars, espallier training system at a distance of 3.5 m x 0.9 m. The treated (two applications/year organophosphate and neonicotinoid against young stages) one 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 for 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. 30m x 30m). 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 we assessed the local contribution to a group (cluster) of relatively high-density (patch) or to a group of zero or relatively small counts (gap). Tests of non-randomness based on the overall index of

aggregation (Ia) and on the average indexes of clustering into patch ($\overline{Vi}$) and into gap ($\overline{Vj}$) were performed ($\alpha = 0.05$) using kriging with SURFER (Golden Software Inc., CO), indexes of local aggregation (vi; vj) and catches were mapped on a two-dimensional map showing their spatial distribution.

Results and discussion

S. titanus adults were captured in both untreated and treated vineyards, but the captures were significantly higher in untreated ones. 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, but some patches were found in treated vineyards, particularly in 2011. In both grape-growing areas a decreasing gradient of *S. titanus* captures were observed from untreated to treated grapevines. Results proved that the vector 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 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 at the end of August. The risk posed by grapevines untreated with insecticides against the *S. titanus* for FD spread in other vineyards is confirmed by the results obtained. These 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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Mark, release and recapture experiments in two model apricot orchards tracking the effects of the insecticide thiacloprid on the dispersal of *Cacopsylla pruni* (Hemiptera: Psyllidae), vector of European stone fruit yellows

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Background and objectives

During the last fifteen years European stone fruit yellows associated with '*Candidatus* Phytoplasma prunorum' has become a major concern in Austrian apricot production (Maier *et al.*, 2013). Well adapted management strategies including control of the disease vector *Cacopsylla pruni* are an urgent need. A mark and recapture study with overwintered *C. pruni* in a model apricot proved a fast and frequent tree to tree movement (Maier *et al.*, 2013). Here we present mark and recapture experiments tracking the effects of the insecticide thiacloprid on dispersal of overwintered *C. pruni* in two apricot orchards.

Materials and methods

Overwintered *C. pruni* adults were collected on *Prunus spinosa* and marked by several fluorescent dyes. Stability of the dyes in the field and effects of the pigment on insect survival were examined as previously described (Maier *et al.*, 2013).

The field experiments were carried out right after flowering in two apricot orchards (orchard 1: spindle shaped trees, within row spacing 2 m, distance between rows 4 m, 15 by 9 trees; orchard 2: standard trees, within row spacing 4-5 m, distance between rows 5-6 m, 5 by 9 trees). Per experiment 600 stained *C. pruni* were freed on one single tree in the middle of the orchard. Insect survival and movement in untreated and insecticide treated orchards were monitored by yellow sticky traps and UV light for one week. In order to estimate the effect of the insecticide on freshly incoming remigrants trees were sprayed before insect release. Thiacloprid (Calypso, Bayer Crop Science, Mannheim, Germany) was applied according to the manufacturer's instructions and trees were allowed to air dry for 4 hours before insects were freed.

Results and discussion

After exposure of marked insects to outdoor conditions for five weeks all dyes were clearly visible in UV light. Laboratory experiments revealed no statistically significant adverse effects of the dyes.

Orchard 1. On untreated trees 37 marked and 128 unmarked *C. pruni* were captured within one week. Already after 24 h some marked specimen 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 2000 *C. pruni* individuals naturally occurred in this orchard. Application of thiocloprid had a striking effect. It decreased the number of stained insects to 10 and the number of unstained individuals to 18. Spatial dispersal of stained individuals was reduced to the

close vicinity of the release tree. Insects were mainly trapped within 24 hours after release, after this period only two specimens were caught.
Orchard 2. The effect of thiacloprid was less prominent although still clearly visible. Before insecticide treatment on average (of 3 repetitions) 17 marked and 40 unmarked individuals were captured allowing the calculation that around 1600 individuals naturally occurred in the orchard. Some insects reached the farthest traps already within 24 h after release and insect movement lasted during the whole observation period. Insecticide treatment decreased the number of stained insects on the traps to 11, the number of unmarked individuals to 18. As in orchard 1 thiacloprid reduced the spatial dispersal and almost all individuals were trapped within 24 h after release.
Our data indicate that mark and recapture experiments could be a helpful tool to monitor the effect on insecticides on vector dispersal.
Thiacloprid significantly reduced insect spread especially in orchard 1. It should therefore also reduce disease spread as long as its insecticide effect lasts. Further experiments, however, are required to explain the apparently lower effect of thiacloprid in orchard 2. The differing tree shapes might result in different quality of insecticide application or influence the trapping success. A repellent effect of thiacloprid could explain the finding that after treatment relatively high numbers of individuals were trapped close to the release point within the first 24 h (in both orchards). Additionally also site or "grower" effect is conceivable.
Although the results of our study might represent a first step towards an appropriate sustainable management of *C. pruni* there is still a long way to go. The flight period of the remigrants lasts at least for 6 weeks, starts early in the year and includes the blossom period. In order to overcome 6 weeks a combination of several insecticides and applications considering 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 field experiments proving the effects of such a vector control strategy on disease spread are necessary.

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

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Background and objectives
Apple proliferation (AP), associated with the presence of '*Candidatus* Phytoplasma mali', is one of the most important phytoplasma diseases in Europe. So far, due to the absence of AP resistant varieties, its management mainly consists in insecticide treatment against the insect vectors and in the eradication of symptomatic plants. These treatments have a strong economic and environmental effect, representing a risk for both operators and final customers. One of the most innovative solutions to develop sustainable approaches is the use of endophytes as inducers of the natural plant defense responses. Bacterial endophytes are plant-associated bacteria that affect the plant life cycles in different manners such as nitrogen fixation or the biocontrol of plant pathogens (Lugtemberg and Kamilova, 2009). 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. In this work, the endophytic bacterial community associated with healthy and AP phytoplasma-infected apple roots was described and characterized in order to find potential biocontrol agents.

Materials and methods
Apple roots were collected from five asymptomatic and five AP-symptomatic plants during field survey conducted in 2010 in North-western Italy. Roots were opportunely sterilized and the total DNA was extracted with 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). Endophytic bacterial community associated with healthy and '*Ca*. P. mali'-infected apple roots was described by cultivation dependent and independent methods (16S rRNA gene library analyses and sequencing). Endophytic bacteria were isolated by cultivation on Tryptic Soy Agar (TSA) and Luria-Bertani (LB) media. Total DNA from bacterial colonies was extracted and the 16S rRNA genes were amplified and sequenced using bacterial universal primers. The endophytic bacteria isolated on culture media were characterized for five beneficial traits related to mineral nutrition (phosphate solubilization, siderophores, nitrogen fixation), development (indolacetic acid synthesis), stress relief (catalase activity), disease control (siderophores).

Results and discussion
The diversity of microbiota associated with healthy and phytoplasma-infected apple roots was investigated both with cultivation-independent and -dependent methods in order to increase the range of diversity explored in a sample. 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 clones from the healthy roots and 44 from the infected ones. 16S rDNA sequence analysis showed the presence of the groups *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Chlamydiae*, and *Firmicutes*. Library analyses underscored 24 and 17 Operational Taxonomic Units (OTUs) in healthy and infected roots, respectively, with a dominance of *Betaproteobacteria*. 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), moreover the influence of pathogen infection on

endophytic bacterial community was reported in healthy and phytoplasma-infected grapevine leaves (Bulgari *et al*., 2011).
Cultivation dependent methods allowed to isolate twelve colonies with different morphology 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*. Six different *Bacillus* species were isolated from healthy apple trees and, among these, *Bacillus amyloliquefaciens* and *Bacillus gibsonii* were found also in infected plants. Interestingly, bacterial strains, here isolated, belong to genera widely studied for developing biocontrol strategies to contain plant pathogens. The endophytic bacteria isolated on culture media were characterized for the five beneficial traits described above in order to select some strains for *in vivo* biocontrol tests. These assays allow to select different strains belonging to the genus *Pseudomonas* and *Lysinibacillus* that showed biocontrol and plant-growth promotion abilities. Future studies will be carried out to investigate the activity of these endophytes to control apple proliferation.

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From genomics to the characterization of virulence mechanisms of phytoplasmas

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Background and objectives

Phytoplasmas are insect-transmitted bacterial pathogens that induce dramatic changes in plant development, including proliferation of stems (witches' brooms) and the 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*., 2011a). In plants, phytoplasmas are restricted to the phloem. The virulence effectors may unload from the phloem and migrate to different plant tissues (Hogenhout and Loria, 2008; Sugio *et al*., 2011a).

Materials and methods

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*., 2011b). A yeast two-hybrid screen was conducted to find targets of SAPs in the plant host (Sugio *et al*., 2011b).

Results and discussion

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) and SAP11 was detected in nuclei of cells beyond the phloem in AY-WB-infected plants suggesting that SAP11 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*., 2011b). 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*., 2011b). 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*., 2011b). SAP11 destabilizes these transcription factors resulting in changes in leaf shape, increased production of stems, downregulation of jasmonic

acid (JA) production and greater susceptibility of plants to AY-WB leafhopper vectors (Sugio *et al*., 2011b). 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' genus

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Background and objectives

The family *Acholeplasmataceae* comprises the genus *Acholeplasma* and the provisory taxon '*Candidatus* Phytoplasma'. Strains of the last one are associated with several hundred of plant diseases worldwide infecting many important crops (Bertaccini, 2007). Phytoplasmas are characterized as obligate parasites of the sieve cells and mainly spread by phloem-sucking insects. Colonization by phytoplasmas can result in manipulation of host and vector by interacting with membrane proteins and/or secretion of effector proteins (Sugio *et al.*, 2011). In contrast, acholeplasmas are widespread saprophytes, not described as primary pathogens. Several *Acholeplasma* species were reported as commensals of plants (*A. laidlawii*, *A. axanthum* and *A. oculi*) and some of them, such as *A. brassicae* and *A. palmae,* refers even by scientific name to the source of isolation (Tully *et al.*, 1994).

Genome research provided a first view on the metabolism of phytoplasmas and was also the starting point for many subsequent studies. Six complete genome sequences from *Acholeplasmataceae* were determined so far comprising two strains from '*Ca.* P. asteris' (OY-M, AY-WB) and '*Ca.* P. australiense' (PAa, SLY), '*Ca.* P. mali' (AT) and *A. laidlawii* (PG-8) (reviewed in Kube *et al.*, 2012; Andersen *et al.*, 2013). Here, we provide insights into the complete genome sequences of *A. palmae* and *A. brassicae*. The comparative analysis provides a more complete picture of the evolution and shared gene content of the *Acholeplasmataceae*.

Materials and methods

A. brassicae strain O502 and *A. palmae* strain J233 were grown at 30°C and 37°C, respectively, in SP4 medium (Tully *et al.*, 1994). Whole genome shotgun sequencing was performed applying clone-based Sanger- and pyro-sequencing. Finishing procedure included manual editing and additional experiments for gap-closure (Kube *et al.*, 2010). Annotation of protein coding genes and structural RNAs was performed and metabolic pathways were reconstructed (Kube *et al.*, 2013).

Results and discussion

Sequencing of the plant-derived *A. brassicae* and *A. palmae* resulted in determination of circular chromosomes with 1,9 Mb and 1,6 Mb in size and a G + C content of 36% and 29%, respectively. Genomes encode above 1,700 and 1,400 proteins, respectively. Comparative analysis of these and recently published genomes of *A. laidlawii*, '*Ca.* P. asteris' strains, '*Ca*. P. australiense' and '*Ca*. P. mali' highlights a limited shared basic genetic repertoire of acholeplasmas and phytoplasmas (Kube *et al.*, 2012; Kube *et al.*, 2013). Besides, acholeplasma chromosomes are characterized by a lower

genetic instability. Rare exceptions are unusual duplications of rRNA genes and independently occurring horizontal gene transfers. Furthermore, acholeplasmas differ for instance by encoding a wide variety of transport systems, a F_0F_1 ATP synthase and a *Rnf*-complex, an additional gene of the *Sec*-dependent secretion system, a rich equipment for carbohydrate, fatty acid, isoprenoid and amino acid metabolism. Important proteins of phytoplasmas such as several transporters, proteins involved in metabolism as well as host-interaction and virulence-associated effector proteins were not identified in acholeplasmas indicating an early evolutionary split of both genera.

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Overtime expression of selected chrysanthemum yellows phytoplasma genes during infection of plant and leafhopper vectors

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Background and objectives

Microarrays analysis suggests that phytoplasma genome expression in the plant can be modulated according to the infection stage (Oshima *et al*., 2011). A real time PCR protocol was set up to study the expression profile of different '*Candidatus* Phytoplasma asteris' Chrysanthemum yellows isolate (CYP) genes during infection of *Arabidopsis thaliana* and the two vector species *Macrosteles quadripunctulatus* and *Euscelidius variegatus*. Target genes were selected among secreted proteins, known effectors, general metabolism and unique CYP ORFs, obtained following Illumina sequencing. A detailed study on gene expression of an obligate parasite in the plant and insect hosts is a prerequisite for the identification of molecular mechanisms involved in phytoplasma perception and regulation of host switch.

Materials and methods

Expression of 14 CYP genes coding three secreted proteins, four generic transporters (a mechanosensitive channel, the translocation component of SecY translocase, a multidrug and an oligopeptide transporters), two specific transporters (for arginine and Zn^{++} ion), a protein involved in phospholipid metabolism and the 30S rRNA ribosomal subunits obtained by the Illumina sequencing of CYP genome, one effector protein (Tengu, Hoshi *et al*., 2009), the major antigenic membrane protein (Amp, Galetto *et al*., 2008), and the immunodominant membrane protein (Imp, Kakizawa *et al*., 2009) was analyzed in the plant (*A. thaliana*) and vector (*M. quadripunctulatus, E. variegatus*) hosts. For each selected target, specific primers were designed and a qPCR protocol with SYBR Green chemistry was optimized. Amplification efficiencies and melting temperatures of the amplicons were obtained by analyses of qPCR amplification plots using serial dilutions of plasmids containing the specific fragment of each target gene. To study phytoplasma gene expression in plant, CYP-infected *M. quadripunctulatus* were fed on 10 *A. thaliana* plants and leaf samples were collected at different days post inoculation (dpi). To study phytoplasma gene expression in the vector, several individuals of the two analyzed species were collected at different days post acquisition (dpa) on the same CYP-infected daisies. Total DNA and RNA were separately extracted from each plant and insect sample. Presence and titre of CYP were determined by PCR (Lee *et al*., 1994) and qPCR (Marzachì and Bosco, 2005), respectively, with total DNA as reaction template. Total RNA was DNase treated, reverse transcribed and amplified by qPCR to quantify the transcripts of each selected gene.

Results and discussion

Phytoplasma specific symptoms appeared on *A. thaliana* plants at two weeks post infection. Fifteen and 83% of the plants were infected at 5 and 25 dpi, first and last sampling dates, respectively. At the first two sampling dates (7 and 14 dpa), 91 and 71% of sampled *M. quadripunctulatus* and *E. variegatus* were already CYP positive. Later on (up to the last sampling date, 35 dpa) all *M. quadripunctulatus* and about 90% *E. variegatus* sampled adults were CYP infected, in line with the

acquisition efficiencies already observed for these species (Bosco *et al*., 2007). CYP titre in *A. thaliana* increased from 5 to 28 dpi, and the final concentration was similar to that observed for other herbaceous hosts (Saracco *et al*., 2006). In the two vector species, CYP titre increased up to 21 dpa. The newly designed primers produced specific amplicons and the standard curves obtained following the amplification of serial dilutions of appropriate plasmids showed R^2 values above 0.99 and efficiencies ranging from 74% to 100%. The expression level of each target gene was calculated with respect to the CYP titre measured in 100 mg of each leaf tissue sample or in each entire insect body at every sampling date. Preliminary results have shown that CYP gene expression in plant and vectors is maximum at the beginning of the infection. Overall, CYP genes were more expressed in *A. thaliana* than in the two vector species. Profiling the expression of the selected CYP genes during infection of different hosts (plant and insect) and of different vector species will improve knowledge on the molecular mechanisms underlying phytoplasma adaptation to different life styles.

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Re-arrangement of sieve element endomembrane network in tomato leaves infected by "stolbur" phytoplasma

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Background and objectives
Despite the economic importance of the diseases associated with phytoplasmas, progress on deciphering the interactions with the hosts has been slow compared to other plant bacterial pathogens; therefore phytoplasmas remain as the most poorly characterized plant pathogens. During infection, phytoplasmas manipulate plant host leading to severely impaired assimilate translocation, being responsible for massive changes in phloem physiology (Musetti *et al*., 2013). But despite this partly negative modulation that causes constricted or blocked vascular system, phytoplasmas systemically spread throughout the plant (Christensen *et al*., 2004). The possibility to move through the sieve elements of the phloem is a first fundamental trait of phytoplasma pathogenicity and one possible mechanism seems to be the actin-based movement (Boonrod *et al*., 2012). Moreover, host plasma-membrane adherence is considered an important factor in pathogenesis (Seemüller *et al*., 2013), as well as the endoplasmic reticulum (ER) (as closely connected to the actin) might play an important role in the complex interactions occurring between phytoplasmas and sieve element endomembrane network. The aim of this work was to give a new insight about the relationships between phytoplasmas and the sieve element plasma-membrane - ER – actin network, using healthy and "stolbur"-infected tomato plants as a model.

Materials and methods
Solanum lycopersicum plants (cv Micro-Tom) were infected with the "stolbur" phytoplasma '*Candidatus* Phytoplasma solani' ('*Ca*. P. solani', Quaglino *et al*., 2013), subgroup 16SrXII-A, isolate V9 (Pacifico *et al*., 2009), by grafting. Approximately 2 months after grafting, when "stolbur" symptoms appeared, phytoplasma presence was assessed in leaf tissues by real time RT-PCR analyses, according with the method reported by Santi *et al*. (2013). To facilitate the accurate observation and the imaging of the above-reported cellular compartments and to evaluate the localization of phytoplasmas in and with sieve element structure, an integrated approach based on the use of epifluorescence microscopy (EFM) and specific protein-binding fluorochromes (Bell *et al*., 2013) and transmission electron microscopy (TEM)-immunogold labeling technique was set up and applied to London Resin White (LRW)-embedded tomato leaf samples (Musetti *et al*., 2002; Hafke *et al*., 2013).

Results and discussion
EFM and specific fluorochromes allowed to detect phytoplasmas, as well as endomembrane –actin filament network in tomato sieve elements. The DNA-specific dye DAPI (4,6-diamidino-2-phenylindole), already extensively used to detect phytoplasmas in fresh plant materials (Musetti *et al*., 2013), resulted to be suitable for the identification of phytoplasmas inside the sieve elements of LRW-embedded samples, both in longitudinal and cross-sections. By using the membrane-soluble fluorescent probe RH-414,[-N-(3-triethylammoniumpropyl)-4-(4-(4-(diethylamino)phenyl) butadienyl) pyridiniumdibromide], a dramatic re-arrangement of host plant endomembranes in the "stolbur"-infected samples was detected, in particular in the sieve element-companion cell complex, as indicated by the increased fluorescent signal. TEM imaging revealed the close connection between phytoplasma cells and sieve

element plasma-membrane, in the form of membrane junctions or overlays. These structural features result common of the class of *Mollicutes*, being present also in animal and human pathogenic Mycoplasmas (Razin *et al*., 1998). The organization of actin filaments has been visualized at EFM using immunofluorescence via α-actin-Texas Red (TR)-conjugated antibody. Differences in the signal intensity and distribution resulted between healthy and "stolbur"-infected samples. Whereas in healthy-control samples actin was mainly localized at the periphery of the cells, the lumen of sieve element appeared to be filled after infection. TEM immunolabeling, by gold-conjugated anti-actin monoclonal antibody, was used to evidence the precise localization of actin in respect to phytoplasma cell. Gold particles were localized on the phytoplasma membrane surface, indicating a direct connection of phytoplasma surface and host plant actin. Moreover, they were invariable positioned on one side of the phytoplasma cell, forming clusters, demonstrating a polarity in the actin and phytoplasma association, as extensively demonstrated in prokaryotic microorganisms (Lybarger and Maddock, 2001). It has been reported that the polar polymerization of the actin drives the motility of the pathogenic intracellular bacteria into the host (Lybarger and Maddock, 2001). As closely connected to the actin (Boevink *et al*., 1998), ER network shows re-organization in infected areas, demonstrating a role in the host-phytoplasma interaction. During the infection process, sieve element ER acts as the site of synthesis, control and secretion of a range of signal molecules (calcium, proteins, etc.; Jelitto-Van Dooren *et al*., 1999), triggering defence responses. In conclusion, our finding shows that "stolbur"-phytoplasma infection results in a tremendous re-organization of *S. lycopersicon* sieve element endomembrane systems, resembling a conserved widespread cellular response common to different classes of pathogens.

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Effect on disease development of suppressive strains of '*Candidatus* Phytoplasma mali' and their molecular identification

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Background and objectives

'*Candidatus* Phytoplasma mali' is the agent associated with apple proliferation (AP) disease. This psyllid-transmitted disorder induces in apple a range of symptoms such as witches' brooms, undersized fruits, foliar reddening, reduced vigor and decline. However, symptom expression is often subject to fluctuation. Following few years of disease, trees may recover and may show no or only mild symptoms for shorter or longer periods, after which severe symptoms may re-appear. From recent work, there is evidence that such fluctuations can be attributed to multiple infections 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 reduced virulence of the infecting phytoplasmas (Seemüller *et al.*, 2010; 2011; 2013). In this communication we provide further evidence of antagonistic interactions between AP phytoplasma strains, focusing on the suppressive action of largely avirulent strains on aggressive strains.

Materials and methods

Several inoculation experiments were carried out with the experimental hosts *Catharanthus roseus* (periwinkle) and *Nicotiana occidentalis* using avirulent/mild '*Ca.* P. mali' strains 1/93Vin and 1/93Tab as suppressors and aggressive strains AT and AP15 as challengers. Graft inoculation was performed by either inoculating the suppressors prior to grafting of the challengers or by co-inoculating suppressor and challenger. The inoculated plants were observed for up to 43 months for symptom development and the presence of suppressors and challengers by multiplex real-time PCR monitoring. The effect of suppressive strains in infected apple trees was examined by comparing disease history and symptomatology at sampling with the presence of suppressive mild strains and nonsuppressive severe strains. These were distinguished based on unique substitutions in the sequence of a variable 5' fragment of AAA+ATPase AP460.

Results and discussion

Periwinkle plants pre-inoculated with strain 1/93Vin and challenged with strains AT or AP15 developed predominantly the mild symptoms of suppressive strain 1/93Vin. Only the growth developed from the challenger scions and leaves below the grafting site showed the typical symptoms induced by strains AT and AP15. They were clearly present for about 10 months. Then the symptoms slowly disappeared. At the end of the observation periods, no differences between healthy controls, pre-immunized and co-inoculated plants were observed. In contrast, all AT- and AP15-inoculated plants were either dead or declining at the final symptom scoring.
In monitoring the occurrence of suppressor and challenger in pre-inoculated periwinkles, suppresser 1/93Vin was detected in all stem and root samples over the entire observation periods of 33 to 43 months whereas challengers AT and AP15 were never detected in the stem and rarely in the roots. Following simultaneous inoculation, the suppressor successively colonized all stem and root regions. One to two years post inoculation, the suppressor was detected in all samples. In contrast, detection of challenger AT steadily decreased but often remained detectable in up to 13% of the stem samples and up to 27% of the root samples. However, the reduced presence of the challenger did not

markedly affect the plants. The trials with *N. occidentalis*, in which strain 1/93Tab was used as suppressor and which were performed in the pre-inoculation modus, yielded results similar to that of the pre-inoculation experiments with *C. roseus*. In the *N. occidentalis* trials, challenger AT was never detected in stem and root samples.

Single-strand conformation polymorphism (SSCP) analysis of cloned fragments of ATPase gene AP460 revealed that suppressive strains 1/93Vin and 1/93Tab show similar profiles that are distinctly different from those of AT, AP15 and other aggressive strains. Both types of profiles were also identified in '*Ca.* P. mali' strains isolated from apple. Nearly all trees examined showed severe symptom once or repeatedly during 15 years of observation and all of them were nonsymptomatic for shorter or longer periods. This indicates shifts in the predominance of mild and severe phytoplasma strains. The symptomatology at sampling corresponded with the virulence of the accessions and with the prevalence of cloned fragments associated with suppression or nonsuppression. In accessions inducing severe symptoms, only fragments showing the profile of nonsuppressive strains were identified whereas in nonsymptomatic trees only suppression-associated fragments were observed. Both types of fragments were detected in trees exhibiting mild symptoms. Based on the SSCP results, selected AP460 fragments were sequenced and subjected to phylogenetic analysis. The phylogram resulted in two homogenous major branches formed by sequences of suppressive mild strains and nonsuppressive severe strains, respectively.

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The antigenic membrane protein Amp of chrysanthemum yellows phytoplasma is involved in transmission by leafhopper vectors

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Background and objectives

The major antigenic membrane protein (Amp) of different isolates of '*Candidatus* Phytoplasma asteris' interacts *in vitro* specifically with cytosolic (actin, myosin) and membrane (ATP synthase) proteins of leafhopper vector species (Suzuki *et al*., 2006; Galetto *et al*., 2011). Such interaction has not been observed with proteins of other phylogenetically-related, non-vector species. Once acquired by the vector upon feeding on an infected plant, phytoplasmas cross the gut epithelium and basal lamina, multiply in the haemolymph and colonize the salivary glands, before being transmitted to a new plant during a successive nutrition (Bosco and D'Amelio, 2010). As these bacterial pathogens lack a cell wall, one or more phytoplasma membrane proteins may interact with cells of the gut and salivary gland epithelia and may be involved in defining specificity of transmission. Aim of this work was to develop a system to evaluate *in vivo* the effect of the most abundant antigenic phytoplasma membrane protein on the acquisition and transmission capabilities by vector leafhoppers.

Materials and methods

To study the *in vivo* role of Amp of the '*Candidatus* Phytoplasma asteris' (chrysanthemum yellows strain, CYP) at the gut level, nymphs of two species (*Macrosteles quadripunctulatus* Kirschbaum ed *Euscelidius variegatus* Kirschbaum), before CYP acquisition on infected daisies, were fed on an artificial medium containing a partial fusion construct of CYP Amp (CYPfAmp, Galetto *et al*., 2008), specific antibody raised against CYPfAmp (AbfAmp), and a mix of CYPfAmp and AbfAmp. At the end of the latency period (LP), adults were singly caged on healthy daisies for the inoculation access period (IAP). After IAP, insects were collected and assayed for phytoplasma presence (Lee *et al*., 1993; 1994). To study the *in vivo* role of CYP Amp at the salivary gland level, adult *E. variegatus* were microinjected with a phytoplasma suspension (Bressan *et al*., 2006; Galetto *et al*., 2009) added with CYPfAmp (Galetto *et al*., 2008) and AbfAmp. Following microinjection, serial inoculation to healthy daisies were performed to establish the length of the LP. After the last IAP, vectors were collected and assayed by PCR to confirm phytoplasma presence (Lee *et al*., 1993; 1994). Following IAP, plants were treated with insecticide and kept under controlled conditions for symptom observation.
Preliminary experiments were carried out to define the optimal protein concentration for the artificial feeding as well as the microinjection experiments, the minimal acquisition length on the infected plant as well as the minimal LP length to obtain efficient phytoplasma acquisition and transmission. ELISA was used to monitor CYPfAmp and AbfAmp persistence in the artificial medium and in the body of microinjected *E. variegatus* adults.

Results and discussion

Sucrose 5% in TE was the artificial substrate which allowed the best survival of both vector species (100% survival for *M. quadripunctulatus* and 80% for *E. variegatus* after 24h), and the fusion protein was assayed at 1 mg/ml in the feeding medium. Under these conditions, survival of both species was over 50% following 24 h artificial feeding and about 80% following abdominal microinjection, in agreement with what previously reported for microinjection of a *Spiroplasma citri* protein in the

Circulifer haematoceps vector (Labroussaa *et al*., 2011). CYPfAmp was detected by ELISA up to 24 h post abdominal microinjection and up to 30 h after the acquisition on the artificial medium. Preliminary experiments under artificial feeding conditions showed that 4 and 6 h AAP were enough to obtain high CYP transmission efficiencies with *M. quadripunctulatus* and *E. variegatus*, respectively. Moreover, LP in the vector in average lasted for 20 (*M. quadripunctulatus*) and 33 days (*E. variegatus*). Following abdominal microinjection, LP decreased to 22 days for *E. variegatus*, about 10 days shorter than required following acquisition by feeding on the infected plant. Preliminary results showed that the mere presence of CYPfAmp in the feeding medium had no effect on phytoplasma acquisition and transmission efficiencies of both vector species. The presence of AbfAmp in the feeding medium had no effect on the transmission of phytoplasmas that were able to pass the gut barrier and colonize the insect body. Reduction of phytoplasma transmission rate of both vector species was recorded after the ingestion of the antibody against CYPfAmp (AbfAmp), possibly due to reduction in phytoplasma acquisition efficiency. This suggests that Amp is involved in the interaction with vector proteins *in vivo* and that this interaction is important for transmission efficiency. Lack of any effect of CYPfAmp on the acquisition and transmission efficiencies of both species may be explained by absence of the *in vivo* interacting domain on the fusion construct (Galetto *et al*., 2008) or by the requirement of more complex protein complexes involving Amp (such as those present in the phytoplasma membrane) for the *in vivo* interaction. Abdominal microinjection experiments to define the role of CYP Amp interaction with vector proteins at the salivary gland level in determining vector efficiency are currently ongoing.

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Adaptation of phytoplasmas responsible for grapevine ''flavescence dorée'' to *Scaphoideus titanus* insect vector: the role of variable membrane proteins VMPs

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Background and objectives
Vineyards of Southern Europe are affected by the "flavescence dorée" (FD) phytoplasma, a quarantine pathogen transmitted by the leafhopper of neartic origin *Scaphoideus titanus*. Phytoplasmas genetically related to FD have been detected in European alders *Alnus glutinosa* and in *Clematis vitalba*. According to multilocus sequence analyses, all constitute a monophyletic cluster within the taxonomic group 16SrV (Arnaud *et al*., 2007; Malembic-Maher *et al*., 2011). Alder phytoplasmas are transmitted by the leafhopper *Oncopsis alni*, which can occasionally inoculate them to grapevine leading to Palatinate Grapevine Yellows (PGY) disease (Maixner and Reinert, 1999; Maixner *et al*., 2000). A survey conducted in five European countries led to the detection of 120 different map genotypes in alders, grapevine, clematis and insect vectors. Only eight genotypes were associated to FD outbreaks, *i.e.* were epidemically propagated in vineyards by *S. titanus*. All alder phytoplasma genotypes transmitted to date to the broad bean *Vicia faba* could not be experimentally transmitted by *S. titanus*. In order to identify genetic determinants responsible for the adaptation of FD phytoplasma strains to the *S. titanus* insect vector we searched the draft genome of the epidemic strains FD92 for genes encoding surface proteins with high genetic diversity.

Materials and methods
Plant and insect DNAs were classically extracted by the CTAB procedure. *Vmp*A and *vmp*B genes were amplified by PCR and sequenced by Beckman Coulter Genomics (Takeley, UK). Raw chromatograms were assembled and edited using the Phred-Phrap-Consed package. Sequences were aligned using CLUSTALW and phylogenetical analysis using maximum parsimony was conducted in MEGA5.
VMPs expression and purification were conducted according to pET28 cloning manual instructions (NOVAGEN). Expression of VMPs in phytoplasma infected faba bean were detected using fluorescent-labelled anti VMP polyclonal antibodies using epifluorescent microscopy or confocal microscopy. Protein-Protein interactions were detected by VMP proteins overlay assays performed on SDS-PAGE separated, membrane transferred insect proteins.

Results and discussion
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, three 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+ bacteria involved in the invasion of eukaryotic cells. Vmps were sequenced among a representative set of 16SrV phytoplasma isolates. VmpA and vmpB sequences form 3 phylogenetic clusters, out of which the cluster II and III corresponded to isolates epidemically propagated by *S. titanus*. As previously reported for the '*Candidatus* Phytoplasma solani' vmp1, vmp 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 deletion and 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 leafhopper vector *Euscelidius variegatus*. The 55 kDa receptor was detected in the ovaries of *E. variegatus*, whereas the 120 kDa receptor was present in testis and salivary glands but not in the ovaries. *S. titanus* protein receptors of equivalent molecular mass were also able to bind VMP-A *in vitro*. VMPs represent valuable marker for the adaptation of "flavescence dorée" phytoplasma to *S. titanus* insect vector and its sequence should be a useful tool for risk assessment of epidemics in the vineyard.

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