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The importance of psyllids (Hemiptera Psyllidae) as vectors of phytoplasmas in pome and stone fruit trees in Austria

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Abstract

To study the occurrence and distribution of psyllids as potential vectors of European stone fruit yellows (ESFY), pear decline (PD) and apple proliferation (AP) a survey was conducted in Austrian orchards during the last years. Specimens were collected by using the beating tray method in apricot, pear and apple tree orchards. The obtained psyllids were analysed by PCR and RFLP assays for the presence of phytoplasmas. Molecular analyses showed few infections of *Cacopsylla pruni* with '*Candidatus* Phytoplasma prunorum' and few individuals of *Cacopsylla pyricola*, *C. pyri* and *C. pyrisuga* were carrier of '*Candidatus* Phytoplasma pyri'. The data presented in this study are a preliminary report because analyses of apple psyllids are still in progress.

Key words: *Cacopsylla* spp., European stone fruit yellows, pear decline, apple proliferation.

Introduction

European stone fruit yellows (ESFY), pear decline (PD) and apple proliferation (AP) are quarantine diseases associated with phytoplasmas ('*Candidatus* Phytoplasma prunorum', '*Ca.* P. pyri' and '*Ca.* P. mali'), which are responsible for great economic losses in fruit production (Seemüller and Schneider, 2004). The spread of these phytoplasmas is due to infected planting material or insect vectors, especially psyllids (Hemiptera Psyllidae). The occurrence of these diseases has been described in Austria by Richter (1999), Spornberger *et al*. (2006), Steffek and Altenburger (2008). A literature review revealed a lack of data on potential vectors in Austria. The aim of this study was to gain more information on the occurrence of these potential vectors and the phytoplasma infection status of psyllids from pome and stone fruit trees in Austria.

Materials and methods

Investigation sites were located in the Eastern part of Austria - 10 apricot orchards in Lower Austria, Burgenland and Vienna, 5 pear orchards and 3 apple orchards in Lower Austria. Samples were collected by using the beating tray method with 100 beats respectively 100 branches (trees) per sampling date and orchard. Psyllid captures were done in the period from March to July, on apricot trees in 2005 and 2006, on pear and apple trees in 2009 and 2010. Collected psyllids were identified according to Ossiannilsson (1992) and Burckhardt and Jarausch (2007).

Molecular analyses for phytoplasma infection of psyllids were carried out with 1 to 8 individuals per sample taken for testing: CTAB-method for the DNA-extraction of psyllids (Maixner *et al*., 1995), qualitative PCR for the detection of phytoplasmas in the psyllid samples using universal primers fU5/rU3 (Lorenz *et al*., 1995) and then for nested PCR using phytoplasma specific primer pairs P1/P7 primer (Deng and Hiruki, 1991; Schneider *et al*., 1995) and f01/r01 primer (Lorenz *et al*., 1995) respectively. RFLP assays using restriction enzymes *Ssp*I and *Rsa*I were applied to discriminate among the three fruit tree phytoplasmas AP, PD and ESFY (Tedeschi *et al*., 2009).

Results

The plum psyllid *Cacopsylla pruni* was found in all investigated apricot orchards. The first report of *C. pruni* on apricot trees in Austria was mentioned in Lethmayer and Hausdorf (2005). Interesting was the high number of *Cacopsylla melanoneura* on apricot trees at some investigation sites which was due to hawthorn hedges near the apricot orchards. The three pear psyllid species *Cacopsylla pyricola*, *Cacopsylla pyri*, *Cacopsylla pyrisuga* and the hawthorn psyllid *C. melanoneura* were the main species on pear trees. *C. pyricola* was the most abundant species. On apple trees the psyllids *C. melanoneura* and *Cacopsylla picta* were mainly captured.

Molecular analyses of *C. pruni* showed five positive samples (with 30 individuals in total) out of 37 tested samples (with 142 individuals in total) with '*Ca*. P. prunorum'. These samples originated from four sites in Lower Austria. All individual of *C. melanoneura* caought on the apricot and pear trees tested negative for phytoplasmas. All three pear psyllid species, *C. pyricola*, *C. pyri* and *C. pyrisuga*, were found infected with '*Ca*. P. pyri' comprising 16 positive samples (with 48 individuals in total) out of 33 tested samples (with 118 individuals in total). Positive samples were obtained at three pear sites in Lower Austria. First analyses showed that the all psyllids collected on apple were infected with '*Ca.* P. mali'. An overview of the molecular analyses is given in table 1.

Other studies have already confirmed that the psyllid species which were tested positive in our study are vectors of the respective phytoplasmas (rewieved by Jarausch and Jarausch, 2010).

Table 1. Results of the RFLP analyses of the *Cacopsylla* samples taken in fruit tree orchards in Austria.

date	orchard/ fruit tree	psyllid species	positive (+)/negative (-) tested for ESFY	PD	AP
2005	apricot	*C. pruni*	+	-	-
2006	apricot	*C. pruni*	-	-	-
2006	apricot	*C. melanoneura*	-	-	-
2009	pear	*C. pyricola*	-	+	-
2009	pear	*C. pyri*	-	+	-
2009	pear	*C. pyrisuga*	-	+	-
2009	pear	*C. melanoneura*	-	-	-
2009	apple	*C. melanoneura*	-	-	-
2010	apple	*C. melanoneura*	-	-	-
2010	apple	*C. picta*	-	-	-

C. pyrisuga has been found infected with '*Ca*. P. pyri' (Kucerova *et al*., 2007) but its ability of transmission is still not verified (Jarausch and Jarausch, 2010). Due to the geographical position of Austria it is interesting which of the two psyllid species found on apple can be identifyind as main vector for transmission of AP in Austria. First investigations did not indicate a particular vector capacity for '*Ca*. P. mali' by *C. melanoneura* or *C. picta* '. However, due to the low number of investigated apple psyllids further analyses are necessary to clarify the vector role and therefore this issue is still in progress.

Discussion

One of the main phytosanitary measures for preventing phytoplasma diseases spread is the control of their vectors. The use of insecticides in sustainable production methods is restricted. Therefore, knowledge on the vectors (mainly psyllids), their distribution and biology is strongly needed for control strategies, especially for new approaches on integrated control strategies.

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'*Candidatus* Phytoplasma mali' infected *Cacopsylla picta* found in apple orchards in South-Western Finland

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Abstract

Apple orchards in four localities in southwestern Finland were surveyed in May 2009 and 2010 to monitor the occurrence of *Cacopsylla picta* (Förster) and *C. melanoneura* (Förster), vectors of apple proliferation phytoplasma. Both psyllid species were present in apple orchards, but *C. picta* prevailed. The number of individuals varied considerably in 2009-2010. The total number of captured overwintering *Cacopsylla* species from the same orchards was 395 in 2009 and 45 in 2010. Individuals of *C. picta* infected with '*Candidatus* Phytoplasma mali' were found from single orchards in Lohja and Parainen. In 2010 the incidence of '*Ca.* P. mali' in *C. picta* individuals collected from apples (*Malus domestica)* was 11.4%.

Key words: apple proliferation, *Malus*, psyllids, PCR, RFLP.

Introduction

Apple proliferation (AP) phytoplasma, classified as '*Candidatus* Phytoplasma mali' (Seemüller and Schneider, 2004) was first described in Italy in the 1950s (Rui *et al.*, 1950) and currently causes severe epidemics and economic losses in many pome fruit growing areas in Europe, including Italy and Germany (Carraro *et al*., 2008; Jarausch *et al*., 2004).

The pathogen is transmitted through infected grafting material, via natural root grafts (Ciccotti *et al.*, 2007) and sap-sucking insects. *Cacopsylla picta* (Förster) has proved to be the most important vector of '*Ca.* P. mali' in Germany and neighbouring regions (Jarausch *et al*., 2007), whereas *C. melanoneura* (Förster) was reported as the main vector of '*Ca*. P. mali' in Northwestern Italy (Tedeschi *et al*., 2002).

C. picta has been known to occur on apple in Southern Finland (Ossiannilsson, 1992) and *C. melanoneura* was found in Finland in the 1990s (Albrecht *et al*., 2003). In the 2000s *C. melanoneura* spread northwards to a greater extent than *C. picta* (Mattila and Söderman, 2011). *C. melanoneura* occurs mainly on hawthorn, but has been found also on apple and pear (Ossiannilsson, 1992). Both psyllid species are presumed to overwinter on conifers in Fennoscandia (Ossiannilsson, 1992).

Apple cultivation has been predicted to expand in Finland in the future as a result of global warming (Kaukoranta *et al.*, 2010). The main area for commercial apple cultivation in Finland is in the south and southwestern parts of Finland, and in the Aland Islands. Apple proliferation phytoplasma has not been surveyed or reported either from apple trees or insects in Finland. The aim of the present study was to confirm the occurrence of *C. picta* and *C. melanoneura* in apple orchards in southern Finland and to determine the incidence of '*Ca.* P. mali' in *C. picta*.

Materials and methods

In May 2009, 18 psyllid samples were collected in Southwestern Finland (Parainen, Piikkiö, Lohja, Jokioinen) from apple (*Malus domestica*) in commercial orchards, research stations and in a few home gardens.

In May 2010, 19 samples were collected in the same orchards. Overwintering adult psyllids were collected using a beating net with an opening of 0.25 m^2. In commercial orchards, one branch from each of 33 randomly selected apple trees was beaten for each sample. Insects were separated, identified and counted by examining male and female terminalia (Ossiannilsson, 1992).

Specimens were stored in 95% ethanol until DNA extraction. In some of the later samples, 1^{st}-3^{rd} nymph stages of *Cacopsylla mali* (Schmidb.) were also present. *C. mali* overwinters as eggs and is the most common psyllid on apple in Finland (Mattila and Söderman, 2011).

Total DNA from adult *Cacopsylla* species was extracted from all individual psyllids from single specimens in 2010, whereas DNA from a third of the psyllids collected in 2009 was extracted from 26 batches of five individuals.

DNA extraction was done using a DNeasy Blood and Tissue Kit (Qiagen) according the manufacturer's instructions. Apple proliferation phytoplasma was detected in the DNA extracts through the use of PCR amplification with AP-specific primers AP5/AP4 (Jarausch *et al*., 1994).

DNA from AP phytoplasma positive samples was analysed further for subtypes by PCR/RFLP using primers AP13/AP10 (Jarausch *et al*., 2000) and amplicons were digested using enzymes *Hinc*I and *Rca*I (New England BioLabs) following the instructions of the manufacturer.

Results

In 2009 a total of 395 *Cacopsylla* adults were collected in the four localities: 294 were identified as *C. picta* and 101 as *C. melanoneura*. In 2010 a total of 45 *Cacopsylla* spp. were collected from the same localities and orchards. Of the 45 identified individuals 44 were *C. picta* and one was *C. melanoneura*.

In 2009, *C. picta* was present in all collected samples, the numbers varying from 1 to 41 per sample, 56% of the 294 were females. *C. melanoneura* was found in half of the samples, 1 to 46 per sample, 65% of the 101 were females. In 2010, *C. picta* was found in 12 samples, 1 to 16 per sample, and 82% were females. *C. melanoneura* was present in only one sample as a single female.

'*Ca*. P. mali' was detected in four groups of the selected 26 groups of *C. picta* samples (4/26) collected in 2009. The characteristic amplicon (483 bp) was amplified from insects using specific primers AP5/AP4. All the positive samples were from one orchard in Parainen.

'*Ca*. P. mali' was detected in five of 45 individuals collected in 2010, all from the same orchard (Lohja). The occurrence of '*Ca*. P. mali' in the specimens of *C. picta* in that orchard was 20.8% (5/24), whereas the incidence in all *C. picta* specimens collected in 2010 was 11.4% (5/44).

AP13/AP10 amplicons and RFLP analyses confirmed that the '*Ca.* P. mali' subtypes present in Finnish orchards were AP and AT-2.

Discussion

Results of this preliminary survey confirmed the incidence of both psyllid vector species of '*Ca.* P. mali', *C. picta* and *C. melanoneura*, in Finnish apple orchards. The number of individuals of both species varied in 2009-2010, but *C. picta* prevailed in both years. It is interesting that the 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 (Vänninen *et al.*, 2011), it may be present here at its northernmost border.

Occurrence of '*Ca*. P. mali' in *C. picta* was determined in samples collected in two localities. One of these orchards (Parainen) represented organic farming, where numerous imported foreign apple varieties were planted. The other orchard (Lohja) represented an old conventional orchard. Observation of abnormal symptoms in apple orchards have not been reported by growers or advisors. '*Ca*. P. mali' infected apple trees have yet to be found and therefore any conclusions based on the present distribution of '*Ca*. P. mali' in Finnish apple orchards are premature.

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Investigation on the apple proliferation epidemics in the orchards of the Pelion Mountain and preliminary observations on possible phytoplasma vectors

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Abstract

The apple producing region in the Pelion Mountain (Magnesia prefecture, Thessaly, Greece) is suffering for more than 10 years from the apple proliferation disease. The orchards are basically planted with cv 'Starking Delicious', are grafted on seedling root-stocks and are older than 40 years. The presence of the '*Candidatus* Phytoplasma mali' was proven by PCR/RFLP analyses as well as by sequencing. '*Ca*. P. pyri' was also detected in at least three different orchards. First attempts to identify the vectors were carried out by monitoring the insect populations in 8 different orchards. one species of psyllids morphologically identified as *Cacopsylla pulchella* (Low) was the most present insect in the orchards all along the two-month monitoring (May/June). *Cacopsylla mali* (Schmidberger) was also present in the orchards but in lower numbers. The monitoring is continued and still in progress.

Key words: apple proliferation, psyllid vector, molecular identification, phytoplasmas.

Introduction

The apple producing region in the Pelion Mountain (Magnesia prefecture, Thessaly, Greece) is suffering for more than 10 years from the apple proliferation disease. The disease was firstly described in Greece in late eighties (Rumbos, 1986) and the '*Candidatus* Phytoplasma mali' was recently detected and identified by molecular methods in infected material (Rumbou *et al*., 2007).

The infected orchards are basically planted with cv Starking Delicious, are grafted on seedling root-stocks and are older than 40 years. Approximately 90% of the apple trees are infected and the yield losses range from 20-40% in the lower elevations to 70-100% in the higher elevations, where apple orchards neighbour beech forest. The last five years and because of the extended epidemics, infected trees are massively removed and replaced with new trees grafted on dwarf rootstocks and planted in palmettos. However, the pathogen has been detected also to those newly planted trees (Rumbou *et al*., 2010a), therefore survey was undertaken to verify the disease presence as well as to identify the insect vector of the disease.

Materials and methods

Samples from apple trees grown in different orchards in the Pelion Mountain and exhibiting apple proliferation symptoms were collected from September to November 2009 and were preserved at −80°C or −20°C. Screening of symptomatic apple material was carried out with total DNA extraction from 24 leaf and root samples from diseased apple trees. DNA extraction was performed in Julius Kühn Institute (Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany) according to a modified protocol following Doyle and Doyle (1990). PCR amplification with P1/P7 was followed by nested PCR with the universal phytoplasma primers fU5/rU3 or with the fruit tree-specific primers fO1/rO1 (Lorenz *et al*., 1995). The PCR products were cloned and sequenced. A second round of PCR amplifications was performed in Max-Planck Institute (Institute for Molecular Genetics, Berlin) with universal primers fU5/rU3. Sequencing was carried out using ABI3730XL capillary systems (ABI 3730xl) and resulted in 20-fold coverage. Sequence quality assessment and assembly were performed using MIRA assembly program and GAP4.

A first attempt to identify the vectors of the pathogen was done in 2006 by monitoring the insect populations in eight different orchards for the flying season.

Results

Positive results were obtained mainly from root samples, in particular nine samples originating from eight different orchards were identified as '*Ca*. P. mali', while three samples originating from three different orchards were identified as '*Ca*. P. pyri'. Two samples from the same orchard were found to be infected by '*Ca*. P. pyri' and '*Ca*. P. mali', respectively (Rumbou *et al*., 2010b).

From the insects trapped, two species of psyllids and one of Jassidae, possible phytoplasma vectors, were identified. From these one species of psyllids, morphologically identified as *Cacopsylla pulchella* (Low), was the most frequently trapped insect in the orchards with 50-1,660 adults/8 traps and flying mainly from 17/5-1/6, with the exception of an orchard in high altitude where

they were present until the 21/6. A non-identified species of Jassidae was the second insect for number of individuals (18-278 adults/8 traps) and present during the two-month monitoring (26/4-21/6). *Cacopsylla mali* (Schmidberger) was also present in the orchards but in lower numbers (1-59 adults/total traps).

Discussion

The presence of the '*Candidatus* Phytoplasma mali' was firstly proven by molecular means in 2005. Lately, the first sequences from local strains were obtained with the use of phytoplasma universal and 16SrX-group specific primer pairs revealing that, apart from '*Ca*. P. mali' also '*Ca*. P. pyri', the agent associated with pear decline, is present in at least three different orchards in the surveyed region.
This year the insect monitoring is repeated and the identification will be done with more accurate tools. The detection of phytoplasma in the insects' body remains to be shown before the transmission trials will be initiated.

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Survey of psyllid vectors of fruit tree phytoplasmas in Bulgaria: a preliminary report

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Abstract

The spread and the frequency of individuals from the psyllid genus *Cacopsylla* has been investigated in spring 2011 in four fruit tree orchards in three different regions of Bulgaria. Insects were captured and identified morphologically for species determination. Phytoplasma infection in psyllid samples was analysed by universal and specific PCR. All psyllid species described as vectors of fruit tree phytoplasmas were present in the investigated areas. Four individuals of *Cacopsylla pruni* from two different regions were carrying '*Candidatus* Phytoplasma prunorum', the agent of European stone fruit yellows (ESFY). This is the first ESFY detection in these regions in Bulgaria.

Key words: '*Candidatus* Phytoplasma prunorum', European stone fruit yellows, psyllid vectors, PCR detection.

Introduction

A few species of the psyllid genus *Cacopsylla* (Hemiptera Psyllidae) have been demonstrated to be vectors of European fruit tree phytoplasmas: '*Candidatus* Phytoplasma mali' associated with apple proliferation (AP), '*Candidatus* Phytoplasma prunorum', the agent of European stone fruit yellows (ESFY) and '*Candidatus* Phytoplasma pyri', the agent of pear decline (PD) (Seemüller and Schneider, 2004). Two psyllids, *Cacopsylla picta* (Foerster) and *Cacopsylla melanoneura* (Foerster) are recognised vectors of '*Ca.* P. mali'. The psyllid *Cacopsylla pruni* Scopoli was described as vector of '*Ca.* P. prunorum' whereas three psyllid species are recognised or presumed vectors of '*Ca.* P. pyri': *Cacopsylla pyri* (L.), *Cacopsylla pyricola* (Foerster) and *Cacopsylla pyrisuga* (Foerster) (reviewed by Jarausch and Jarausch, 2010).

ESFY and PD have been first detected in Bulgaria near Plovdiv (Topchiiska *et al*., 2000). But so far no report exists concerning cases of infected psyllid species in Bulgaria. AP has been reported to occur in Bulgaria since long time (www.eppo.org); however the *Cacopsylla* species vectoring fruit tree phytoplasmas were described in Bulgaria before they have been identified as phytoplasma vectors (Harizanov, 1966a; 1966b; 1982). However almost nothing is known about the incidence and the spread of these quarantine diseases in Bulgaria. Therefore, the aim of the present work was to gain first information on the spread and frequency of psyllid vector species and to determine their natural infection status in selected fruit tree orchards in Bulgaria.

Materials and methods

A survey was conducted in spring 2011 in four fruit tree orchards in three different regions in Bulgaria. Insects were caught using sweep-netting. Captured psyllids were frozen at −20°C and psyllid species identification was done using different determination keys (Hodkinson and White, 1979; Ossiannilsson, 1992; Burckhardt and Jarausch, 2007). DNA was extracted from single psyllid individuals with a CTAB-based protocol as described by Maixner *et al.* (1995).

PCR amplification of phytoplasma DNA was achieved with universal ribosomal primers fU5/P7 (Lorenz *et al.*, 1995; Schneider *et al.*, 1995). For specific PCR of positive *C. pruni*, ESFY-specific non-ribosomal primers ECA1/ECA2 were applied (Jarausch *et al.*, 1998).

Results and discussion

In the surveyed fruit tree orchards all known and putative psyllid vectors of fruit tree phytoplasmas were identified: *C. pruni, C. picta, C. melanoneura, C. pyri, C. pyrisuga* and *C. pyricola*. They were captured in three different region: Dupnica, Sofia (Gorni Lozen, Vrajdebna), and Plovdiv. (figure 1, table 1).

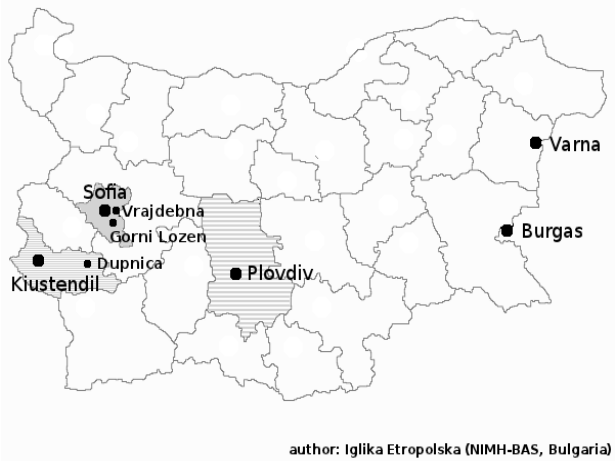

Figure 1. Location of fruit tree orchards surveyed in spring 2011 in Bulgaria.

Table 1. Number of individuals of *Cacopsylla* species captured in fruit tree orchards and results of phytoplasma detection by PCR in individual insects.

Psyllid species	Phytoplasma	Region			
		Dupnica	Gorni Lozen	Vrajdebna	Plovdiv
Cacopsylla pruni	'*Ca.* P. prunorum'	3 / 94 (3.2%)*	1 / 16 (6.3%)	nt	nt
Cacopsylla picta	'*Ca.* P. mali'	0 / 3	0 / 0	nt	0 / 1
Cacopsylla melanoneura	'*Ca.* P. mali'	0 / 23	0 / 1	nt	0 / 11
Cacopsylla pyri	'*Ca.* P. pyri'	0 / 0	0 / 0	0 / 181	nt
Cacopsylla pyricola	'*Ca.* P. pyri'	0 / 2	0 / 0	0 / 1	nt
Cacopsylla pyrisuga	'*Ca.* P. pyri'	0 / 84	0 / 6	0 / 1	nt

* PCR positive versus total number of individuals tested; nt = not tested.

In total, 440 individuals of the six *Cacopsylla* species were collected in the different fruit tree orchards and individually analysed for phytoplasma presence. Among the different known and putative vector species, only 4 individuals of *C. pruni* were found to be infected by phytoplasmas with universal ribosomal primers. The specific PCR revealed the presence of '*Ca.* P. prunorum' in all four phytoplasma-infected *C. pruni* insects. Interestingly, the infected specimens originated from two different collection sites. This is the first report of ESFY detection in these regions.

Discussion

Psyllid species described in Bulgaria by Harizanov (1966a, 1966b, 1982) were from different regions from those investigated in the present study. In all surveyed orchards an important number of psyllid vector species was found; the collected specimens showed variations in presence and abundance at the different sites. In two of the investigated pear orchards (Dupnica and Gorni Lozen, table 1) only *C. pyrisuga* and *C. pyricola* were present while at the location of Vragdebna *C. pyri* was the only pear psyllid species found. Despite abundances of *C. pyri* and *C. pyrisuga*, none of the individuals captured during this preliminary survey was phytoplasma infected. The detection of '*Ca.* P. prunorum' in its vector species in two different regions indicates a broader distribution of ESFY in Bulgaria as known so far. The future investigations will aim to monitor regularly insects and plants in these regions and other fruit tree growing areas in Bulgaria for possible phytoplasma infection.

Acknowledgements

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Effects of possible repellents on feeding and survival of *Cacopsylla pruni* (Scopoli)

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Abstract

During the last years *Cacopsylla pruni* has become a major concern in Austria because of its vectoring of Stone fruit yellows in apricots. Products based on kaolin, paraffin oil, orange oil and extract of fennel oil as active ingredients were tested for their ability to repel the disease vector *C. pruni* from landing and feeding on *Prunus armeniaca*. In free choice experiments all products showed significant repellency to adults 24 h after start of the tests. After 72 h, however, fennel extract oil and orange oil had lost their effect as compared with the untreated control, whereas plants treated with kaolin or paraffin oil were barely colonized.

In no choice experiments kaolin and paraffin oil significantly influenced feeding behaviour. All tested products significantly reduced the number of surviving insects in comparison to water treated controls.

Key words: *Cacopsylla pruni*, kaolin, paraffin oil, repellent.

Introduction

Wachau is one of the most important apricot growing areas in Austria. Apricot gardens dominate the banks of the river Danube. The river valley is adjoined by areas of higher altitude covered by forests. In European Union law the apricot produced there are designed as "Wachauer Qualitätsmarille" a product with protected designation of origin (PDO). Fruits are produced mainly of the local cultivar 'Klosterneuburger Marille' (a clone of 'Hungarian Best') and the rootstocks are apricot seedlings, myrobalans and other plums. In addition to its commercial value, apricot farming in the Wachau is very important for tourism. The Wachau was inscribed as "Wachau Cultural Landscape" in the UNESCO List of World Heritage Sites in recognition of its architectural and agricultural history. The spring landscape is characterized by wide expanses of flowering apricot trees.

During the last decade European stone fruit yellows caused by '*Candidatus* Phytoplasma prunorum' has become a major concern in the Wachau area. Due to the high susceptibility of the predominant cultivar, the small scale structured apricot gardens and the close vicinity of conifers as putative overwintering hosts for the vector *Cacopsylla pruni* (Carraro *et al.*, 1998) disease incidences are high. Tree losses are a daily occurrence. It is well established that apricot cultivars differ in regard to their disease sensitivity and disease incidence might also depend on a grower effect (Thébaud *et al.*, 2006). In the case of the 'Wachauer Qualitätsmarille', however, a switch to less sensitive cultivars is not in accordance with the PDO status. The small scale structured apricot gardens are a part of the protected landscape and should therefore be kept as they are. All these factors make the development of control strategies very difficult.

Previous studies point out that overwintered adult *C. pruni* returning to apricot orchards in spring are the most efficient disease vectors (Thébaud *et al.*, 2009). The aim of our present work is the development of strategies to repel the vector *Cacopsylla pruni* from landing and feeding on apricot plants. Therefore, we investigated the repellency to *C. pruni* of commercially available products (based on kaolin, mineral oil and plant extracts) under experimental conditions.

Materials and methods

Cacopsylla pruni adults used in this study were field collected on *Prunus spinosa* in March and April 2011. One year old *Prunus armeniaca* (cv. 'Klosterneuburger Marille') grafts in pots and excised *P. armeniaca* branches (cv. 'Klosterneuburger Marille') inserted into water were used for the experiments. The plants were maintained under outdoor conditions but protected from rain.

Concentrations and manufacturers of the products evaluated in this study are indicated in table 1. Deonized water was used for untreated controls. The apricot grafts and excised branches were sprayed to run off with the tested products and allowed to air dry before placement of insects.

In free choice assays excised branches treated with the mentioned products were simultaneously placed into insect proof cages (40x35x40 cm). 100 insects were introduced into the cages and allowed to freely move around in the cage. Numbers of adults present on each branch and on the cage surface were counted daily. Eggs present on 5 randomly selected leaves per treatment were also counted.

In no-choice assays insects were kept in cylindrical cages (diameter 9 cm, height 25 cm) on potted grafts and excised branches. 10 individuals were introduced into each cage. Numbers of feeding and surviving adults were counted daily. Numbers off eggs were also recorded.

All experiments were repeated four times. Statistical analyses (ANOVA, least significant difference test) were performed by aid of the statistics program SPSS 12.0 (SPSS, Chicago, Illinois, USA).

Table 1. Plant protection products and concentrations used in this study.

Product name	Active ingredient	Concentration	Manufacturer
Cutisan	Kaolin	5% (w/v)	Biofa, Münsingen, Germany
Prev-B2	Orange oil, fatty alcohol ethoxylate	0.4% (v/v)	Biofa, Münsingen, Germany
Nu Film 17*	Pine oil	0.15 (v/v)	Löffler, Leopoldsdorf, Austria
Promanal	Paraffin oil	2% (v/v)	Proagro, Abenberg, Germany
HF Pilzvorsorge	Extract of fennel oil	0.4% (v/v)	Biofa, Münsingen, Germany

*, only used in combination with Cutisan.

Results

In free choice experiments all tested products significantly influenced host plant choice of *C. pruni* adults 24 h after start of the experiment. On average of 72% of adults had landed on untreated controls, 8% on plants treated with fennel oil extract, 20% on plants treated with orange oil. No insects were observed on kaolin or paraffin oil treated plants. After 72 h, however, fennel extract oil and orange oil had lost their repellency as compared with the untreated control, whereas only few individuals were present on plants treated with kaolin or paraffin oil.

In no choice experiments the active ingredients kaolin and paraffin oil significantly influenced feeding behaviour. One day after treatment on average of 53% of insects fed on water treated control plants but only 10% on paraffin oil treated plants and 12.5% on kaolin treated plants. All tested products significantly reduced the number of surviving insects in comparison to water treated controls. In the control treatments on average of 83% of insects were still alive after an observation period of 6 days. On plants treated with kaolin in combination with pine oil 22.5% of insect were still alive after this period, on plants treated with kaolin alone 19%, on fennel oil extract treated plants 53%, on orange oil treated plants 57% and on paraffin oil treated plants 21%.

Discussion

In the present study especially kaolin (Cutisan with and without addition of Nu Film) and paraffin oil (Promanal) had a prominent repellent effect on *C. pruni* adults both in free-choice and in no-choice experiments. Therefore, they might have a great potential for the use in integrated pest management programs targeted against *C. pruni*. Our experiments, however, were conducted under experimental conditions excluding rainfall. Thus, the repellent effects of these products to *C. pruni* must be verified under field conditions. Kaolin was sucessfully used in laboratory and field experiments against the European pear sucker, *Cacopsylla pyri* (L.) significantly reducing the population density of this polyvoltine species (Daniel *et al.*, 2005; Erler and Cetin, 2007). These experiments show that the kaolin particle film is stable under field conditions. In the mentioned experiments with *C. pyri*, however, attention was mainly paid on the effects of kaolin on oviposition and development of nymphs. In case of the univoltine *C. pruni* repellency to incoming adults as the most effective vectors would be required. Thus, long term experiments proving the effect of these products not only on vector density but also on disease incidence are desirable. Besides this, more products should be screened for their ability to repel *C. pruni* from landing and feeding on apricot trees.

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The use of *Spiroplasma melliferum* as a model organism to study the antagonistic activity of grapevine endophytes against phytoplasma

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Abstract

The objective of the research was to isolate from grapevines endophytes with antagonistic activity against phytoplasmas. In order to overcome the inability of grow phytoplasmas *in vitro*, the antagonistic activities of endophytes isolated from various grapevines on *Spiroplasma melliferum*, a phylogenetic close and cultivable Mollicute as a model organism was tested. No correlation was found so far between the inhibitory activity and the different plant sources, i.e. healthy, recovered or infected vines.

Key words: *phytoplasmas, Spiroplasma melliferum, endophytes, recovery*.

Introduction

The recovery phenomenon and the various degrees of susceptibility of grapevine cultivars to yellows disease may indicate a possible involvement of endophytes in the mechanism of plant resistance.

The objective of the research project was to isolate from grapevines endophytes with antagonistic activity against phytoplasmas. However, the inability to cultivate phytoplasmas on artificial medium prevent from a direct trial. In order to overcome this problem, the antagonistic activities of endophytes isolated from various grapevines on *Spiroplasma melliferum*, a phylogenetic close and cultivable Mollicute as a model organism was tested.

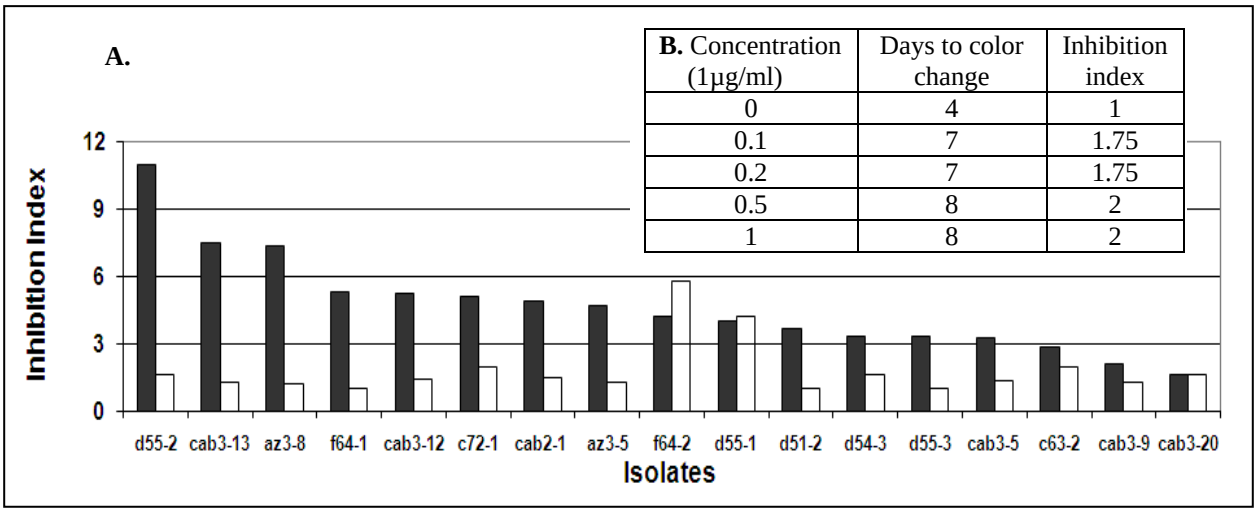
Materials and methods

Isolates from infected, recovered and healthy Cabernet-Sauvignon vines and from deserted vines were isolated in the early and late summer by placing sterilized discs of canes on nutrient agar and on PDA. The percentage of discs from which fungi or bacteria developed was recorded (table 1).

To test the antagonistic activity of the isolated endophytes, the different isolates were grown in a modified spiroplasma broth for 10 days (Trachtenberg and Gilad, 2001). After centrifugation (4,000 rpm; 30 min) and filtration, spiroplasma cells were added to either 50% diluted or 100% of the supernatant to make an initial concentration of ca. 10^6 cells/ml and incubated at 29°C for five days. Spiroplasma growing in fresh modified broth served as a positive control and the inhibitory effect of 0.5 µg/ml oxy-tetracycline was used as a reference. To monitor spiroplasma development in the filtrates we inoculated fresh spiroplasma medium with 1 µl of the incubated filtrate. The fresh medium was supplemented with phenol red as a color marker for cells growth (Trachtenberg and Gilad, 2001). Spiroplasma cell growth causes a pH decrease that changes the color of the medium from red to yellow. The time required to color change is correlated with the initial spiroplasma concentration and was therefore used as a quantitative parameter for the inhibitory effect of the filtrates. Inhibition index was defined as the ratio between the number of days to color change in the filtrate and the time required in the positive control. The inhibition index of 0.5 µg/ml oxy-tetracycline was 2. Thus, an isolate was considered inhibitory if the inhibition index of its filtrate was >=2 (figure 1A and B).

Table 1. Percent of discs with endophytic fungi or bacteria from four collection dates. Discs were cut from deserted grapevines and from healthy, recovered 1y, recovered 2y and infected Cabernet-Sauvignon grapevines.

Date of isolation	Deserted		Healthy		Recovered 2y		Recovered 1y		Infected	
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria
5.2009	1.7	45.6	0.0	6.0	1.9	20.0	2.9	20.9	0.8	10.0
7.2009	35.8	7.8	10.4	2.7	4.9	2.8	5.9	0.8	9.5	1.7
5.2010	22	0.6	1.2	0.7	2.5	6.5	2	0.8	1.0	4.0
8.2010	69.3	3.7	8.0	2.0	6.3	9.3	16.7	13.3	24.7	3.3
Average	34.5	6.9	4.9	2.87	3.9	9.7	6.9	9.5	9.0	4.8



B. Concentration (1µg/ml)	Days to color change	Inhibition index
0	4	1
0.1	7	1.75
0.2	7	1.75
0.5	8	2
1	8	2

Figure 1. Inhibition index for spiroplasma of different isolates (Mean of 2-15 replications).
A. In: ■ -100% filtrate;□ -50% diluted filtrate.
B. Inhibition index of oxy-tetracycline.

Results and discussion

In general, the highest number of populated discs was found in deserted vines (34.5% fungi, 6.9 bacteria) in contrast to healthy vines with the lowest percentage of isolates (avg. 4%). Endophytes developed from 4.8-9.7% of the discs cut from canes of recovered and infected vines. In the first date of collection we isolated more bacteria than fungi while in the other three dates the number of fungal isolates was higher.

Using this method, one fungus and several bacteria (out of 300 tested isolates) showed a relatively high inhibition activity against spiroplasma (figure 1). Growing in 100% filtrates caused 2-10 folds inhibition of spiroplasma cell growth relative to the positive control and was similar or higher compared to inhibition activity of 0.5 µg/ml oxy-tetracycline. However, the inhibitory effect of the diluted (50%) filtrate was much lower. No correlation was found so far between the inhibitory activity and the different plant sources, i.e. healthy, recovered or infected vines. This study shows that spiroplasmas can serve as an initial model system to test the effect of different compounds on phytoplasma development. In further studies the filtrates will be tested on phytoplasma in nurse culture *in vitro*.

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'*Candidatus* Phytoplasma phoenicium'-related strains infecting almond, peach and nectarine in Lebanon

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Abstract

Genetic diversity among '*Candidatus* Phytoplasma phoenicium'-related strains infecting almond, peach and nectarine plants in diverse geographic regions of Lebanon was investigated by virtual restriction fragment length polymorphism (RFLP) analysis of 16S rDNA nucleotide sequences. Calculation of virtual restriction similarity coefficients indicates the presence of two new subgroups, -F and -G, in group 16SrIX. Obtained results open new opportunities for in-depth studies on the distribution of '*Ca.* P. phoenicium' strains in plant hosts and insect vector populations from different geographic areas of Lebanon.

Key words: single nucleotide polymorphisms, genetic diversity, phytoplasma classification, restriction fragment length polymorphism.

Introduction

'*Candidatus* Phytoplasma phoenicium' strains, belonging to subgroups 16SrIX-B and -D, are associated with a lethal devastating disease of almond trees (almond witches broom, AlmWB) in Lebanon (Abou-Jawdah *et al.*, 2002). By the year 2002, more than 100,000 almond trees had died by AlmWB in Lebanon; in 2009, '*Ca.* P. phoenicium' was identified also in association with a severe disease of peach and nectarine in southern Lebanon (Abou-Jawdah *et al.*, 2009).

The rapid spread of '*Ca.* P. phoenicium' over large geographical areas in North Lebanon suggested the presence of an efficient vector (Abou-Jawdah *et al.*, 2009). However, this vector has not been identified yet. In order to have a better understanding of the disease epidemiology and achieve an effective disease management, a development project financed by Italian Cooperation is being implemented by AVSI (Association of Volunteers in International Service) Foundation in Lebanon. In the present study, data on genetic diversity among '*Ca.* P. phoenicium' strains infecting almond, nectarine and peach plants from diverse Lebanese regions are reported.

Materials and methods

Leaf samples were collected in 15 orchards from 24 plants (table 1) showing symptoms such as witches' broom, phyllody, virescence and chromatic alterations. Total DNA was extracted from 100 mg of leaf veins and used for phytoplasma detection by 16S rDNA amplification in nested PCRs primed by phytoplasma-universal primer pairs P1/P7 and R16F2n/R16R2 (Gundersen and Lee, 1996).

Amplicons from nested PCRs were sequenced, assembled, and compared with the GenBank database with the aim of searching possible identity. A total of 37 16S rDNA sequences of phytoplasma group 16SrIX (13 from GenBank and 24 obtained in this work), plus sequences from phytoplasma strains representative of known 16Sr subgroups, were analyzed through an automated *in silico* restriction assay, as described by Wei *et al*. (2007).

Results and discussion

Primer pair R16F2n/R16R2 primed amplification of DNA from templates derived from all samples studied. Phytoplasma strains identified here shared a 99-100% of sequence identity with '*Ca*. P. phoenicium' (accession number AF515636). Visualization and comparison of virtual gel plotted images revealed three different RFLP patterns (table 1). One pattern, indistinguishable from that characteristic of strains classified in the subgroup 16SrIX-D, was exhibited by DNAs from 15 '*Ca.* P. phoenicium' strains (figure 1). The remaining two virtual RFLP patterns differed from that of the previously described subgroup IX-D (figure 1), and shared similarity coefficients of 93 to 97%, confirming their affiliation with group 16SrIX. Actual RFLP analyses confirmed the recognition of two new subgroups in group 16SrIX. Prior to this work, five subgroups had been described in the group 16SrIX; the results of this study add two new, confirmed by real RFLP subgroups -F (two strains) and -G (seven strains) from almond, nectarine and peach plant hosts. The data evidenced extensive diversity of '*Ca*. P. phoenicium' in Lebanon, particularly in Sarada regions, where three 16SrIX subgroups (-D, -F, and -G) co-exist and infect nectarine plants.

Table 1. '*Ca.* P. phoenicium' strains, belonging to distinct 16SrIX subgroups, in orchards of Lebanon regions.

Strain	Origin	Orchard No.	Host	Subgroup 16SrIX
SarN1-2	Sarada	1	nectarine	-G
SarN5	Sarada	1	nectarine	-F
SarN8-1	Sarada	2	nectarine	-D
SarN9-7	Sarada	1	nectarine	-D
SarN10-8	Sarada	3	nectarine	-D
SarP10(297)	Sarada	4	peach	-D
MarN13-1	Marjayoun	5	nectarine	-D
MarN14-1	Marjayoun	6	nectarine	-D
MarN27-2	Marjayoun	7	nectarine	-F
MarN28-1	Marjayoun	7	nectarine	-D
FegA1-1	Feghal	8	almond	-G
FegA11-4	Feghal	9	almond	-D
FegA13-1	Feghal	9	almond	-G
FegA16-4	Feghal	8	almond	-D
FegA18-1	Feghal	10	almond	-G
FegP1-2	Feghal	11	peach	-D
FegP2-6	Feghal	11	peach	-D
FegP3-1	Feghal	11	peach	-G
FegPL3-1	Feghal	11	almond	-D
FegA3	Feghal	12	almond	-G
FegA4	Feghal	13	almond	-G
KKN18-1	Kerbet Kanafar	14	nectarine	-D
KKN19-1	Kerbet Kanafar	14	nectarine	-D
KKN29-1	Kerbet Kanafar	15	nectarine	-D

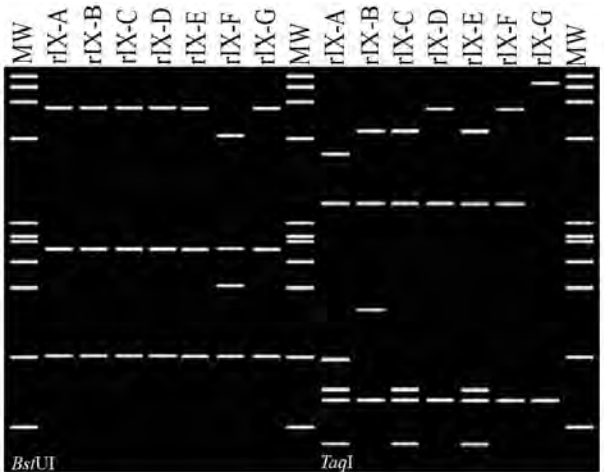

Figure 1. Virtual R16F2/nR2 RFLP patterns by key enzymes *Bst*UI and *Taq*I for distinguishing among 16SrIX subgroups.

Conclusions

The broad genetic diversity among '*Ca*. P. phoenicium'-related strains suggests possible influence of different ecological and/or climatic niches on phytoplasma population composition. In particular, it would be interesting to investigate whether particular '*Ca*. P. phoenicium' subgroup(s) could be correlated with certain biological properties and different species of insect vector. These investigations will be crucial for a better understanding of the disease epidemiology and achieving an effective disease management.

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Monitoring *Scaphoideus titanus* for IPM purposes: results of a pilot-project in Piedmont (NW Italy)

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Abstract

A pilot-project was conducted in Piedmont to determine if insecticidal sprays against *Scaphoideus titanus*, the vector of grapevine 'flavescence dorée' disease, can be decreased in case of low populations of the leafhopper. Field samplings included a sequential sampling of nymphs on leaves and yellow sticky traps for capturing adults in vineyards subject to different pest management strategies. Population levels of *S. titanus* were lower in conventional than in organic vineyards, the latter being always more variable, suggesting some problems in the distribution of the active ingredient. The vineyards that were under the proposed threshold received just one spray, however no increase in the presence of *S. titanus* was observed. As a result, provided a suitable sampling plan, it is possible to reduce insecticidal sprays against this leafhopper vector.

Key words: grapevine, 'flavescence dorée', leafhopper vector, mandatory phytosanitary procedures.

Introduction

The nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) is the main vector for 'flavescence dorée' (FD) phytoplasmas (16SrV, subgroups C and D) (Boudon-Padieu, 2003). In Italy, the control of *S. titanus* and FD is mandatory, and include insecticidal treatments and removal of infected plants. Usually, at least two-three sprays are necessary in conventional and organic farming, respectively. However, if no or little FD occurs, they may be dropped to one, provided the population levels of the leafhopper are low enough, in order to preserve non-target organisms. This research consists in a pilot-project for targeting the efforts in controlling *S. titanus* in grapevine growing areas of Piedmont (North-Western Italy).

Materials and methods

Data were collected in 2009-2010 in 22 vineyards situated in 12 districts of the Cuneo Province (44.38271-44.56752 °N; 7.85674-7.98295 °E), and subject to traditional or organic pest management against *S. titanus*: active ingredients used included thiamethoxam (TH), organophosphates (OP), etofenprox (E), pyrethroids (P), and natural pyrethrum (NP). All the vineyards were of cv "Dolcetto", except one (cv "Chardonnay"), and their size range was 0.5-0.9 ha. Plants with FD symptoms were detected only in one of them (no. 12) and were up to 2% of the total.

Nymphs of *S. titanus* were sampled at the middle of June, before insecticidal sprays, with direct observations on the leaves following a sequential sampling plan with a fixed precision level of 0.75 (Lessio and Alma, 2006): counts were interrupted either when the stop was reached, or if the pest density was lower than 0.02 nymphs per 5 leaves per plant (no more than one nymph on 137 plants). Adults were sampled with yellow sticky traps, 3 per vineyard, placed at the beginning of July and changed every 10 days until the middle of October. In Piedmont, the thresholds for dropping to one insecticidal spray are < 0.02 nymphs, and ≤ 2 adults on 3 traps/ha. Data were analysed with a hierarchical cluster method, using the level of nymphs and adults as variables, in order to find a trend in clustering of vineyards subject to different PM strategies.

Results

During 2009, nymphs and adults of *S. titanus* were found in 15 and 18 vineyards out of 22, respectively; three vineyards were under the threshold of 0.02 nymphs and 2 adults. In 2010, no nymphs were found in 5 vineyards, whereas adults were captured in different numbers in all vineyards; 2 vineyards were under the threshold. The highest presence of both nymphs and adults was recorded in the same vineyard (no. 5), that was subject to organic farming, in both years. The cluster analysis showed the presence of 5 and 6 groups in 2009 and 2010, respectively (table 1, figure 1). Cluster 1 was the most represented, and included vineyards with low populations of nymphs and adults. As a result, NP-treated vineyards were almost evenly distributed within different clusters, indicating a great variability in the effectiveness of this active ingredient, probably depending on the accuracy of distribution (e.g. sprays during day or evening, pH of the solution, etc.) or on the date of application. In 2009, the majority of vineyards (13) were in cluster 1, and adopted a PM strategy based on TH + OP. In 2010, PM strategies were more heterogeneous due to the fact that many farmers applied just one spray, provided low population levels of the vector were detected; however 16 vineyards were in cluster 1 (figure 1).

Table 1. Results of hierarchical cluster analysis of vineyards with different levels of *S. titanus* nymphs (mean density calculated with sequential sampling plan) and adults (mean captures per 3 traps).

Cluster	2009			2010		
	No. vineyards	Nymphs (mean)	Adults (mean)	No. vineyards	Nymphs (mean)	Adults (mean)
1	16	0.06	3.19	13	0.08	4.57
2	3	0.05	26.67	4	0.03	12
3	1	0.65	91	1	0.33	39
4	1	0.11	55	1	0.11	32
5	1	1	475	1	0.71	91
6	-	-	-	1	0.71	216

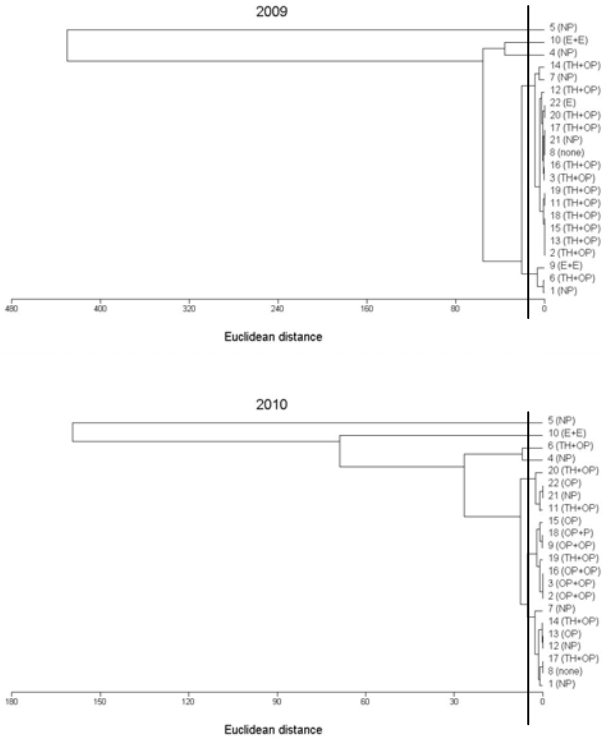

Figure 1. Dendrogram showing the vineyards subject to different PM strategies (see text for details) and falling into different clusters of *S. titanus* density. The vertical line indicates the points of dendrogram cutting.

Discussion

This pilot-project showed how a correct monitoring of the leafhopper vector, *S. titanus*, is important for targeting pest management strategies. When populations at the nymphal stage are very low (<0.02), one spray is enough to prevent infestation and therefore disease outbreaks if no FD is present within the vineyard. However, monitoring with traps permits to shift to 2 sprays within the season in case of an increase of adults' captures. The reduction of spraying should also have a benefit for honeybees and foraging insects, especially given the high toxicity of certain active ingredients Iwasa *et al*., 2004). The reduction cannot be applied in the case of organic pest management, as pyrethrum has no or little persistence and must be repeated at least 3 times during the season, however it has in any case a low environmental impact. Another risk factor is the presence of wild grapevine close to the vineyards, which can host both *S. titanus* and 16SrV phytoplasmas (Lessio *et al*., 2007; Lessio *et al*., 2011). In this case, decreasing insecticidal sprays should be considered carefully.

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On the distribution of '*Candidatus* Phytoplasma pyri' in the European Union based on a systematic literature review approach

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Abstract

The present study was conducted within the framework of a European Food Safety Authority (EFSA)-funded project (Prima phacie). The objective was to determine the current status of '*Ca*. P. pyri' in the fruit growing areas of the European Union (EU) based on a systematic literature review approach (SLR). The results show that '*Ca*. P. pyri' occurs in 15 out of the 27 EU countries, including the most important pear production areas in the EU. '*Ca.* P. pyri' is not reported from ten pear producing countries, only two of which declared the absence on the basis of official surveys. In the eight others, official surveys for pest freedom are not available, thus contributing to the uncertain pest status in those countries. Within the Prima phacie project, the results of the SLR are used when analyzing the risk of entry, establishment, spread and impact of '*Ca*. P. pyri' in regions where it is not present. Furthermore, the results are used to test the effectiveness of different options in reducing the risk of introduction and spread.

Key words: Pear decline, pest risk assessment, literature search.

Introduction

Prima phacie is a European Food Safety Authority (EFSA)-funded project to improve methodology in pest risk assessment and in the evaluation of the effectiveness of risk reduction options (MacLeod *et al*., 2010). Risk assessment methods being developed within the project are tested by using ten case study pests, among them '*Candidatus* Phytoplasma pyri', which is associated with pear decline (PD) disease. This is a destructive disease of pear (Jarausch and Jarausch, 2010), which is currently regulated within the European Plant Health Directive 2000/29 EC. Obtaining up to date information on the present distribution of a pest, by countries and areas within countries, is essential in pest risk assessments. The objective of this study was to determine the current status (presence/absence) of '*Ca*. P. pyri' in the fruit growing areas of the EU. The information obtained is based on a systematic literature review (SLR), including results of a questionnaire sent to the country representatives of EFSA's scientific network for risk assessment in plant health.

Materials and methods

Following the principles of the EFSA Guidance on application of systematic review methodology (EFSA, 2010), the key steps to conduct a SLR were applied: a clearly formulated question was developed *a priori* ("What is the distribution of '*Ca.* P. pyri' in the fruit growing areas of the EU?"), search terms were defined and combined (Pear decline OR Phytoplasma pyri OR Parry's disease OR *Cacopsylla pyri* OR *Psylla pyri* OR *Cacopsylla pyricola* OR *Psylla pyricola* OR *Cacopsylla pyrisuga* OR *Psylla pyrisuga* = Set 1; Set 1 AND occur*, Set 1 AND distribute*, Set 1 AND presen*, Set 1 AND spread, Set 1 AND monitor*, Set 1 AND survey) to search for articles in scientific abstracting databases (AGRICOLA, Agris and CAB Abstracts, searched on 08.04.2010; Web of Science, searched on 21.06.2010). The searches were not restricted concerning language and were traced back to the first findings of PD in the early 20th century.

In addition, a hand search was conducted, because not all relevant literature was expected to be included within electronic databases. For this purpose the following sources were used: EPPO Reporting Service (back to 1967, accessed 26.06.2010), EPPO-PQR (version 4.6; 07-2007), meeting reports (e.g. COST Action FA0807, Sitges, Spain, February 2010). Furthermore, in 2010 a questionnaire on the current status (the presence or confirmed absence) of '*Ca.* P. pyri' in different fruit growing areas was sent to the delegates of EFSA's scientific network for risk assessment in plant health.

In a two step screening procedure all abstracts from databases and hand searches were checked for relevance to the question by two reviewers. In the first step, abstracts were filtered out which do not address the two predefined criteria. 1. Does the abstract describe the distribution of the phytoplasma diseases and/or the vector? 2. Does the abstract describe primary research (as opposed to a review?). For all abstracts, meeting these eligibility criteria, full text papers were obtained from library services and evaluated in a second stage by using the following question: Does the paper clearly specify the fruit growing region for which the results are applicable (e. g. region Emilia-Romagna, province of Parma, municipalities x and y; as opposed to "Northern Italy").

Results

Searches with the key word combinations in the databases resulted in 477 abstracts (after removal of duplicates). 50 (10.5%) of them have been selected for the second stage screening. The main reason for papers not to be included was that the distribution of PD in fruit growing areas of the EU was not addressed, as the majority of these papers focused on diagnosis, biology or management of the phytoplasma and/or its vector. In the second stage, 24 full papers (out of 50) were selected to be included in the SLR. The main reason for discarding information was that more recent data for a specific fruit growing area was available. Furthermore, the results of 17 questionnaires (Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, Germany, Italy, Latvia, Malta, Poland, Portugal, Slovenia, UK + questionnaires from pest experts in Greece and Cyprus), 9 citations in the EPPO Reporting Service and 5 papers through hand searching were included in the SLR.

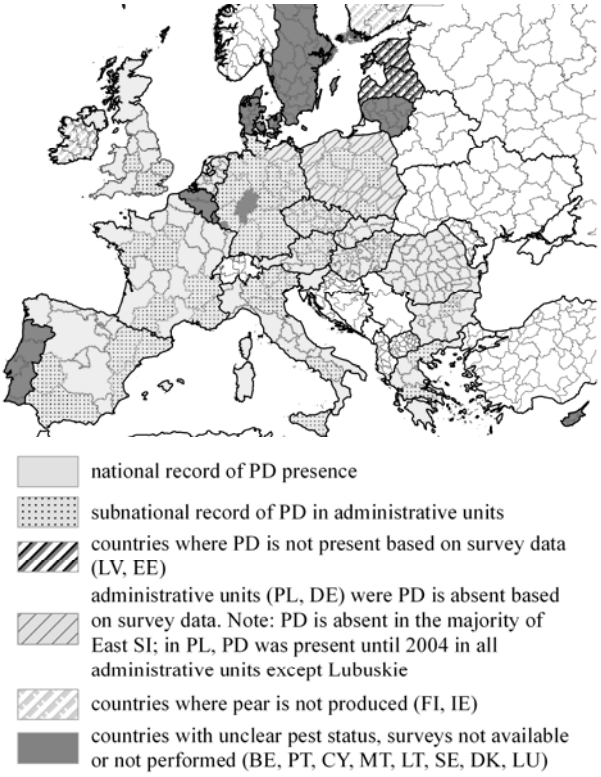

Figure 1. Status of '*Ca*. P. pyri' in the EU.

The results of the SLR are shown in figure 1. '*Ca*. P. pyri' is a native European species, which is very widespread and has been reported in 15 out of the 27 EU countries, including the largest pear production areas in Italy and Spain. In Poland and Germany, PD is officially absent in few administrative units.

'*Ca*. P. pyri' is not reported from ten pear producing countries. Of these countries only Latvia and Estonia declared the absence based on official surveys. Portugal and Belgium, two major pear producing countries are currently surveying the pest status. In Denmark and Cyprus, the disease is assumed to be absent; however, surveys have not been undertaken so far. No data were available from Lithuania, Sweden, Malta and Luxembourg, but these countries have only limited area of pear production.

Discussion

The study applies a SLR to assess the distribution of '*Ca*. P. pyri' in the EU. The methodology of SLR was found particularly useful to answer a specific question in the pest risk assessments of '*Ca*. P. pyri' as it presents an exhaustive, transparent and unbiased way of collecting, reporting and analyzing data. The results show that this pest is widespread in the EU; however, in a few countries/regions the disease appears to be absent. Within the Prima phacie project the results of the SLR are used when analyzing the risk of '*Ca*. P. pyri' entering new areas. Entry comprises different elements, such as the association of the pest and the host plant at the place of origin, the volume of trade of the commodity, the survival of the pest along the pathway and the transfer to a suitable host. By testing different risk assessment methods, particular emphasize is given to the introduction and impact of '*Ca*. P. pyri' in regions where PD has not been reported. Moreover, the effectiveness of risk reduction options on the introduction and spread is assessed.

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Response of apple proliferation-resistant *Malus sieboldii* hybrids to multiple infections with latent apple viruses

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Abstract

Apple proliferation (AP) is the most important phytoplasma-associated disease affecting apple in Europe. The failure in controlling this disease by standard means strongly increased the importance of adopting resistant genotypes. About 6000 seedlings were obtained from a breeding programme crossing *M. sieboldii,* donor of resistance to AP, with standard apple rootstocks (M9 mainly) as donor of agronomic value. Resistance screening showed that the trait was inherited to the progenies and trials are in progress to test the agronomic value of these genotypes. In an additional trial, the response of AP-resistant genotypes to a superinfection with different latent apple viruses was investigated. For this, *M. sieboldii*-derived first and second generation hybrids were analysed. In summer, three repetitions for each genotype were inoculated with *apple chlorotic leaf spot* (ACLSV), *apple stem grooving* (ASGV) and *apple stem pitting* (ASPV) virus. The two following springs after infection, the presence of the viruses was assessed by ELISA test and virus-specific symptom recording on young leaves. In parallel, the reaction of the plants to infections with Trentino strains of '*Candidatus* Phytoplasma mali' was evaluated. AP-susceptible *Malus* x *domestica* genotypes were considered as controls. The results confirmed an incidence of the viral infections on *Malus sieboldii* as it was reported in the past. However, the *M. sieboldii* hybrids showed a high variability of response ranging from no viral symptoms to severe symptoms. Nevertheless, highly phytoplasma-resistant genotypes which showed no presence of viral superinfections could be identified in these experiments.

Key words: '*Candidatus* Phytoplasma mali', *apple stem grooving* virus, *apple stem pitting* virus, resistance screening, breeding.

Introduction

Apple proliferation (AP) is one of the most important phytoplasma diseases in Europe that causes considerable economic losses. It is transmitted by grafting, insect vectors and root bridges (Ciccotti *et al*., 2007). The failure in controlling this disease by standard means strongly increased the importance of adopting resistant genotypes. Previous work indicated that, due to the colonization behavior of the associated agent, the disease can be controlled by the use of resistant rootstocks (Seemüller *et al.,* 1984). While extensive screening revealed no satisfactory resistance in established rootstocks (Kartte and Seemüller, 1991), substantial levels of resistance were identified in experimental rootstocks derived from crosses of the apomictic species *Malus sieboldii* and genotypes of *M.* x *domestica* and *M.* x *purpurea* (Bisognin *et al.,* 2008a and b; Seemüller *et al*., 2008).

As these experimental rootstocks had poor agronomic values, a breeding programme was started ten years ago in order to develop commercial AP-resistant apple rootstocks exploiting the natural resistance found in *Malus sieboldii* (Bisognin *et al.,* 2009). Resistance screening showed that the trait was inherited by the progenies and trials are in progress to test the agronomic value of these genotypes (Jarausch *et al.,* 2010). Moreover, some apomictic rootstocks budded with a virus-contaminated scion source revealed great differences in susceptibility to such viruses that include *apple chlorotic leaf spot virus* (ACLSV), *apple stem pitting virus* (ASPV) and *apple stem grooving virus* (ASGV) (Seemüller *et al*., 2008). In the present study, the response of different *Malus sieboldii* hybrids to infection with three different latent viruses was investigated and compared with phytoplasma resistance of these genotypes to two Trentino strains of '*Candidatus* Phytoplasma mali'.

Materials and methods

Healthy one-year-old micropropagated plants of *M. sieboldii*-derived first and second generation hybrids, *M. sieboldii*, 4551, D2212, H0909, H0801 o.p., Gi 477/4 o.p., C1907 o.p, 4551 o.p. (Ciccotti *et al.*, 2008) and selected hybrids obtained from the crosses 4551xM9, D2212xM9, H0909xM9 and M9xD2212 (for details see Bisognin *et al*., 2009), were inoculated in pots *ex vitro* during summer 2008. Some AP-susceptible genotypes were taken as control.

In a first experiment three replicates for each genotype were separately inoculated by chip budding with *apple chlorotic leaf spot* (ACLSV), *apple stem grooving* (ASGV) and *apple stem pitting* (ASPV) virus. In a second experiment three replicates for each genotype were contemporary inoculated with the three viruses to evaluate the reaction of the plants to superinfection. Trials were conducted in an insect-proof screenhouse. In spring 2010 ELISA test was used to evaluate the presence of the viruses and symptoms were recorded on young leaves. Symptom incidence of the viruses was evaluated as follows: 0 = no symptoms, x = low incidence, xx = moderate incidence, xxx = high incidence.

The same genotypes were evaluated in a parallel experiment for AP resistance. *Ex vitro* plants were inoculated by grafting with phytoplasma infected scions with two '*Ca*. P. mali' strains PM6 and PM11 isolated in Trentino, Northern Italy. Three repetitions for each

genotype-strain combination were performed. The second autumn after inoculation, phytoplasma infection was evaluated and expressed by a disease index based on incidence of specific symptoms such as enlarged stipules, witches brooms, foliar reddening, stunting (index values ranged from 0 = no symptoms to 4 = high presence of symptoms). In the same period '*Ca.* P. mali' concentration in the roots was also evaluated by real time quantitative PCR (data not shown).

Results and discussion

In the first experiment single infections with the latent apple viruses ACLSV, ASGV and ASPV were difficult to evaluate as more than 50% of the plants were not infected as assessed by ELISA. In contrast, the multiple infections of the *M. sieboldii* hybrids with all three viruses yielded an incidence of the viral symptoms ranging from no to severe symptoms. Indeed, sensitivity of apomictic rootstocks to latent apple viruses was already observed by Schmidt (1988) as stunting and chlorosis. Seemüller *et al.* (2008) observed a poor development and stunting of *M. sieboldii* and 4,551 seedlings inoculated accidentally with both, phytoplasma and latent viruses. Our results showed that plants of *M. sieboldii* and 4,551 selections were slightly to moderate affected by the multiple presence of viruses alone. In contrast, D2212 which was less affected in the work of Seemüller *et al.* (2008) showed no symptoms of virus infections and behaved as the tolerant *M.* x *domestica* control M9. The same was observed for plants of the apomictic selections like C1907 and Gi 477/4 which were originally derived from open pollination. Interestingly, the sensitivity to latent apple viruses was expressed very heterogeneously in the progeny of the crosses made with D2212, H0909 and 4551. The progeny genotypes showed either no viral symptoms or were much more severely affected as the parental *M. sieboldii*-derived genotypes.

The objective of the work to find a rootstock resistant to AP and tolerant to the latent apple viruses was achieved by apomictic genotypes like D2212 and C1907 o.p. as well as by some selected progeny of the breeding programme as tested here. These genotypes showed no or only mild symptoms upon inoculation with the Trentino strains of '*Ca.* P. mali' and exhibited no viral symptoms after multiple infections.

These finding should be confirmed in further trials in which breeding genotypes will be used as rootstocks of commercial varieties in order to follow the influence of virus infections also in the production of the plants. After this step the response to infection will be completely understood.

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Breeding apple proliferation-resistant rootstocks: durability of resistance and pomological evaluation

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Abstract

To develop apple proliferation (AP) resistant rootstocks, a breeding program was initiated in 2001 employing apomictic *Malus sieboldii* and *M. sieboldii*-derived hybrids as donors of the resistance trait and mainly standard apple rootstock *Malus* x *domestica* cv. M9 as donor of agronomic values. Examination of the experimentally inoculated progeny of seven crossings made in 2001 and 2002 over a period of seven to eight years showed that inheritance of resistance differs considerably among the parental lines by yielding between 10 to 55% of resistant offspring. Resistant rootstocks were characterized by poor host properties for the AP phytoplasma. This is evidenced by lower phytoplasma titers in resistant genotypes than in standard stock M9 and by preventing detectable phytoplasma development in the top grafted susceptible cv. Golden Delicious. At the end of the observation period, 80% of the root samples collected from resistant rootstocks tested PCR-negative. Size and productivity of trees grown on resistant rootstocks varied over a wide range. A preliminary pomological evaluation was done on the cv. Golden Delicious which has been grafted as infected scion on the progeny genotypes to test. Comparisons with trees grown on M9 rootstocks indicated that there are genotypes among the offspring examined that fulfill the requirements of commercial apple growing.

Key words: Apple proliferation, apple rootstocks, *Malus sieboldii*, phytoplasma titer, resistance breeding.

Introduction

Apple proliferation (AP) is induced by the wall-less bacterium '*Candidatus* Phytoplasma mali' and is widespread in several major fruit-growing areas in Europe. AP is of considerable economic importance due to its negative effect on tree productivity and fruit quality.

The disease is difficult to control. Because the recommended measures are often not satisfactory, the most promising approach to control AP appears the use of resistant plants. Previous work indicated that, due to the seasonal fluctuation of '*Ca*. P. mali' between stem and roots of infected apple trees, growing the scion cultivars on resistant rootstocks can prevent the disease (Schaper and Seemüller, 1982; Seemüller *et al.*, 1984).

However, examination of many established and experimental rootstocks, which were mostly based on *Malus x domestica*, and a large number of other *Malus* taxa revealed that there is no satisfactory resistance in these groups (Kartte and Seemüller, 1991; Seemüller *et al.*, 1992). Suitable resistance was observed in some experimental rootstock selections derived from the apomictic species *M. sieboldii* (Bisognin *et al.*, 2008; Seemüller *et al.*, 2008).

Because trees on the resistant genotypes were more vigorous and less productive than trees on standard stock M9, a breeding program was initiated to develop resistant rootstocks with satisfactory pomological properties by using *M. sieboldii*-based parental lines from the above screening as donors of resistance and M9 and other dwarfing stocks as donors of pomological values (Bisognin *et al.*, 2008; Bisognin *et al.*, 2009; Jarausch *et al.*, 2007; Jarausch *et al.*, 2010). Here we present results of long-term observations on level and durability of resistance, the relationship of phytoplasma concentration to resistance, and on vigor and productivity of trees grown on selected offspring.

Materials and methods

In 2001 and 2002 the following crossings were made that resulted in a substantial number of offspring (see Bisognin *et al.*, 2009 for details): 4551 (Laxton's Superb x *M. sieboldii*) x M9; 4608 (*M. purpurea* 'Eleyi' x *M. sieboldii*) x M9; H0909 [(Laxton's Superb x *M. sieboldii*) x M9)] x P22; H0909 x M9; *M. sieboldii* x M9; D2212 [(Laxton's Superb x *M. sieboldii*) x o.p.)] x M9; M9 x D2212.

All progenies were grown in pots in the greenhouse. Sets of 5 to 6 locus-specific simple sequence repeats (SSR) markers were employed to distinguish sexually derived seedlings from apomictically derived seedlings (Bisognin *et al.*, 2009). In July, preferentially recombinant seedlings, for comparison also a representative number of motherlike (apomictic) plants, were graft-inoculated with cv. Golden Delicious infected with severe strains of '*Ca.* P. mali'. The following spring, inoculated plants were transplanted to the nursery where they were observed for 2 to 3 years for symptom expression on Golden Delicious. All trees on recombinant seedlings that never developed symptoms or only temporarily mild symptoms such as foliar reddening or enlarged stipules were considered to be resistant and were transplanted for further evaluation under commercial growing conditions. In addition, some trees on

motherlike seedlings were also transplanted. Symptom development and yield were recorded annually for 4 to 5 years. Vigor as expressed by trunk diameter in 40 cm height was determined in fall of 2010.

Quantitative real-time PCR was performed to determine presence and concentration of '*Ca.* P. mali' in inoculated trees as described (Bisognin *et al.*, 2008).

Results and discussion

From the crossings made, approximately 3,000 offspring were obtained. SSR genotyping revealed that the majority of them derived from apomixis. Some 750 seedlings were inoculated of which 535 were recombinant.

Screening during the nursery phase revealed considerable differences in the inheritance of AP resistance of the various apomictic parents used. The best donors of this trait were selections 4608 and D2212. Crossings of these genotypes with M9 yielded 60 to 70% recombinant offspring classified as resistant. In the progenies of the other crossings resistance ranged between 20 and 30%.

At the end of the nursery growing phase, 207 trees on recombinant rootstocks and 47 trees on motherlike rootstocks were selected and transplanted for further evaluation. In the following field observation period, 70 to 80% of the trees on both recombinant and motherlike rootstocks continued to show excellent resistance properties whereas the remaining trees showed, mostly temporarily, mild to moderate symptoms. Only the trees on rootstocks derived from selection H0909 depicted lower values on the persistence of resistance, being in the range of 40%. In contrast to selected trees from resistant parents, all transplanted control trees on M9 rootstocks showed permanently moderate to severe AP symptoms.

Quantitative RT-PCR showed that the phytoplasma titer in *M. sieboldii*-derived resistant rootstocks is usually in the range from 10^4 to 10^6 cells/g phloem. In the roots of M9 stock the titer was 10 to 1,000 times higher. This is confirming previous findings (Bisognin *et al.*, 2008). Accordingly, the phytoplasma titer in roots of severely affected progeny genotypes was found to be about 10 times higher than in offspring that was not or only slightly affected. Furthermore, there is indication that the phytoplasma infection in resistant roots is eliminated or reduced to an undetectable level. Eight years post inoculation, 80% of the root samples collected from resistant stocks tested PCR-negative.

At the end of the observation period the selected trees on recombinant rootstocks differed considerably in size. Most of them were too vigorous for the commercial growing of culinary apples. However, in all progenies recombinant stocks were identified that mediate satisfactory dwarfism to the scion cultivar. E.g., more than 10% of the trees on resistant genotypes of the 4608 x M9 progeny were similar in size to trees on M9.

The correlation of productivity and vigor known from established rootstocks also applies for the selected resistant stocks. Regarding trees on resistant stocks derived from the most successful crossing 4608 x M9, the cumulative yield ranged from 1.6 to 5.0 kg/cm^2 cross section of the trunk. Comparison showed that the average yield of trees on dwarfing stocks was 3.7 kg/cm^2 whereas that of trees on stocks too vigorous for commercial growing was only 2.4 kg/cm^2.

Promising genotypes are currently multiplied by micropropagation for further agronomic evaluation.

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First data obtained by shotgun proteomics from *Nicotiana occidentalis* infected by '*Candidatus* Phytoplasma mali'

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Abstract

The protein content of *Nicotiana occidentalis* infected by the non-cultivable phytopathogenic mollicute '*Candidatus* Phytoplasma mali' strain AT was determined by shotgun proteomics. 102 out of 497 predicted phytoplasma proteins were identified as expressed in shoot tissue. In addition, 940 proteins of *N. occidentalis* were detected. Results demonstrate the successful application of LTQ Orbitrap XL ETD™ mass spectrometer in detecting phytoplasma-specific proteins in protein mixtures. A high portion of proteins with unknown function was identified beside prominent proteins involved in translation. Several of the proteins with unknown function contain a signal peptide suggesting a potential pathogen-host interaction.

Key words: phytoplasma, apple proliferation, proteomics.

Introduction

'*Candidatus* Phytoplasma mali' is the agent associated with apple proliferation disease which represents one of the most economically important phytoplasma diseases in Europe. Infection results in impaired fruit quality and productivity of the apple trees. '*Ca.* P. mali' belongs together with '*Ca.* P. pyri', '*Ca.* P. prunorum' and few other phytoplasmas to the apple proliferation group, which forms a distinct major subclade within the phytoplasmas (Seemüller and Schneider, 2004). The genome sequence of '*Ca.* P. mali' strain AT was determined and highlighted an unusual linear chromosome organisation and a low G+C content of 21.4% (Kube *et al.*, 2008). The condensed genome with a size of 602 kb encodes 497 protein coding genes.

This study aims to evaluate the application of shotgun proteomics to '*Ca.* P. mali' strain AT infected plant material and thereby provide additional information and evidence for the expression of the predicted proteins. The shotgun proteomics approach was successfully used for the identification of proteins assigned to the mulberry dwarf phytoplasma by comparing mass spectra with the proteins of all *Mollicutes* (Ji *et al.*, 2009). Due to the complete genome sequence of '*Ca.* P. mali' a more stringent assignment is possible. Here we present the first experimental derived proteome data of this study.

Materials and methods

Protein isolation from plant material

Nicotiana occidentalis greenhouse plants were inoculated with '*Ca.* P. mali' strain AT as previously described (Kube *et al.*, 2008) at the Julius Kühn Institute Dossenheim. One plant showing distinct symptoms of infection such as little leaves and witches` broom was chosen for the initial experiment. Proteins were isolated from shoots by two different methods in parallel. First approach (I) started by freezing the tissue in liquid nitrogen followed by TissueLyser treatment (Qiagen), suspension in SDS lysis buffer containing protease inhibitors (unpublished) and sonification. For the second approach (II), shoots were disrupted in meshbags (extraction Bags, Bioreba) and proteins isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) according to the manufacturer's instruction. Proteins were separated in 12.5% SDS-PAGE gel and stained by Coomassie G250 for visualisation. After destaining in 20% methanol, 7.5% acetic acid gel lanes were cut into 16 slices. Reduction and alkylation was performed. Proteins were digested within the gel slices with trypsin (Roche Diagnostics) for 16 h at 37°C in humidified atmosphere. Peptides were extracted and vacuum dried. Afterwards, samples were resuspended in 5% acetonitrile with 2% formic acid.

LC-MS/MS measurement

LC-MS/MS was performed on LTQ Orbitrap XL ETD™ mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray ion source (Thermo Fisher Scientific) and an Agilent 1200 Series HPLC- System (Agilent Technologies). The System was fitted with a self-packed C18 RP column (0.15 mm × 150 mm *PicoFrit*, New Objective; *ReproSil- Pur C18- AQ* Dr. Maisch). Buffers A (2% acetonitrile + 0.1% formic acid) and B (98% acetonitrile + 0.1% formic acid) served as mobile phase and the peptides were eluted via a gradient of 2.7% to 90% buffer B over a period of 150 min with a flow rate of 0.2 µl/min. Full-scan mass spectra were detected with the Orbitrap mass spectrometer. The ten most intense peptides were selected for CID MS/MS scans in the linear ion trap. Each sample has been injected two times.

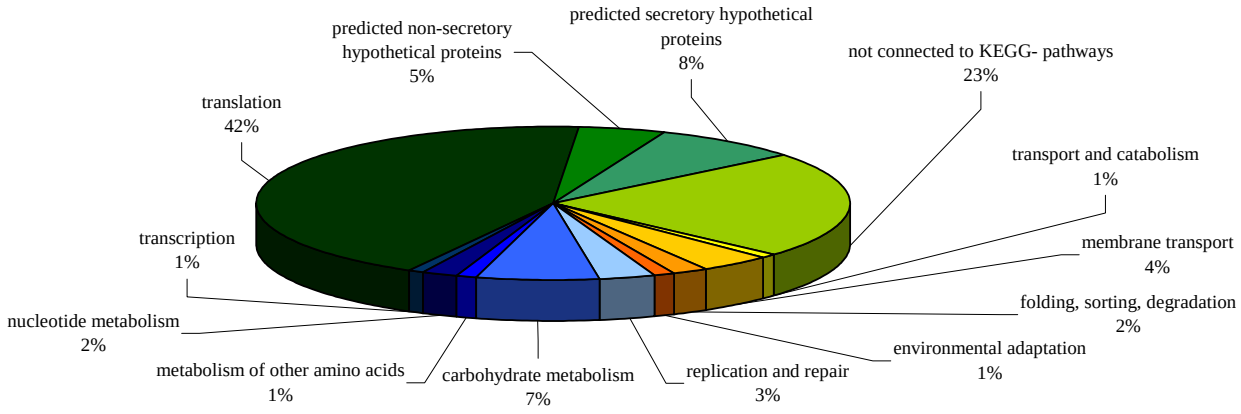

Figure 1. Functional categories of the 102 identified '*Ca*. P. mali' strain AT proteins according to their connection to KEGG pathways. Proteins with assigned function but no connection to KEGG were grouped in "not connected to KEGG pathways" whereas those with no assigned function were grouped in "predicted secretory/non-secretory hypothetical proteins".
(In colour at www.bulletinofinsectology.org)

Protein identification

Protein identification has been carried out using the Andromeda search engine (Cox *et al.*, 2011) in the MaxQuant V1.1.1.25 environment (Cox *et al.*, 2008) with a FDR at peptide and protein level of 1% and a maximum mass deviation for the fragment ions of 0.5 Da. A target-decoy database was constructed comprising 9002 *N. occidentalis* (http://www.ncbi.nlm.nih.gov/) and 497 '*Ca.* P. mali' protein sequences (CU469464). In addition, 13,080 open reading frames (ORFs) with a minimum length of 20 amino acids were calculated from the "*Ca.* P. mali" strain AT genome sequence and also implemented in the database. A set of common contaminant proteins (http://www.maxquant.org/) like keratines has been included. A maximum of three missed cleavages was allowed for the protease trypsin. Oxidation of methionine and acetylation of the N-terminus have been applied as variable modifications. Carbamidomethylation was set as fixed modification. A protein hit has been considered as valid, if the protein was identified by at least two peptides, of which one had to be unique.

Results and discussion

Within these first experiments 102 proteins of '*Ca*. P. mali' and 940 *N. occidentalis* were identified. While the plant derived proteins are still under investigation, a preliminary analysis of the '*Ca*. P. mali' proteins was performed. Genes assigned to the KEGG (Kanehisa *et al.*, 2010) based functional category translation (figure 1) dominate the overall number of proteins. The nearly complete set of ribosomal proteins dominates this category. However, proteins of unknown function represent the third largest group of the identified proteins. A high portion of predicted secretory proteins without assigned function is remarkable and indicates the lack of information in phytoplasmas. As supposed the immunodominant membrane protein Imp was identified.

First results clearly indicate that the proteomic shotgun approach is successful applicable for the identification of expressed '*Ca*. P. mali' proteins in plants. Analysis of the plant-derived proteins and of additional tissue from other *N. occidentalis* plants representing biological replicates is in progress.

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TAL effectors from *Xanthomonas*: design of a programmable DNA-binding specificity

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Abstract

Xanthomonas spp. are Gram-negative bacteria with powerful molecular weapons to attack their plant hosts. Key for pathogenicity of *Xanthomonas* is a type III secretion system that injects a cocktail of effector proteins into plant cells to function as potent virulence factors. TAL (transcription activator-like) effectors from *Xanthomonas* function as transcriptional activators of plant genes in the plant nucleus. They contain a central domain of tandem, near-identical 34 amino-acid repeats. Each repeat recognizes a single base pair in a contiguous DNA sequence and two adjacent hypervariable amino acids per repeat specify the base that is bound. This modular DNA-binding code allows a simple reprogramming of DNA-binding specificity, a feature with high potential for biotechnology. We developed a method called "Golden TAL Technology" that allows a flexible assembly of TAL proteins with a designed order of repeats.

Key words: Transcription factor, AvrBs3, zinc-finger proteins, golden gate cloning, TALE.

Introduction

Pathogenicity of plant pathogenic *Xanthomonas* relies on the delivery of virulence proteins, so called effectors, into target plant cells. Transport of effectors is facilitated by a specialized type III secretion system (Hrp) which spans both bacterial membranes and employs a hollow exterior conduit (the Hrp-pilus) that traverses the plant cell wall and delivers effectors across the plant plasma membrane via a translocon (Büttner and Bonas, 2010).

Typically, *Xanthomonas* strains harbor approx. 30 different effectors. In most cases their molecular activities are still unknown. TAL (transcription activator-like) effectors form a large and important effector family that is nearly exclusively found in *Xanthomonas* (Boch and Bonas, 2010). They function as transcription factors of plant genes and several TAL effectors induce expression of sugar exporters (Chen *et al.*, 2010).

TAL effectors contain a central domain of tandem near-identical 34-amino acid repeats (2 to 34). Each repeat binds to one DNA base pair in a contiguous sequence. Specificity of the repeats is determined by two hypervariable amino acids per repeat. Rearranging the repeats yields novel and predictable DNA-binding specificities (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). TAL-nuclease fusions have been used to specifically edit the human genome (Miller *et al.* 2010).

Materials and methods

Golden TAL Technology

Four key TAL repeat types (NI, HD, NN, NG; table 1) were chosen that specifically recognize the four bases of the DNA. Individual TAL repeats were cloned with flanking *BpiI* sites. One to six repeats are ligated into an assembly vector replacing a *lacZ* selection marker by *Bpi*I cut-ligation (step I). Several repeat assemblies are combined together with fragments encoding N- and C-terminus of the TAL effector, respectively, into a target expression vector by *Bsa*I cut-ligation (step II).

Cut-ligations were set up with 50-100 ng of each plasmid, 1 µl restriction enzyme (*Bpi*I or *Bsa*I), 2 µl ATP (10 mM), 2 µl restriction buffer no. 4 (NEB), 1 µl T4-ligase (5 u/µl) in a 20 µl reaction. Reactions were incubated for 1 h at 37°C followed by 20 min. at 70°C to inactivate the enzymes. For further details please see (Geißler *et al.*, 2011).

Table 1. DNA-specificity of individual TAL effector repeats.

TAL repeats[a]	DNA specificity[b]
NI	A
HD	C
NN	G/A
NK	G
IG	T
NG	T
HG	T

[a] According to amino acids 12 and 13 per repeat.
[b] Only upper strand base is shown.

Results

Construction of designer TAL effectors

TAL effector repeats are highly repetitive on protein as well as DNA level which complicates cloning approaches. Our initially constructed artificial TAL effectors with novel repeat arrangements were based on

a random assembly of individual modules of four key repeats encoding a specificity for the four DNA bases, respectively (table 1). This cloning was possible, because exactly one restriction site for the type IIs restriction enzyme *Esp3*I is naturally present in each TAL repeat. Type IIs restriction enzymes have separate recognition and restriction sites, which lead to non-palindromic overhangs and a linear unidirectional ligation of TAL repeat fragments (Boch *et al.*, 2009).

To construct TAL effectors with a given order of repeats and, therefore, a programmed DNA-specificity, it was essential to align TAL effector repeats in a more controlled fashion. We improved our initial construction method and modified a method termed golden gate cloning (Engler *et al.*, 2008). In essence, golden gate cloning describes the assembly of a series of fragments containing specific overhangs that have been generated with flanking type IIs restriction enzymes. The fragments are designed such that correct ligation products lack the applied restriction site and are enriched in the reaction.

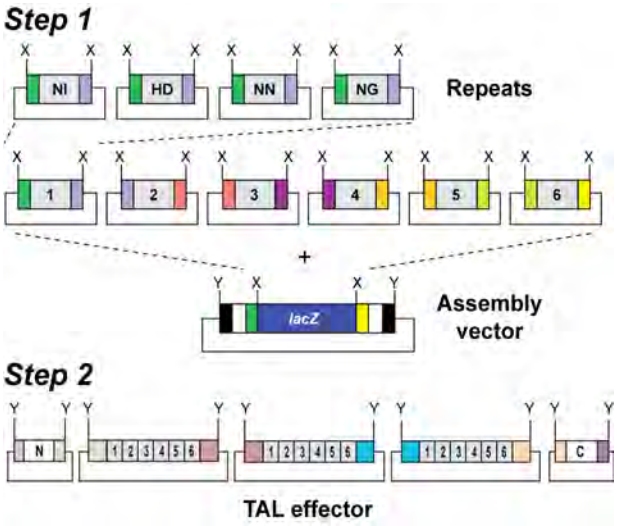

Figure 1. The golden TAL technology. TAL effectors with a designed repeat composition were assembled in two cut-ligation steps. X, *Bpi*I; Y, *Bsa*I. Same colors indicate matching overhangs.
(In colour at www.bulletinofinsectology.org)

TAL proteins with a desired order of repeats were constructed using a two-step assembly technique. First, a library of four key repeats with specific flanking type IIs restriction sites was constructed. Up to six repeats were combined into an assembly vector (figure 1). Second, several assembly vectors were combined with fragments encoding N- and C-terminal regions of TAL effectors into an expression vector (Geißler *et al.*, 2011).

Our setup allows a very flexible generation of TAL effectors with any designed order of repeats. Generation of programmable DNA-binding specificities is now easily possible.

Discussion

The controlled assembly of TAL effectors with a designed repeat composition is key to use these proteins as programmable DNA-targeting devices. Our golden TAL technology allows an easy assembly of TAL effectors with designed repeats. In addition, it is flexible to incorporate N- and C-terminal variations, as well as different tags as modules in the cut-ligation reaction. This should greatly facilitate the adoption of this technique also for the generation of fusion proteins (e.g. TAL-nucleases) for biotechnology.

Several groups have recently developed similar techniques. The tools that are currently generated will enable a broad applicability of the TAL technology for different biological fields.

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European interlaboratory comparison and validation of detection methods for '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum' and '*Candidatus* Phytoplasma pyri': preliminary results

THE EUPHRESCO FRUITPHYTOINTERLAB GROUP

Abstract

A working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of '*Candidatus* Phytoplasma prunorum', '*Ca*. P. mali' and '*Ca*. P. pyri' in fruit trees. Four molecular protocols were submitted to interlaboratory trials starting from extracted DNAs prepared in four laboratories. The tested molecular protocols consisted in universal and group-specific real time and conventional nested PCR assays. A good agreement among laboratories was obtained and high values of diagnostic sensitivity were revealed for all tested protocols. The preliminary analysis of the results also highlighted some diagnostic specificity problems that require further investigations.

Key words: ringtest, '*Candidatus* Phytoplasma prunorum', '*Candidatus* P. mali' and '*Candidatus* P. pyri'.

Introduction

In the competitiveness of agricultural products the phytosanitary quality is of increasing importance and harmonized protocols had taken an active role in the agricultural food chain.

In this context a working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of '*Candidatus* Phytoplasma prunorum', '*Ca*. P. mali' and '*Ca*. P. pyri' in fruit trees. These phytoplasmas are agents of relevant diseases inducing severe crop losses in fruit trees and particularly, the last two phytoplasmas are included in the EPPO A2 List of quarantine pests.

Protocols based on conventional and real time PCR for the detection of above mentioned phytoplasmas were selected and submitted to ringtest trials performed in 22 European laboratories. Validation data (diagnostic sensitivity and specificity) were calculated and statistically analysed according with the UNI CEI EN ISO/IEC 17025.

In the present paper the preliminary results of the collaborative studies are reported.

Materials and methods

Design of the study

The ringtest program was scheduled from April 2010, for exchange of procedures, to February 2011 for laboratories trials and statistical analysis.

The 22 participant laboratories (table 1) analysed an identical series of 30 blind samples following the provided working protocols. The involvement of each participant laboratory in the ringtest is reported in table 2. In the analysis of the results all the participant laboratories are reported with anonymous number.

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

Institution	Contact person	Location
AGES	Helga Reisenzein	AT
CRA-W	Stephen Steyer	BE
ILVO	Kris de Jonghe	BE
CLPQ	Zhelju Avramov	BG
ACW	Santiago Schaerer	CH
State Phytosan. Admin.	Gabriela Schlesingerova, Hana Orsagova	CZ
JKI	Bernd Schneider	DE
Aarhus Univ.	Mogens Nicolaisen	DK
DAR	Ester Torres, Joan Bech	ES
IRTA	Assumpcio Batlle, Amparo Laviña	ES
UPVLC	Isabel Font	ES
CRA-PAV	Graziella Pasquini, Luca Ferretti	IT
Phytosanitary Lab. Lombardy	Marica Calvi	IT
University of Bologna	Samanta Paltrinieri, Assunta Bertaccini	IT
BIOFORSK	Dag-Ragnar Blystad, Sonja Sletner Klemsdal	NO
PPS	Linda Kox, Jeanette Teunisse, Bart van de Vossenberg	NL
Main Inspect. of Plant Health	Ewa Hennig, Justyna Moszczynska	PL
INRB/L-INIA	Esmeraldina Nascimento Agostinho de Sousa, Eugenia Andrade	PT
UKSUP	Lubomir Horvath, Michaela Hudecoba	SK
NIB	Marina Dermastia, Natasa Mehle	SI
GDAR	Nursen Ustun, Aydan Kaya	TR
FERA	Adrian Fox, Anna Skelton	GB

Table 2. Numbers of laboratories involved in each tested protocol.

Number of labs involved	Nested PCR	Specific qPCR (Nikolic *et al.*, 2010)	Universal qPCR (Christensen *et al*., 2004)	Universal qPCR (Hodgett *et al*., 2009)	Plasmid
6	X	X	X	X	X
8	X	X	X	X	
2	X	X	X		
4	X				
1	X		X	X	
1	X	X		X	

To avoid manipulation of living quarantine organisms, the samples to be tested were constituted by extracted DNAs. Standards of a cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca.* P. pyri' in concentration from 10^7 to 10^1 were also included in the trials.

In order to standardize the experiments Taq DNA polymerase and real time master mix brand were specified and Taqman probes were supplied by the organizer.

Samples

A series of 30 samples, target (symptomatic and asymptomatic infected plants) and non-target (healthy plants and closely related bacteria) have been selected to perform all experiments (table 3).

Table 3. List of tested samples and their origin.

N°	Specie	Sanitary status	Origin
1	apple	healthy	JKI
2	apple	'*Ca*. P. mali'	JKI
3	apple	'*Ca*. P. mali'	JKI
4	apricot	'*Ca*. P. prunorum'	CRA-PAV
5	apricot	'*Ca*. P. prunorum'	CRA-PAV
6	plum	healthy	DAAM
7	pear	healthy	JKI
8	plum	healthy	DAAM
9	pear	'*Ca*. P. pyri'	JKI
10	extracted DNA	bacteria	FERA
11	extracted DNA	bacteria	FERA
12	apple	'*Ca*. P. mali'	JKI
13	pear	'*Ca*. P. pyri'	JKI
14	extracted DNA	bacteria	FERA
15	apple	'*Ca*. P. mali'	JKI
16	apple	'*Ca*. P. mali'	JKI
17	extracted DNA	bacteria	FERA
18	pear	'*Ca*. P. pyri'	JKI
19	apple	healthy	JKI
20	pear	'*Ca*. P. pyri'	JKI
21	plum	healthy	DAAM
22	pear	'*Ca*. P. pyri'	JKI
23	extracted DNA	bacteria	FERA
24	extracted DNA	bacteria	FERA
25	pear	healthy	JKI
26	apricot	'*Ca*. P. prunorum'	CRA-PAV
27	pear	healthy	JKI
28	apricot	'*Ca*. P prunorum'	CRA-PAV
29	pear	healthy	JKI
30	plum	'*Ca*. P. prunorum'	DAAM

From each tested sample the total DNA was extracted from midribs using CTAB protocol (Doyle and Doyle, 1990) in four laboratories (DAAM, JKI, CRA-PAV, FERA) and sent to each partner as dried DNA. All the laboratories were asked to re-suspend it in 500 µl of distilled water.

In all tested protocol undiluted and tenfold diluted extracted DNA from each sample was used as template.

Tested protocols. Four molecular protocols were submitted to interlaboratory trials:

1. AP group specific nested PCR (nested PCR);
2. real time for the specific detection of '*Ca*. P. mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum' (Nikolic *et al*., 2010) (specific qPCR);
3. real time PCR for the universal detection of phytoplasmas (Christensen *et al*., 2004) (universal qPCR-C);
4. real time PCR for the universal detection of phytoplasmas (Hodgetts *et al*., 2009) (universal qPCR-H).

Nested PCR (1)

The protocol is based on a direct PCR using the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by a group specific nested PCR performed with the primer pairs fO1/rO1 (Lorentz *et al*., 1995). Direct and nested PCR were performed in a 25 µl mixture containing: 1X Green GoTaq reaction buffer, 200 µM dNTPs (Promega), 0.4 µM of each primer, 0.625 U of GoTaq DNA polymerase (Promega), 1 µl of extracted DNA for direct PCR and 1 µl of P1/P7 amplicons diluted 1:30 for nested PCR (for 50 µl mixture: 2 µl DNA/diluted PCR product).

The cycling parameters included an initial denaturation step at 94°C for 2 min, followed by 36 and 38 cycles, for direct and nested PCR, respectively: 1 min at 94°C (denaturation), 1 min at 55°C and 50°C, for direct and nested PCR, respectively (annealing), 2 min at 72°C (extension) and a final extension step at 72°C for 8 min.

Specific qPCR (2)

The protocol consisted in a TaqMan real time PCR (qPCR) employing three specific FAM-MGB probes (AP-P, ESFY-P and PD-P) in separate reactions, using chemicals and amplification conditions reported in Nikolic *et al*. (2010).

Universal qPCR-C (3)

The protocol consisted in a TaqMan qPCR employing a FAM-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported in Christensen *et al*. (2004).

Universal qPCR-H (4)

The protocol consisted in a TaqMan qPCR employing a VIC-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported in Hodgetts *et al*. (2009). All the participants included their own positive and negative template controls. By qPCR, all samples were tested in duplicate. COX or human 18S rRNA (Applied Biosystem) was used as endogenous quality control of DNA extraction. qPCR were performed in 10 or 25 µl reactions.

Processing of the result data

The participants were asked to provide only '+' or '-' results for nested-PCR. Ct values for each replicate were asked for qPCR protocols, specifying threshold and baseline (manual or automatic).

The following parameters were calculated, using the R statistical framework (2010), to analyze the result:

- Agreement between laboratories - measured by calculation of the Kappa coefficient (Fleiss *et al*., 2003) and interpreted as reported in Landis and Koch (1977). The output included also significance tests for the kappa index (the null hypothesis is a zero kappa value).
- Diagnostic sensitivity (SE) - an estimation of the ability of the method to detect the target.
- SE=100xTP/(TP+FN) (table 4);
- Diagnostic specificity (SP) - an estimation of the ability of the method not to detect the non-target.
- SP= 100xTN/(FP+TN) (table 4).

If a laboratory had more than 10% of no-coincident replicates or more than 10% of unexpected differences between undiluted and diluted sample for one method, all the results obtained were omitted in the statistical analysis.

Table 4. Parameters for calculation of diagnostic specificity and sensitivity.

TP – true positive	positive detected from positive expected
FN - false negative	negative detected from positive expected
FP - false positive	positive detected from negative expected
TN - true negative	negative detected from negative expected

Results

All the participant laboratories carried out analysis on all DNA samples according to provided protocols. Results obtained with endogenous controls included in real time PCR protocols confirmed the good quality of all extracted DNAs. Analytical sensitivity obtained from standards of the cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' is reported in table 5. No relevant differences were observed among the protocols.

Agreement among laboratories. Fleiss' kappa index showed a 'almost perfect' agreement for all tested protocols (table 6), and p-values were almost 0. Diagnostic sensitivity and diagnostic specificity. SE and SP were calculated for each protocol and for each laboratory and mean values are reported in table 6. SE values resulted high for all protocols, ranging from 99.3% to 100%, whereas the specificity ranged from 93.8% to 99.7%.

Table 5. Analytical sensitivity of tested protocols calculated using serial dilutions of P1/P7 cloned fragment from '*Ca*. P. mali' and '*Ca.* P. pyri'. The analysis was not performed for universal qPCR-H, that amplify a region different from 16S-23S.

Laboratories	Nested-PCR		Specific qPCR		Universal qPCR-C	
	'*Ca*. P. mali'	'*Ca*. P. pyri'	'*Ca*. P. mali'	'*Ca*. P. pyri'	'*Ca*. P. mali'	'*Ca*. P. pyri'
1	n.t.	n.t.	10^1	10^1	10^1	10^2
8	n.t	n.t	10^1	10^1	10^1	10^1
11	n.t.	n.t.	n.t.	10^1	10^1	10^1
12	10^3	10^1	n.t.	n.t.	10^1	10^2
13	n.t	n.t	10^1	10^1	10^1	10^1
16	10^1	10^1	n.t	n.t	n.t	n.t

Table 6. Diagnostic sensitivity (SE), Diagnostic specificity (SP) and agreement values calculated by the Fleiss' kappa index for each tested protocol.

Method	Number of laboratories considered	Mean		Kappa index*
		SE	SP	
Nested-PCR	20	99.3	97.7	0.940
Universal real time-C	10	100.0	96.0	0.926
Universal real time-H	12	99.4	97.2	0.945
Specific qPCR – '*Ca*. P. pyri'	12	100.0	99.7	0.980
Specific qPCR – '*Ca*. P. mali'	12	100.0	98.7	0.924
Specific qPCR – '*Ca*. P. prunorum'	13	100.0	93.8	0.840

*, Kappa values interpreting: <0 poor agreement; 0.00-0.20 Slight agreement; 0.21-0.40 Fair agreement; 0.41-0.60 Moderate agreement; 0.61-0.80 Substantial agreement; 0.81-1.00 Almost perfect agreement (Landis and Koch, 1977).

Discussion

The results obtained in the interlaboratory trials showed that all four tested protocols resulted sensitive. The robustness of the protocols was also supported by the agreement levels for the different participants using different thermo cycler.

Nevertheless, diagnostic specificity values resulted affected by some unexpected results that open important questions and make necessary further investigations. Particularly, some non-target samples (plants assumed as healthy and phytoplasma related bacteria) gave positive results in different experiments and laboratories. In case of related bacteria the positive reactions can be explained as 'laboratory contamination', whereas in case of samples assumed as negative this result indicates the necessity to establish if the samples are 'true negative' or if they have a low titre of phytoplasma, detectable only by highly sensitive techniques.

Finally, in this ringtest the DNA extraction step have not been taken into consideration because living quarantine pathogens have to be manipulated as reported in CE 95/44 directive. Therefore the results are only related to the reliability of the amplification procedures, even if the nucleic acid extraction should be considered a critic step in phytoplasmas detection.

All the questions and problems derived from the preliminary analysis of the results have induced the participants to implement the experiments and further trials are in progress.

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Highlights on some EPPO activities in plant quarantine

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Abstract

One of the aims of EPPO is to help its member countries to prevent entry or spread of dangerous pests (plant quarantine). The Organization has therefore been given the task of identifying pests which may present a risk (early warning), evaluating their risk for the region and making proposals on the phytosanitary measures which can be taken against them (Pest Risk Analysis). To perform these activities, much information on pests presenting a risk to the EPPO region is required and is collected by the Organization and made available to its member countries. In addition, as identifying the pests is important EPPO has established since 1998, a work programme in the area of diagnostics to harmonize procedures across the region. The different activities conducted in the framework of this programme are presented.

Key words: pests, emergence, alert systems, diagnostics, accreditation.

Emerging plant diseases

Human societies have throughout their histories faced the emergence of new plant diseases which damaged crops or the environment. In plant pathology, the classical example remains the disastrous consequences of the introduction of potato late blight which caused famine in Ireland in the 1840s and now causes problems in potato production worldwide. In more recent history, many new plant diseases have emerged in different parts of the world, and this phenomenon seems to have accelerated. Although there is no agreed definition of what is an emerging plant disease, it can correspond to an already known disease whose incidence or geographical distribution is notably increasing but it may also be caused by newly described pathogens. The causes of plant disease emergence are multiple and quite complex, but it is generally accepted that human activities (e.g. trade of plants, accidental introduction of vectors, modifications of agricultural practices or land use) play an important role.

In the European and Mediterranean region, agriculture is an economically important sector covering a large variety of plants which are subject to an ever increasing trade and at the same time potentially threatened by a wide range of pests and diseases. Therefore, it is essential for Plant Protection Services to avoid the introduction and spread of new pests via commercial exchanges. Over the years, EPPO has made recommendations to its fifty member countries on phytosanitary measures which should be implemented to avoid the introduction of damaging pathogens (e.g. *Xanthomonas citri* pv. *citri*, *Liberibacter* species associated with citrus huanglongbing which are currently emerging in the Americas) or to prevent further spread of diseases which already occur in the region (e.g. Citrus tristeza virus, Plum pox virus). However, these existing phytosanitary measures can be challenged by the emergence of new diseases. In the EPPO strategy, it is felt essential to assess the risks associated with emerging diseases and, whenever appropriate, to propose management measures (i.e. restrictions on trade) against them. EPPO has elaborated a Pest Risk Analysis (PRA) scheme which will be presented. When new diseases are emerging, it is also important to provide early warning to Plant Protection Services so that they can put into place import inspections and surveillance programmes on their territories. Since 1998, EPPO has set up an Alert List on its website (www.eppo.org) to provide data on emerging diseases (e.g. stolbur phyoplasmas, *Chalara fraxinea*, *Fusarium oxysporum* f. sp. lactucae, *Phytophthora kernoviae*, *Pseudomonas syringae* pv. actinidae, viroids of solanaceous plants, new tomato viruses). Some of these emerging pathogens may later be submitted to a PRA and eventually be recommended for regulation as quarantine pests. When a quarantine status is considered appropriate for an emerging pathogen, EPPO Standards can also be developed in order to provide guidance on diagnostics, certification schemes, eradication and containment programmes.

Diagnostics

Since 1998, EPPO has established a work programme in the area of diagnostics to harmonize procedures across the region. The different activities conducted in the framework of this programme are presented.

Diagnostic protocols

In 1998, a programme was initiated to develop diagnostic protocols for as many as possible of the pests of the EPPO A1 and A2 lists (Zlof *et al.*, 2000). The preparation of protocols involves close collaboration between different Panels composed of diagnostic experts: the Panels on Diagnostics (coordination role), on Bacterial Diseases, on Nematodes, on Certification of Fruit Crops and the European Mycological Network. Each first draft is prepared by an individual expert according to a common format and should contain all the information necessary to detect and positively identify a particular pathogen or pest. The draft is then reviewed by relevant Panels. 92 diagnostic protocols for specific pests and 3 horizontal standards have now been

approved as (see www.eppo.org). 15 protocols are in different stages of preparation.

A survey on the use of the protocols was conducted in 2008 on a selection of 58 protocols in all disciplines of plant health diagnosis (Petter and Suffert, 2010). Laboratories registered in the EPPO database on Diagnostic Expertise (see below) were asked to indicate the number of samples that they tested in 2007 and which test they used. From this survey it can be concluded that many of the tests for detection mentioned in EPPO diagnostic protocols are widely used in laboratories in the EPPO Region.

Accreditation and quality management

In 2003, a separate Panel was created to develop standards on quality assurance (two standards have been developed so far OEPP/EPPO, 2007 and 2010). A joint informative document between EPPO and EA (European Co-operation for Accreditation, the European network of nationally recognised accreditation bodies) states that "EA will recommend that assessors from Accreditation Bodies take note of EPPO documents when evaluating plant pest diagnostic laboratories". It is also envisaged to create a database where validation data from laboratories could be shared between EPPO countries. EPPO also organized two workshops on quality assurance in 2007 and 2009, to allow experts to share their experience on quality assurance and accreditation.

EPPO database on diagnostic expertise

In 2004, EPPO Council stressed that the implementation of phytosanitary regulations for quarantine pests was jeopardized by decreasing knowledge in plant protection. The Panel on Diagnostics proposed that an inventory should be made of the available expertise on diagnostics in Europe. The database on Diagnostic Expertise was created (Roy *et al*., 2010) to allow identification of experts who can provide diagnosis of regulated species and those who can help in the identification of new or unusual species. EPPO member countries were contacted and as of May 2010, 70 laboratories from 25 countries have provided data corresponding to more than 500 experts. These results are available in a searchable database on the EPPO website. The database can also help national accreditation bodies identify auditors for pest diagnostic laboratories for accreditation.

The EPPO Secretariat considers that these initiatives and future plans will aid the optimization of diagnostic activities in laboratories in the EPPO region.

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Difficulties with conventional phytoplasma diagnostic using PCR/RFLP analyses

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Abstract

Polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) analysis is often used for phytoplasma identification and classification. Although these techniques are very sensitive and specific, in some cases, nonspecific reactions, false positives and negatives results, as well as unusual or illegible profiles after RFLP analyses, amplification of plant host´s DNA or other difficulties occurred. Experiences with suitability of positive and negative controls integration in PCR, evaluation of critical samples and other difficulties in phytoplasma PCR/RFLP identification are reported.

Key words: DNA extraction, PCR, primers, RFLP, positive and negative controls, critical samples.

Introduction

The '*Candidatus* Phytoplasma' taxon comprises prokaryotic wall-less pathogens of the class *Mollicutes* that inhabit plant phloem and insects. Polymerase chain reaction (PCR) with primers from sequencing of randomly cloned phytoplasma DNA, from 16S rRNA, from ribosomal protein gene sequences, from SecY and Tuf genes, and from membrane associated protein genes opened paths for phytoplasma finer identification and classification. Restriction fragments length polymorphism (RFLP) analysis together with the sequencing of 16Sr phytoplasma genes was the first step on this way enabling the construction of phylogenetic trees of many micro-organisms especially in the *Mollicutes* taxon (Bertaccini, 2007). However, sequence similarity of phytoplasma with hosts plants or other micro-organisms genes, their low concentration and uneven distribution as well as presence of phenolic substances and other inhibitors, especially when the extraction is performed from woody plants can make their detection difficult. Experiences with phytoplasma detection and identification using PCR/RFLP analyses to show several difficulties and their resolution is demonstrated and discussed.

Materials and methods

The nucleic acid extraction was performed from the following phytoplasma reference strains in *Catharanthus roseus* kindly provided by A. Bertaccini: peanut witches´ broom, PnWB (16SrII-A), peach X-disease, CX (16SrIII-A), German stone fruit yellows 1, GSFY/1 (16SrX-B), German stone fruit yellows 2, GSFY/2 (16SrX-B), Moliére disease, MOL (16SrXII-A). Phytoplasma strains: aster yellows, AY (16SrI-B, host: *Callistephus chinensis*), clover phyllody, CPh (16SrI-C, host: *C. roseus*), clover yellow edge, CYE (16SrIII-B, host: *Trifolium pratense*), apple proliferation, AP (16SrX-A, host: *Malus x domestica* ´Matčino´), pear decline, PD (16SrX-C, host: *Pyrus communis*) previously identified in our lab, and tissues from 18 healthy *C. roseus* plants were also used for DNA extraction. A phenol/chloroform method, a CTAB method and commercially available kits were tested. PCR assay was carried out with different primer pairs combination. To amplify region that includes the 16S rRNA gene, the spacer region, and the start of 23S rRNA gene of the phytoplasma genome, the primer pairs P1/P7 and P1A/P7A were used in direct PCR. PCR products were diluted with sterile distilled water (1: 29) prior to amplification by nested PCR using P1A/P7A, F1/B6, R16(I)F1/R, fU2/P7, fU5/rU3, 16R758F/16R1232R, F1/R0, Pc399 /Pc1694, R16F2n/R2 and F1/B6, R162n/R2 primer pairs, respectively. Double nested PCR was carried out by several ways with subsequent primer pairs combinations: P1/P7–P1A/P7A–R16F2n/R2, P1/P7–F1/B6–R16F2n/R2, P1/P7-R16F2/R2-R16(I)F1/R1, P1/P7–F1/B6–16R758F/16R1232R, and P1/P7–F1/B6–fU5 /rU3. About 20 ng of each DNA preparation in water were added to the PCR mix (Schaff *et al.*, 1992) in a final reaction mixture volume of 25 µl. The DNA was amplified by 35 cycles in a MJ Research thermocycler (Watertown, MA, USA). To reduce handling errors, in some cases PCR reactions were repeated up to 6 times. Approximately 200 ng of DNA of each positive PCR product from positive controls and DNA originating from 5 asymptomatic *C. roseus* plants, which revealed often positive signals in PCR, were separately digested from R16F2n/R2 amplicons. Digestions were carried out with 2.5 U of *Mse*I, *Alu*I, *Hha*I and *Rsa*I restriction enzymes. Restriction patterns obtained were compared with positive controls and with those described in the literature (Lee *et al*., 1998) after electrophoresis through an 8% polyacrylamide gel in 1x TBE buffer followed by staining with ethidium bromide and visualization under an UV transiluminator.

Results

DNA extracted by phenol/chloroform or CTAB techniques diluted in distilled water reacted positively also

after 5 years of storage in refrigerator. DNA extracted by used-friendly and quick commercial kits showed lower concentration and also low bands intensity under UV transilluminator after one month of storage in the same conditions as above or in conditions recommended by manufacturer. Some of these positives samples did not work in PCR or produced bands of nonspecific length after one year of storage.

Primers P1/P7–R16F2n/R2 and P1A/P7A-R16F2n/R2 amplified specifically DNA of all positive controls in nested PCR. Highly specific and sensitive was also the double nested PCR using P1/P7-F1/B6-R16F2n/R2 primer combination; no product was obtained by amplification of DNA from all 18 healthy *C. roseus* plants as well as from water. RFLP profiles with*Mse*I, *Alu*I, *Hha*I and *Rsa*I were in agreement with literature (Lee *et al*., 1998). However the DNA from 5 asymptomatic out of 18 *C. roseus* examined, give amplification with some other primers combination. False positives were obtained sporadically using primer pairs combination P1/P7-fU5/P7, P1/P7-fU5/rU3 and exceptionally P1/P7-P1A/P7A. DNA amplicons from 5 healthy *C. roseus* plants, which gave positive reactions up to 9 primer combinations, were choose for RFLP. RFLP with all four endonucleases employed showed R16F2n/R2 patterns different from those characteristic for phytoplasmas. After repeated digestion, a very weak profile corresponding to ribosomal subgroup 16SrI-B was observed in one sample, with *Mse*I. The sequencing of this amplicon (1,500 bp) confirmed no phytoplasmas (data not shown).

Discussion

Though PCR/RFLP analyses are routine techniques for phytoplasma detection and identification, their still meet some difficulties, at least with some primers: several primer pairs and their combination are recommended (Heinrich *et al.*, 2001). Moreover, in some papers, nonspecific PCR amplifications are mentioned. For example, Siddique *et al*. (2001) described after PCR amplification with P1/P6 primers besides the band of expected size, additional bands of different sizes. The same was observed with primer pairs Pc399/Pc1694, P1/U3 and M1/P7 in our analyses. According to Heinrich *et al.* (2001), some primers can induce dimers, bands of nonspecific sizes. In these cases, false positives can be expected. In our hands, nested PCR with primer combination P1/P7–16R758F/16R1232R amplified products not only from all positive controls and asymptomatic *C. roseus* plants, but also with water used as template or when only master mix and primers were used for PCR amplifications. Similar reactions were observed using P1/P7-Pc399/Pc1694 and P1/P7-fU5/rU3 primer pairs in nested PCR. In the contrary, the same DNA samples amplified for example with P1/P7-P1A/P7A, P1A/P7A-R16F2n/R2 or P1/P7-P1A/P7A-R16F2n/R2, P1/P7-F1/B6-R16F2n/R2 never reacted with DNA from healthy *C. roseus* plants or with water controls.

According to our knowledge, it seems that in the case of phytoplasma positive samples, the primers preferentially amplified phytoplasma sequence of expected sizes, exceptionally, also additional bands could be observed. In the case of DNA isolated from healthy plants, some primers can react probably with sequences of plant genome or dimers and false positives could be observed. That is one of the reasons for including DNA originating from corresponding healthy plants and also water controls in PCR assays. In some cases, no visible products were obtained not only from healthy controls, but also from phytoplasma positive samples. This could be caused by inhibitor presence. In this case, higher dilution of DNA is advised (Heinrich *et al.*, 2001). The PCR alone is not sufficient enough for phytoplasma detection. Subsequent confirmation of phytoplasma presence and its identification must be accomplished at least by RFLP analyses using at least two or more endonucleases. In the case of critical samples, different primer pair combination, RFLP with more enzymes and also sequencing should be used for elucidation of phytoplasma presence.

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Sharing information and collections on phytoplasmas: from QBOL to QBANK

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Abstract

A total of 154 phytoplasma strains from 15 ribosomal groups were employed for barcode sequences production. Besides strains in periwinkle, 36 strains in natural infected plants such as napier grass, grapevine, plum, jujube, apple, pear, spartium, pine tree, hibiscus and erigeron were employed. Barcode sequences were produced for 16SrDNA, tuf and SecA gene for 36 phytoplasma strains, while for 54 strains 16SrDNA sequences were obtained, and for 118 and 89 strains respectively the tuf and secA barcode. All obtained sequences and protocol for extraction and PCR amplification will be available in Qbank.

Key words: phytoplasmas, collection, barcode, detection, quarantine.

Introduction

The increased international trade of plant material is increasing also exchanges of possible quarantine pathogens and a quick and reliable system for their identification is of the utmost importance.

DNA barcoding is a generic diagnostic method that uses short standardised genetic markers to aid species identification. The first genetic marker to be described as a "barcode" was the mitochondrial cytochrome *c* oxidase I (COI) gene which is used for species identification in the animal kingdom (Herbert *et al.* 2003). Among quality standards requirements for "barcodes" sequences are that the sequence data must be obtained from fully identified and vouchered specimens from a known origin with a unique identifier; sequence data must be at least 500 bp long and must be associated with trace files for the forward and reverse sequencing runs, and of forward and reverse primers used; primer sequences must be trimmed from the barcode sequence data (Hanner, 2009).

DNA barcoding protocols are under development and validation within the Quarantine organisms Barcoding of Life (QBOL) project financed by 7th framework program of the European Union. Using the developed DNA barcoding protocols, sequence data of EU regulated plant pests and phylogenetically related species was generated and will be made publicly available using the Q-Bank database.

The list of phytoplasma diseases that are of EU quarantine are summarized in table 1. It is important to underline that the quarantine list a number of diseases but it is well known that different phytoplasmas are associated to the same disease in diverse parts of the world. Therefore, phytoplasmas listed in table 1 were selected as main representatives of those associated with the same disease worldwide. Based on this knowledge and in order to provide the most useful and robust tool possible a number of phytoplasma strains belonging to the majority of described taxons were barcoded and will be deposited in the QBank.

Materials and methods

Fresh or frozen phytoplasma infected plant material from periwinkle or from natural plant hosts was used after a chloroform/phenol extraction of total nucleic acid (Prince *et al*., 1993). This DNA was then employed for sequencing the marker regions selected for reliable identification of quarantine phytoplasmas; for all markers a 400 - 600 bp fragment is suggested for the use of the barcode system.

A total of 154 phytoplasma strains from 15 ribosomal groups were employed for barcode sequencing. Besides strains in periwinkle also 36 strains in natural infected plants such as napier grass, grapevine, plum, jujube, apple, pear, spartium, pine tree, hibiscus and erigeron were employed. Strains belongs to the official collection of micropropagated phytoplasmas (Bertaccini, 2010), or were collected or provided by colleagues listed below. Specific protocol and primers are in validation phase in order to provide a quick and reliable tool for identification of quarantine phytoplasmas. The selected barcode regions are within the 16Sr DNA, tuf and SecA genes. The produced barcode sequences will be uploaded in the publicly available database that is in preparation (Qbank) where protocols for nucleic acid extraction and primers for amplification will also be available after the end of the validation process.

Table 1. List of EU quarantine phytoplasma-associated diseases.

Name of the disease	'*Candidatus* Phytoplasma'	16Sr DNA grouping of phytoplasmas Qbol target*	Other 16Sr DNA phytoplasmas associated with the disease*
Elm phloem necrosis	'*Ca.* P. ulmi'	16SrV-A	None
Peach rosette		16SrIII	16SrI
Peach X disease		16SrIII-A	None
Peach yellows		16SrIII	16SrI-B/16SrXII
Strawberry witches' broom		16SrI-C	'*Ca*. P. fragariae', 16SrI-B, 16SrXII
Apple proliferation	'*Ca.* P. mali'	16SrX-A	None
Apricot chlorotic leafroll	'*Ca.* P. prunorum'	16SrX-B	None
Pear decline	'*Ca.* P. pyri'	16SrX-C	None
Palm lethal yellowing		16SrIV	16SrI/16SrXXII
Witches' broom on *Citrus*	'*Ca*. P. aurantifolia'	16SrII-B	16SrVI, 16SrIX
Grapevine flavescence doreé		16SrV-C/-D	None
Potato stolbur		16SrXII-A	16SrI-A, 16SrI-C, 16SrII
potato purple top wilt	'*Ca*. P. americanum'	16SrXVIII-A	16SrVI

* Groups and subgroups are according with Lee *et al*., 1998 and as in Bertaccini and Duduk, 2009.

Results

For a total of 26 phytoplasma strains all the three barcode sequences were produced, while for 54 strains 16SrDNA sequences were obtained, and for 118 and 89 strains, respectively, tuf and secA barcode sequences were obtained.

For the naturally infected plants, 4 strains were sequenced in the 16S rDNA, while 74 sequences were obtained for tuf gene and 54 sequences for the secA gene. The project is still in progress and more sequences are under production.

Discussion

The three barcode sequences employed in the project will allow to unambiguously detect and identify quarantine phytoplasmas in a short time and with no need of specific phytoplasma expertise in the laboratory. From the research carried out, the tuf gene fragment was shown to be able to differentiate the majority of phytoplasmas enclosing those in the EU quarantine list (table 1) and together with the 16S rDNA appears to be very helpful in barcoding of phytoplasmas. Further work is in progress toward increasing number of sequences to be deposited in the Qbank.

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