Phytopathogenic Mollicutes
Vol. 1(2), December 2011, 00-00

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Research Article

The most widespread phytoplasmas, vectors and measures for disease control in Slovenia

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Received : Accepted :

Abstract

Phytoplasmas, as fastidious wall-less mollicutes, colonize phloem tissue of many plant species, including grapevine and fruit trees. They are transmitted between plants by vegetative propagation and by sap-sucking insect vectors, which enable spread of the diseases. In Slovenia in the period 2001-2010, 3,189 plant and 109 insect samples were collected in targeted surveys and analyzed for the presence of phytoplasmas by molecular methods. Phytoplasmas were found in 1,708 plant samples; in 19 cases mixed phytoplasma infection was also identified. The majority of the samples were collected within systematic surveys conducted to determine the prevalence of phytoplasmas in Slovenia. '*Candidatus* Phytoplasma mali', associated with apple proliferation (AP), '*Ca.* P. prunorum', associated with European stone fruit yellows (ESFY) and '*Ca.* P. pyri', associated with pear decline (PD), were detected in several fruit-growing areas of Slovenia. The most widespread phytoplasma on grapevine in Slovenia was the stolbur phytoplasma, ('bois noir': BN). The same phytoplasma was also identified in *Convolvulus arvensis* and *Lycopersicon esculentum*. Since 2005, the 'flavescence dorée' phytoplasma (FD), associated with serious disease of grapevine, has been reported in Slovenian vineyards. The same phytoplasma was also detected in *Clematis vitalba*. During the surveys, several known and putative vectors of phytoplasmas were identified in Slovenia.

Keywords : Phytoplasma, Slovenia, vectors, hosts, detection, prevention measures

Introduction

Phytoplasmas are cell wall-less Gram-positive bacteria of the class *Mollicutes*, and both their cell and genome size are the smallest among bacteria. They are obligate intracellular parasites, generally restricted to sieve elements of the infected plants, and transmitted by phloem-feeding leafhoppers (Cicadellidae), planthoppers (Fulgoromorpha, Cixiidae), and psyllids (Psyllidae) of the order Hemiptera. In addition, they are also transmitted by the majority of the dodder species, by micropropagation, grafting and cutting techinques, and the possibility of phytoplasma transmission by seed may not be excluded (Bertaccini, 2007; Calari *et al.*, 2011). Determination of the taxonomic status of phytoplasmas by the traditional methods is not possible, however by molecular methods phytoplasmas were demonstrated to represent a clearly distinct monophyletic cluster within the class *Mollicutes*. For classification of phytoplasmas the '*Candidatus* Phytoplasma' genus has been proposed based on phylogeny produced on 16S ribosomal DNA. One sequence from each group of '*Ca.* Phytoplasma' was selected as representative, with the reference strain deposited in GenBank (IRPCM, 2004). Phytoplasmas are associated with more than a thousand diseases of wild and cultivated plants (Seemüller *et al.*, 2002). An

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overview of phytoplasmas identified in fruit trees and grapevines as well as some insect vectors in Slovenia is provided in this paper.

Phytoplasmas associated with important diseases of grapevine and fruit trees

In Europe, fruit trees of the family *Rosaceae* are seriously affected by phytoplasmas of the apple proliferation group (AP, 16SrX group) (Lee *et al*., 1995). The AP group includes two phytoplasmas from the lists of pests recommended for regulation (EPPO, 2010), '*Candidatus* Phytoplasma mali' and '*Ca*. P. pyri', as well as the widespread '*Ca.* P. prunorum'. These are the agents associated with apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY), respectively (Seemüller and Schneider, 2004; Marcone *et al*., 2010). Although '*Ca.* P. mali' infection prevails in the genus *Malus*, it has also been occasionally identified in plants other than the typical host, for example stone fruits and both European pear (*Pyrus communis*) and Asian pear (*Pyrus pyrifolia*) (Lee *et al*., 1995; Del Serrone *et al*., 1998; Seemüller and Schneider, 2004). '*Ca.* P. pyri' is associated with the genus *Pyrus* (Seemüller and Schneider, 2004). '*Ca.* P. prunorum' causes economically important disorders in apricot (*Prunus armeniaca*), Japanese plum (*Prunus salicina*) and peach (*Prunus persica*) (Carraro and Osler, 2003). European plums (*Prunus domestica*) as well as some other wild *Prunus* species (*P. spinosa*, *P. cerasifera*, *P. insititia*) are susceptible to the infection, but generally do not show symptoms representing a dangerous source of infection (Carraro and Osler, 2003; Carraro *et al*., 1998a, 2004). On the other hand, *Prunus avium* has demonstrated a high level of resistance to '*Ca.* P. prunorum' (Jarausch *et al*., 1999). Phytoplasmas from the AP group have also been detected in hazel (*Corylus avellana*), ash (*Fraxinus excelsior*), dog rose (*Rosa canina*), hackberry (*Celtis australis*), hawthorn (*Crataegus monogyna*), oak (*Quercus robur* and *Quercus rubra*), hornbeam (*Carpinus betulus*) and bindweed (*Convolvulus arvensis*) (Seemüller and Schneider, 2004). Psyllids seem to play a crucial role in the transmission of phytoplasmas from the AP group (Tedeschi and Alma, 2004). '*Ca.* P. prunorum' is transmitted to the host plants of *Prunus* spp. by the vector *Cacopsylla pruni* (Carraro *et al*., 1998b). Additionally, the leafhopper *Asymmetrasca decedens* (synonym *Empoasca decedens*) has been suggested as a potential vector of this phytoplasma (Pastore *et al*., 2004). '*Ca.* P. pyri' is transmitted to the host plants by *Cacopsylla pyricola* (Davies *et al*., 1992), and *Cacopsylla pyri* (Carraro *et al*., 1998c). Known psyllid vectors of '*Ca.* P. mali' are *Cacopsylla picta* (synonym *C. costalis*) (Frisinghelli *et al*., 2000; Jarausch *et al*., 2003) and *C. melanoneura* (Tedeschi and Alma, 2004). Besides psyllids, some other insects have been reported as vectors of '*Ca.* P. mali', including the spittlebug *Philaenus spumarius*, the leafhopper *Artianus interstitialis* (Hegab and El-Zohairy, 1986) and *Fieberiella florii* (Krczal *et al*., 1988; Tedeschi *et al*., 2004).

On grapevine (*Vitis vinifera*) phytoplasmas are associated with severe and worldwide present symptomatology named grapevine yellows (GY). Although GYs are associated with different phytoplasmas, infected plants show the same symptoms of leaf rolling and curling, brittleness of leaves along with yellowing or reddening, lack of cane lignifications and desiccation of grape clusters. Leaf necrosis and bark splitting may also develop (Lee *et al*., 2000; Prince *et al*., 1993). The symptoms usually appear in late spring or in the summer, and some vines may die in the following years. In Europe the most important GYs are 'bois noir' (BN) and 'flavescence dorée' (FD), which are indistinguishable by symptoms. While the phytoplasma BN belonging to stolbur or 16SrXII subgroup is widespread, that of FD belonging to elm yellows subgroups 16SrVC/D has quarantine status in the European Union. It is also recommended for regulation as a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO).

The confirmed vector of BN is *Hyalesthes obsoletus* Signoret (Maixner, 1994; Sforza *et al*., 1998). However, it is supposed that this vector cannot efficiently transmit BN from grapevine to grapevine and cannot survive on the grapevine, which is a dead-end host for BN phytoplasma. The natural reservoirs for this phytoplasma are weeds, such as bindweed and nettle. Few other species have been identified as potential vectors, including *Pentastiridius beieri* and *Reptalus quinquecostatus* (Gatineau *et al*., 2001; Holzinger *et al*., 2002; Trivellone *et al*., 2005). The main known natural vector of FD is an ampelophagous leafhopper *Scaphoideus titanus* Ball (Schvester *et al*., 1961, 1963). *Dictyophara europea* has recently been confirmed to be able to transmit FD from clematis to grapevine under greenhouse conditions (Filippin *et al*., 2009). *Orientus ishidae* has been shown to be infected with different strains of FD (Mehle *et al*., 2010a). Other

phytoplasmas associated with GYs such as phytoplasmas belonging to subgroups 16SrI-A, 16SrI-B, 16SrIII-I, 16SrVII-A, 16SrX-B; and 16SrXII-B, were sporadically reported in grapevine or are present in particular geographical regions (Alma *et al.*, 1996; Davis *et al.*, 1997, 1998; Angelini *et al.*, 2007; Gajardo *et al.*, 2009).

Methodological approaches for phytoplasma detection and diagnosis

Reliable detection of phytoplasmas is notoriously difficult and relies mainly on molecular techniques. The reasons are various and include their inability to grow *in vitro*, besides the low annually and seasonally fluctuating titres of the pathogens and their uneven distribution in infected plants. The detection techniques include conventional PCR, usually followed by RFLP for specific determination of phytoplasma 16S rRNA group or species (Deng and Hiruki, 1991; Lorenz *et al.*, 1995, Schneider *et al.*, 1995; Daire *et al.*, 1997; Angelini *et al.*, 2001; Clair *et al.*, 2003; Filippin *et al.*, 2009). In addition, several protocols based on real-time PCR have been proposed recently for the universal or group-specific determination of phytoplasmas in either single or multiplex assays (Baric and Dalla Via, 2004; Bianco *et al.*, 2004; Christensen *et al.*, 2004; Jarausch *et al.*, 2004; Galetto *et al.*, 2005; Torres *et al.*, 2005; Angelini *et al.*, 2007; Babini *et al.*, 2008; Pignatta *et al.*, 2008; Hodgetts *et al.*, 2009; Margaria *et al.*, 2009; Pelletier *et al.*, 2009; Yvon *et al.*, 2009). The advantages of the real-time PCR assays lie in their higher sensitivity combined with three-fold shorter processing time in comparison with conventional PCR (Fig. 1).

A real-time PCR assay using TaqMan minor groove binder probes was recently designed for the general presence of phytoplasmas as well as for the species or group-specific detection of FD and BN (Hren *et al.*, 2007), aster yellows group (Nikolić *et al.*, 2009) and AP group (Nikolić *et al.*, 2010).

Both the conventional PCR and the real-time PCR approach have been applied for reliable determination of phytoplasmas in Slovenian plants and their insect vectors.

In Slovenia, 3,298 samples were analyzed for the presence of phytoplasmas between 2001 and 2010 (Table 2, 3). These were collected within systematic surveys of the Phytosanitary Administration of the Republic of Slovenia, carried out to determine the presence of phytoplasma diseases in Slovenia. The majority of samples derived from fruit trees of the *Rosaceae* family and grapevine (*Vitis vinifera* L.). Other samples were either from other plant species or from insect vectors. For the analysis, DNA was extracted

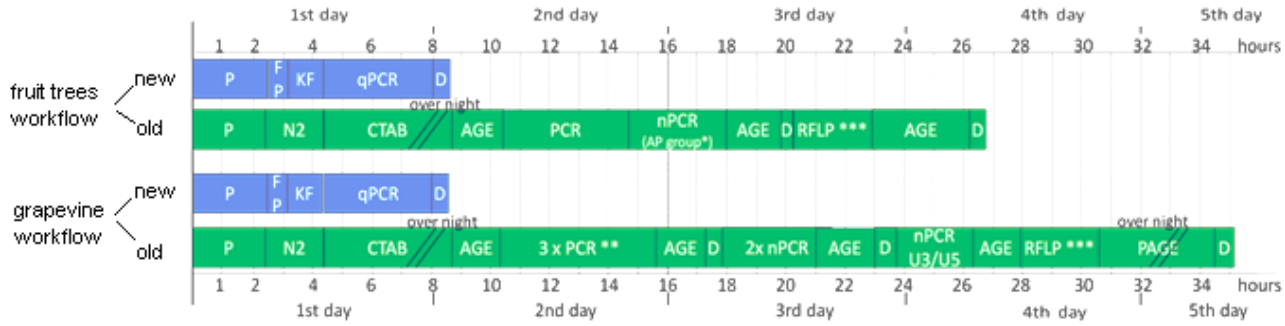

Figure 1. Comparison of new and old workflows for phytoplasma detection. Workflow time scales are approximately determined for 10 samples.
*The time scale of fruit tree work flow is for positive samples, and in the case of negative results of nested PCR for AP group phytoplasma another nested PCR with universal U3/U5 primers for phytoplasma detection is required, which is not included in this time scale.
**Time for the old workflow depends on the number of PCR machines available and on the infection status of samples (only negative samples or also positive ones). This time scale is for 3 simultaneous PCR reactions (using universal phytoplasma, BN and FD-specific primers) and in the case of positive and negative samples.
***RFLP only for positive samples.
Abbreviations: P-sample preparation; FP-fast prep homogenization; KF-Kingfisher DNA extraction; qPCR-real time polymerase chain reaction; D-data analysis; N2-homogenization in liquid nitrogen; CTAB-CTAB extraction; AGE-agarose gel electrophoresis; PCR-polymerase chain reaction; nPCR-nested PCR; RFLP-restriction fragment length polymorphism.

from insects, roots in the case of asymptomatic samples, or shoots of symptomatic samples using a CTAB extraction procedure, modified from a protocol by Ahrens and Seemüller (1992). Since 2009, a new, faster DNA extraction procedure based upon the binding of DNA to magnetic beads (Pirc *et al*., 2009; Boben *et al*., 2007) has been implemented in the detection procedure. In 2010, the time required for detection was additionally reduced by implementation of an automated simple homogenization step instead of manual homogenization (Fig. 1).

Phytoplasmas of the AP group associated with diseases of fruit trees in Slovenia

In the period 2001-2010, 1,405 samples from fruit trees in production and mother plant orchards from different regions of Slovenia were tested for the presence of '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum'. All three phytoplasmas were detected in several areas in Slovenia where fruit trees are cultivated (Table 1, 2). Specifically, '*Ca*. P. mali' is present at low prevalence, and '*Ca*. P. pyri' is found only in some areas where pear is grown (Primorska, Savinjska valley, Posavje, Štajerska). Similarly, '*Ca*. P. prunorum' is present in the Primorska and Notranjska regions where host crops are grown, but may be also found at low prevalence in the Savinjska, Štajerska and Prekmurje regions. All three '*Ca*. Phytoplasma' species are under official control. Testing for '*Ca*. P. mali' in mother plants every six years is obligatory in certified and standard material (Regulation, 2006), and for '*Ca*. P. prunorum' national control measures against phytoplasma and its vector *Cacopsylla pruni* are prescribed (Regulation, 2004).

Disease symptom expression is highly variable, and sometimes symptoms become visible only as a result of special weather conditions or of significant changes in cultivation practices. It is noteworthy that many cultivars especially in the first years of tree development do not show typical symptoms. Moreover, several symptomless trees were proven to be latently infected (Lešnik *et al*., 2007; Ambroziè Turk *et al*., 2008). Such trees may be a hidden source of infection, and its early detection and consequent tree removal is as important as intensive vector control (Ambroziè Turk *et al*., 2008; Mehle *et al*., 2010b).

'*Candidatus* Phytoplasma mali'

Infection of apple trees (*Malus domestica*) with '*Ca*. P. mali' was found in 76 samples (Table 2). In general, infected symptomatic plants have altered size of leaf stipules and witches' broom formations. Nevertheless, some of the infected apple trees did not show typical symptoms, and some were even symptomless. Positive results were obtained for 39% of symptomatic and 3% of symptomless trees.

The main transmission path of '*Ca*. P. mali' is by sap-sucking insect vectors, but transmission may also occur through grafting of infected propagation material (Kartte and Seemüller, 1988). Although there are little firm data available about the transmission of '*Ca*. P. mali' via natural root grafts (Baric *et al*., 2008), such transmission has been confirmed in Slovenia M9 apple rootstock (Lešnik *et al*., 2008).

Besides being present in its natural host apple trees, '*Ca*. P. mali' was also detected in four cherry (*Prunus avium*), two apricot (*P. armeniaca*) and one plum (*P. domestica*) sample (Table 1) (Mehle *et al*., 2007).

'*Candidatus* Phytoplasma pyri'

'*Ca*. P. pyri' was identified in 81 samples of pear tree (*Pyrus communis*) (Table 2). Seventy-five positive samples derived from symptomatic trees and 6 samples from trees without any symptoms. Thirty-eight per cent of symptomatic trees were positive, while 26% of symptomless trees tested positive. The typical symptoms were reddening and curling of leaves, and sometimes lines of necrotic tissue in the bark.

'*Candidatus* Phytoplasma prunorum'

Two hundred four samples of stone fruit trees (*Prunus* sp.) were infected with '*Ca*. P. prunorum' (Table 1). Forty-seven percent of symptomatic trees were positive, and 27% of symptomless trees. Typical symptoms were reddening and curling of leaves, and sometimes lines of necrotic tissue in the bark. Among 53 apricot (*Prunus armeniaca)* trees, phytoplasma was detected in 53% of the samples (64% of 36 symptomatic and 29% of 17 asymptomatic trees). In the case of peach (*Prunus persica*) and nectarine trees '*Ca*. P. prunorum' was detected in 41% of 314 sampled trees. Among 59 symptomless samples of peaches, 3% were positive. On the other hand, symptoms correlated well with the infection; thus '*Ca*. P. prunorum' was confirmed in 50% of samples from symptomatic peach plants. Although the total number of tested samples from Japanese plum trees (*Prunus salicina*) was low, all tested samples were proven to contain '*Ca*. P.

Table 1. Phytoplasma detected in Slovenia

Phytoplasma	Plant host	Incidence	Symptoms	Region/s
FD	*Vitis vinifera*	In 2005 individual infected plants; in 2010 first outbreaks in two vineyards, under official control	Leaves turn yellow or red depending on the cultivar. They roll downward and become brittle. Shoots show incomplete lignification and rows of black pustules develop on the green bark along the diseased branches. Grape yield greatly decreases	SE, SW, NE Slovenia
	Clematis vitalba	Widespread; also present in areas where FD has never previously been recorded in grapevine	Reddening, yellowing and rolling of the leaves at the end of the summer. Sometimes does not show symptoms.	Slovenia
BN (stolbur)	*V. vinifera*	Widespread; planting material under official control	Impossible to distinguish from the symptoms of FD-infected grapevine	all wine-growing regions in Slovenia
	Lycopersicon esculentum	individual infected plants	Stunting, dying of plants	W Slovenia
	Convolvulus arvensis	widespread	Yellowing	Slovenia
'*Ca*. P. prunorum'	*Prunus persica*	present only in areas where host crops are grown; planting material under official control	Premature leaf reddening or yellowing, leaf curling, line of necrotic tissue in the bark, blossoming out of the usual time, fruit malformations, tree dieback	W Slovenia (Primorska, Notranjska)
	Prunus armeniaca		Premature leaf reddening or yellowing, leaf curling, line of necrotic tissue in the bark, premature budding in late winter	NE Slovenia: Present at low prevalence (Savinjska valley, Štajerska, Prekmurje)
	Prunus domestica		Generally does not show symptoms	
	Prunus salicina		Premature leaf reddening or yellowing, leaf curling, tree dieback	
'*Ca*. P. pyri'	*Pyrus communis*	present only in some areas where host crops are grown; planting material under official control	Premature leaf reddening, leaf curling, premature leaf drop, line of necrotic tissue in the bark, tree dieback	W Slovenia (Primorska), E Slovenia (Savinjska valley, Posavje, Štajerska)
'*Ca*. P. mali'	*Malus domestica*	Present; planting material under official control	Witches' broom at the end of shoots, enlarged stipules, early leaf reddening, small fruits	Slovenia
	Prunus avium[a]	individual infected plants	Wilting, dying, floral and phloem necrosis	SW Slovenia
	P. armeniaca[a]	individual infected plants	Stem necrosis and leaf wilting	SW Slovenia
	P. domestica[a]	individual infected plants	Late blooming	SW Slovenia
'*Ca*. P. asteris'	*Echinacea purpurea*[b]	Present in several gardens	Plant weakness, leaf yellowing, floral malformations, such as virescence and phyllody	E Slovenia (Savinjska valley)

[a]Mehle *et al*., 2007; [b]Radišek *et al*., 2009

prunorum'. The very high infection rate of this plant species is in accordance with the situation in Japanese plum orchards elsewhere (Marcone *et al*., 2010). In European plum tree (*Prunus domestica*) samples the incidence of phytoplasma was 37%. It is worth noting that in most infected plants the disease symptoms were not expressed, which is in accordance with the reports in the literature (Carraro *et al*., 1998a). Sweet cherry trees (*Prunus avium*) and sour cherry trees (*Prunus cerasus*) were also checked for the presence of '*Ca*. P. prunorum' (Table 1). Although natural infections with this phytoplasma have been observed in this species (Marcone *et al*., 2010), the presence of '*Ca*. P. prunorum' in cherry has not been confirmed in Slovenia.

Insect vectors of phytoplasmas from the AP group in Slovenia

The known and putative insect vectors of phytoplasmas from the AP group have not been systemically sampled and analyzed in Slovenia. However, considering that some insect species that are known or putative vectors of these phytoplasmas are widespread in Slovenia (Seljak, 2006), their possible involvement in phytoplasma transmission should not be neglected (Table 3).

The number of phytoplasma-infected insect samples was loosely correlated with the number of plant samples confirmed to be infected with specific phytoplasmas. For instance, six out of eight tested specimens of *Cacopsylla pruni*, vector of '*Ca*. P. Prunorum' (Carraro *et al*., 1998b), were infected with this phytoplasma (Table 3). Testing of two samples of a putative vector of '*Ca*. P. prunorum', *Asymmetrasca decedens* (Pastore *et al*., 2004), did not yield positive result (Table 2).

Both vectors of '*Ca*. P. pyri', *Cacopsylla pyricola* (Davies *et al*., 1992) and *Cacopsylla pyri* (Carraro *et al*., 1998c), are widespread in Slovenia (Seljak, 2006). Only one sample of *C. pyri* was examined and the presence of '*Ca*. P. pyri' in that sample was confirmed (Table 2).

There are several reported vectors that transmit '*Ca*. P. mali'. Among them are species that are widespread in Slovenia (Seljak, 2006, Holzinger and Seljak, 2001, Seljak, 2004), for example *Cacopsylla picta* (Frisinghelli *et al*., 2000; Jarausch *et al*., 2003), *C. melanoneura* (Tedeschi and Alma, 2004), *Philaenus spumarius* (Hegab and El-Zohairy, 1986), *Artianus interstitialis* (Hegab and El-Zohairy, 1986) and possibly *Fieberiella florii* (Krczal *et al*., 1988; Tedeschi and Alma, 2004). However these insects were not yet included in the Slovenian surveys. In areas with high infection pressure due to common occurrence of host plants and phytoplasma insect vectors, the maintenance of healthy mother plants in the open field is quite difficult, so the use of insect-proof net-houses was suggested and confirmed to be appropriate for stone fruit mother plant cultivation (Ambroziè Turk *et al*., 2010).

Phytoplasmas associated with grapevine yellows diseases in Slovenia

In 1,679 tested symptomatic grapevine (*Vitis vinifera*) samples 7% percent of samples were infected with FD and 70 % with BN (Table 2).

Bois Noir

At the beginning of the survey in 2001, all symptomatic grapevine plants tested were positive for BN phytoplasma that is widespread in all winegrowing regions of Slovenia (Table 1). During the surveys BN was also detected in 13 out of 30 samples of bindweed (*Convolvulus arvensis*) tested (Table 2). Phytoplasma closely related to BN and belonging to the stolbur group were found in one out of six tested tomato plants (*Lycopersicon esculentum*) expressing stunting and dying symptomatology (Table 1, 2).

The main vectors of BN occur in Slovenia (Table 3). A test for BN presence in *Hyalesthes obsoletus* (Maixner *et al*., 1995) revealed 18 positive samples out of 23. BN was also found in other insects, including *Euscelis incisus*, *Reptalus panzeri*, *Reptalus cuspidatus* and *Scaphoideus titanus*. Among these, only *R. panzeri* has been confirmed as a vector of stolbur phytoplasmas in corn (Jović *et al*., 2007).

Flavescence dorée

The first finding of FD was in the coastal area in the south-western part of Slovenia in 2005, in the following years FD was also detected in other winegrowing regions. It was usually found in individual plants in vineyards. However, in 2010 an epidemic spread was observed in the south-eastern and south-western areas of Slovenia, where a heavy infection rate of symptomatic grapevine plants was observed. Since the disease is under official control in Slovenia all FD infected plants and plants showing symptoms must be removed from vineyards.

Table 2. Number of sampled and infected host plants for symptomatic and asymptomatic samples, obtained in targeted inspections and sampling in the period 2001 – 2010 in Slovenia.

Phytoplasma	Plant host	symptoms	number of samples	positive samples	
				no.	%
FD[a]	*Vitis vinifera*	yes	1,679	123[b]	7%
	Clematis vitalba	yes	52	40	77%
		no	17	9	53%
BN (stolbur)	*V. vinifera*	yes	1,679[c]	1,173[b]	70%
	Lycopersicon esculentum	yes	6	1	17%
	Convolvulus arvensis	yes	30	13	43%
'*Ca*. P. prunorum'	*Prunus persica*	yes	255	127	50%
		no	59	2	3%
	Prunus armeniaca	yes	36	23	64%
		no	17	5	29%
	Prunus domestica	yes[d]	38	9	24%
		no	77	33	43%
	Prunus salicina	yes	3	3	100%
		no	2	2	100%
	Prunus cerasus	no	2	0	0%
	Prunus avium	yes	15	0	0%
		no	1	0	0%
'*Ca*. P. pyri'	*Pyrus communis*	yes	197	75	38%
		no	23	6	26%
'*Ca*. P. mali'	*Malus domestica*	yes	163	63	39%
		no	473[e]	13	3%
	P. avium[f]	yes	44	4	9%
	P. armeniaca[f]	yes	29[g]	2	7%
	P. domestica[f]	yes	34[g]	1	3%

[a]the first finding was in 2005; [b]19 samples were infected with both BN and FD; [c]samples were also tested for FD; [d]almost symptomless; [e]several symptomless apple trees were tested because testing of mother plants for '*Ca*. P. mali' is obligatory every six years in Slovenia; [f]Mehle *et al.*, 2007; [g]samples were also tested for '*Ca*. P. prunorum'

Obligatory vector treatment and a ban on use of material for propagation are imposed (Regulation, 2009). Once FD is confirmed, further removal of all symptomatic plants from infected vineyards can be done without sampling/testing. If more than 20 % of plants show symptoms, the entire vineyard is uprooted.

Molecular characterization of FD genetic clusters in grapevines by RFLP and sequencing showed that in Slovenia about two thirds of tested infected grapevines harboured the FD2 strain (Mehle *et al.*, 2011), which correlates well with data from France and Italy (Arnaud *et al.*, 2007; Filippin *et al.*, 2009). However, about one third of isolates from grapevine belonged to the genetic cluster FD3 (Mehle *et al.*, 2011). In Slovenia no grapevine infected with FD1 was detected (Mehle *et al.*, 2011).

FDp was also detected in 49 out of 69 samples of *Clematis vitalba* (Table 1, 2), including in areas away from vineyards and areas where FD has not been found in grapevine. The infected clematis plants either showed the symptoms of reddening or yellowing of the leaves or were symptomless. Notably, all isolates

Table 3. Recognized and putative phytoplasma vectors identified in Slovenia. They were captured on yellow sticky traps or by sweep net sampling and then stored in 96% ethanol until DNA extraction.

Insects	Population density	Phyto-plasma	Vector	positive/ total[a]	Feeding plants
Dictyophara europaea	moderate; widely distributed in winegrowing regions	FD	putative	0/0	*Amaranthus retroflexus, Urtica dioica*
Orientus ishidae	moderate; limited distribution	FD	putative	5/7	various woody and herbaceous plants, *Malus, Salix, Alnus, Diospyros kaki, Prunus spinosa*
Scaphoideus titanus	low to high; established in all winegrowing regions, but not in all vineyards	FD	yes	1/57[b]	*Vitis vinifera*
		BN	no	3/57	*V. vinifera*
Hyalesthes obsoletus	moderate; widely distributed	stolbur	yes	18/23	*Urtica dioica, Convolvolus arvensis,* occasionally *V. vinifera*
Euscelis incisus	high; very common	stolbur	putative	1/2	herbaceous plants
Reptalus panzeri	low; widely distributed	stolbur	yes	2/7	herbaceous plants, occasionally *V. vinifera*
Reptalus cuspidatus	locally common; widespread in SW Slovenia	stolbur	putative	1/1	herbaceous plants
Reptalus quinquecostatus	low; very local distribution	stolbur	putative	0/1	herbaceous plants
Cacopsylla pruni	high; widely distributed	'*Ca*. P. prunorum'	yes	6/8	*Prunus* spp, especially *P. domestica, P. institita, P. spinosa*
Empoasca decedens	moderate to high; locally common	'*Ca*. P. prunorum'	putative	0/2	*Prunus persica, P. domestica, P. armeniaca, Pyrus* spp., *Malus* spp., *Salix* spp. etc.
Cacopsylla pyri	high; widely distributed	'*Ca*. P. pyri'	yes	1/1	*Pyrus communis*
Cacopsylla pyricola	low to moderate; widely distributed	'*Ca*. P. pyri'	yes	0/0	*Pyrus communis, P. pyraster, P. nivalis*
Cacopsylla picta	low to moderate; widely distributed	'*Ca*. P. mali'	yes	0/0	*Malus* spp.
Cacopsylla melanoneura	high; widely distributed	'*Ca*. P. mali'	yes	0/0	*Rosaceae,* mainly *Crataegus* spp., *Malus* spp.
Philaenus spumarius	high; very common	'*Ca*. P. mali'	putative	0/0	herbaceous plants
Artianus interstitialis	absent; in Slovenia replaced by *A. manderstjernii*	'*Ca*. P. mali'	putative	0/0	–
Fieberiella florii	moderate, widely distributed	'*Ca*. P. mali'	yes	0/0	bushy and tree vegetation

[a]Number of positive samples/number of all samples tested from 2001 to 2010; [b]samples were tested also for BN phytoplasma

from clematis plants belonged to the FD3 genetic cluster (Mehle *et al*., 2011) in accordance with reports of other FD infected clematis plants (Angelini *et al*., 2004; Filippin *et al*., 2009).

Insect vectors of FD

It is interesting that until 2010 the presence of FD was confirmed only in 1 out of 57 tested samples of *S. titanus*, the main natural vector of FD (Table 3). *S. titanus* has been identified in western Slovenia since 1983 and in eastern Slovenia only since 2003. Considering that it is already widespread it is very likely that this low rate of detected infections is due to low levels of FD in vineyards. In 2009, adults of another leafhopper, *Orientus ishidae*, were captured on bushy vegetation in areas distant from vineyards. They were confirmed to be infected with phytoplasmas similar to FD from grapevine (Mehle *et al*., 2010a). In *O. ishidae* FD1, FD2 and FD3 were detected (Mehle *et al*., 2010a; 2011). Further research is needed to shed light on the role of *O. ishidae* in the possible transmission of FD.

Additionally, alder (*Alnus glutinosa* and *A. incana*) may harbour phytoplasma isolates that are very similar to FD1, FD2 and FD3 strains and are transmitted by *Oncopsis alni* (Filippin *et al*., 2009; Arnaud *et al*., 2007; Mehle *et al*., 2011).

Other phytoplasmas associated with grapevine yellows diseases

GY diseases is are associated with a wide variety of phytoplasmas. For instance in the Republic of South Africa, Italy, Tunisia, Israel, North America and Chile it is associated in some cases also with aster yellows phytoplasmas from the 16SrI group (Prince *et al*., 1993; Alma et al., 1996; Carstens, 2008: Gajardo *et al*., 2009). In the Slovenian survey of phytoplasmas an isolate from this group was detected in purple coneflower (*Echinacea purpurea*) (Radišek *et al*., 2009).

Conclusion

Presence of '*Ca.* P. mali', '*Ca.* P. prunorum' and '*Ca.* P. pyri' were confirmed in several fruit-growing areas of Slovenia. The most widespread phytoplasma on grapevine in Slovenia is BN phytoplasma, stolbur phytoplasma was also identified in bindweeds and tomato plants. Since 2005, the phytoplasma FD that causes the most serious disease of viticulture, 'flavescence dorée', has been identfied in Slovenian vineyards. FDp was also confirmed in Clematis plants and Aster yellows phytoplasma was detected in purple coneflower. Several known and putative vectors of these phytoplasmas were identified in Slovenia and in some of them particular phytoplasmas were also detected. For quarantine phytoplasma diseases, several measures are prescribed by national regulation.

Acknowledgements

The analysis of the samples was financed by the Ministry of Agriculture, Forestry and Food. The authors would like to thank the phytosanitary inspectors, expert surveyors and their coordinator at Phytosanitary Administration RS, mag. Erika Orešek, for providing the samples and Jana Frank and Nina Prezelj for excellent laboratory work. Many thanks to colleagues who were involved in developing the diagnostic methods and research of phytoplasma: Dr. Nataša Petroviè, Dr. Jernej Brzin, Dr. Matjaz Hren, Dr. Jana Boben, Dr. Petra Nikolić and Matevz Rupar. Figure 1 was prepared mainly by Nina Prezelj and Dr. Petra Nikolić.

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