Identification of fruit tree phytoplasmas and their vectors in Bosnia and Herzegovina

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Surveys were carried out in autumn 2004 and spring 2005 in the traditional areas dedicated to pome and stone fruit cultivation in Bosnia and Herzegovina to assess the presence, distribution and incidence of phytoplasma diseases in fruit trees. The occurrence of psyllid vectors was also considered. The detection of phytoplasmas in plant and insect samples and their identification were carried out by symptom observations in the field, double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA), nested polymerase chain reaction (nested-PCR) and restriction fragment length polymorphism (RFLP) analyses. Laboratory analyses showed the presence of phytoplasmas belonging to: (i) 16SrX group, subgroup A ('*Candidatus* Phytoplasma mali') in 23 out of 25 apple samples, in 4 groups out of 18 of *Cacopsylla picta* (synonym *Cacopsylla costalis*) and in 2 groups out of 9 of *Cacopsylla melanoneura*; (ii) 16SrX group, subgroup C ('*Candidatus* Phytoplasma pyri') in 11 out of 30 pears samples and in 2 groups out of 9 of *Cacopsylla pyri*; (iii) 16SrX group, subgroup B ('*Candidatus* Phytoplasma prunorum') in 4 apricots, 2 peaches out of 42 stone fruit samples and in 1 group out of 14 of *Cacopsylla pruni*. The presence of different subtypes of *Candidatus* Phytoplasma mali, both in apple trees and in insects, was proven.

Introduction

Phytoplasma diseases cause serious damage to fruit trees in areas with a temperate climate. Phytoplasma-like symptoms have been observed on fruit trees in Bosnia and Herzegovina during recent years. No phytoplasma expertise and detection facilities were available locally, thus other biotic or abiotic factors were usually held responsible for the diseases. Only recently has the presence of pear decline been confirmed by PCR and RFLP analyses (Duduk *et al*., 2005a) in Bosnia and Herzegovina.

Phytoplasma diseases – apple proliferation associated with '*Candidatus* Phytoplasma mali', pear decline associated with '*Candidatus* Phytoplasma pyri' and European stone fruit yellows associated with '*Candidatus* Phytoplasma prunorum' – have been detected in neighbouring countries (Saric & Cvjetkovic, 1985; Tanaskovic & Carraro, 2003; Duduk *et al*., 2005b) with which plant material is frequently exchanged. Taking this into account, the main objective of the present paper was to detect the presence and quantify the incidence of phytoplasma diseases in fruit trees in Bosnia and Herzegovina.

A further objective was to assess the presence of the known psyllid vectors of fruit tree phytoplasmas, because phytoplasma diseases are particularly dangerous in areas where both infected plants (sources of inoculum) and insect vectors are present.

Materials and methods

Field surveys

Surveys were carried out during autumn (October 2004) and spring (April 2005). Stone fruit, apple and pear trees were checked for symptoms of phytoplasma infection. The symptoms considered were: leaf roll, yellowing and phloem necrosis for European stone fruit yellows; witches' broom, small leaves, enlarged stipules and reddening for apple proliferation; leaf roll, small leaves, yellowing/reddening and decline for pear decline. Several hectares of orchards in the north-western (Gradiska-Banjaluka), southern (Mostar) and central (Sarajevo-Maglaj) areas of Bosnia and Herzegovina were inspected. Samples were collected from cultivated apple (*Malus pumila*), pear (*Pyrus communis*), peach (*Prunus persica*), apricot (*Prunus armeniaca*), European plum (*Prunus domestica*) and from wild myrobalan (*Prunus cerasifera*) and blackthorn (*Prunus spinosa*). Apple and pear samples were only collected from symptomatic trees. Different cultivars on different rootstocks from private or commercial orchards were chosen; some bordering trees were also considered.

To better understand the epidemiology of fruit tree phytoplasmas in Bosnia and Herzegovina, surveys were also carried out during April 2005 in the same areas to detect the presence of known psyllid vectors. *Cacopsylla picta*, *Cacopsylla*

melanoneura, *Cacopsylla pyri* and *Cacopsylla pruni* were collected using the beating tray method. One old pear orchard was chosen for collecting *C. pyri*. *C. picta* and *C. melanoneura* were captured in abandoned apple orchards, and *C. pruni* in abandoned plum and apricot orchards. After identification of the collected insects under a binocular microscope, they were split into groups of 10 and 20 individuals and kept at −20 °C (in 70% ethanol) for successive laboratory analyses.

Serological and PCR/RFLP analyses

DAS-ELISA was carried out to detect '*Ca*. P. mali' in the apple samples, using the 1F4/1E2 monoclonal antibody (Loi *et al*., 2001).

Two procedures were used for DNA extraction. The phytoplasma enrichment procedure modified by Malisano *et al*. (1996) was adopted for DNA extraction from leaves collected from different trees. The DNA extraction from insects was carried out according to Doyle & Doyle (1990). Nested PCR was employed for the detection of phytoplasmas both in plants and psyllids using the universal primers P1/P7 (Deng & Hiruki, 1991) followed by R16F2n/R16R2 (Gundersen & Lee, 1996). The R16F2n/R2 amplicons were then digested with *Mse*I restriction enzyme. Another nested PCR procedure using 16SrX phytoplasma group specific primer pair f01/r01 (Lorenz *et al*., 1995) was performed, in which the PCR products were digested with the endonucleases *Bsa*AI and *Ssp*I.

'*Candidatus* Phytoplasma mali' subtypes

Both apple trees and psyllid vectors *C. picta* and *C. melanoneura* were analysed for the presence of '*Ca*. P. mali' subtypes. Two PCR/RFLP procedures were adopted: the method described by Jarausch *et al*. (2000) based on nonribosomal DNA fragments that distinguish the subtypes AP, AT-1 and AT-2; the method developed by Martini *et al*. (2005), based on ribosomal protein (rp) gene sequences *rpl22* and *rps3*. The rp genes-based method distinguishes four different '*Ca*. P. mali' subtypes, indicated as rpX-A, rpX-B, rpX-C and rpX-D, by RFLP analysis using the *Alu*I enzyme.

Results

Field surveys

Phytoplasma symptoms were observed on fruit trees in all orchards surveyed, with the exception of apricot and peach orchards in Banjaluka where symptomatic plants were not found. The percentage of symptomatic trees was: 20–90% in apple orchards; 6–70% in pear orchards; 0–50% in stone fruit orchards (Tables 1, 2 and 3). The wild *Prunus* species as well as the European plum trees were symptomless. Altogether, 97 samples were collected: 25 symptomatic apples, 30 symptomatic pears and 42 stone fruits.

Table 1 The presence of '*Ca*. Phytoplasma mali' in apple trees in different locations in Bosnia and Herzegovina and samples analysed using two nested PCR/RFLP procedures and ELISA. The percentage of symptomatic plants in the surveyed orchards is also reported

Location	Symptomatic plants (%)	N° of samples positive/ tested using phytoplasma universal primers	N° of samples positive/ tested using phytoplasma group specific primers	N° of samples positive/ tested using ELISA
Gradiska	20	7/7	7/7	6/7
Srbac	n.p.	2/2	2/2	2/2
Laktasi	n.p.	2/2	2/2	1/2
Sarajevo	90	7/8	7/8	4/8
Maglaj	90	5/6	5/6	4/6
Total	/	23/25 (92%)	23/25 (92%)	17/25 (68%)

n.p., not performed.

Table 2 '*Ca*. Phytoplasma pyri' found in pear trees in different locations in Bosnia and Herzegovina and analysed using two nested PCR/RFLP procedures. The percentage of symptomatic plants in the surveyed orchards is also reported

Location	Symptomatic plants (%)	N° of samples positive/ tested using phytoplasma universal primers	N° of samples positive/ tested using phytoplasma group specific primers
Gradiska	60	6/12	6/12
Srbac	6	0/3	0/3
Banjaluka	70	2/3	2/3
Sarajevo	30	2/3	2/3
Zenica	n.p.	0/1	0/1
Maglaj	30	1/8	1/8
Total	/	11/30 (37%)	11/30 (37%)

n.p., not performed.

Table 3 '*Ca*. Phytoplasma prunorum' present in *Prunus* species in different locations in Bosnia and Herzegovina and analysed using two nested PCR/RFLP procedures. The percentage of symptomatic plants in the surveyed orchards is also reported

Location	*Prunus* spp.	Symptomatic plants (%)	N° of samples positive/ tested using phytoplasma universal primers	N° of samples positive/ tested using phytoplasma group specific primers
Banjaluka	apricot	0	1/5	1/5
	peach	0	1/5	1/5
Mostar	apricot	50	2/7	2/7
	peach	20	2/4	2/4
Sarajevo	myrobalan	0	0/12	0/12
Mostar, Doboj	European plum	0	0/3	0/3
Sarajevo, Doboj	blackthorn	0	0/6	0/6
Total		/	6/42 (14%)	6/42 (14%)

Table 4 Presence of '*Ca*. Phytoplasma mali' in *C. picta* and *C. melanoeura*, '*Ca*. Phytoplasma pyri' in *C. pyri* and '*Ca*. Phytoplasma prunorum' in *C. pruni*. All the psyllids were collected in orchards of Bosnia and Herzegovina and grouped in batches of 10–20 individuals

Psyllid[a]	N° insects/ group	N° tested groups	N° of phytoplasma positive/tested groups
C. picta	10	8	3/8
	20	10	1/10
C. melanoneura	10	6	1/6
	20	3	1/3
C. pyri	10	4	0/4
	20	5	2/5
C. pruni	10	13	1/13
	20	1	0/1

[a]A total of 280 *C. picta*, 120 *C. melanoneura*, 140 *C. pyri* and 150 *C. pruni* were collected.

In April 2005, a total of 690 individuals of *C. pruni*, *C. picta*, *C. melanoneura* and *C. pyri* were captured (Table 4). *C. pyri* had reached the end of its annual life cycle (overwintering generation) and therefore was not very abundant. In stone fruit orchards, psyllids were mostly captured from suckers (*P. domestica*, *P. cerasifera*) or from wild *P. spinosa*. In some of the orchards where psyllids were collected, pear decline, apple proliferation and European stone fruit yellows were present.

Detection and identification of phytoplasmas in fruit trees and psyllids

Using ELISA (Table 1), 17 out of 25 (68%) of the tested apple trees were positive for the presence of '*Ca*. P. mali'. Tables 1, 2, 3 and 4 show the results obtained using two PCR procedures (with universal and group specific primer pairs) for the detection of the three phytoplasmas in both plants and insects. The two PCR procedures adopted were equally sensitive. All the R16F2n/R2 amplicons, when digested with *Mse* I restriction enzyme, showed the typical restriction profile of the 16Sr-X phytoplasma group, both in plants and insects. RFLP analyses of f01/r01 PCR products using *Bsa*AI and *Ssp*I enzymes confirmed the presence of: '*Ca*. P. mali' in apple samples, *C. picta* and *C. melanoneura*; '*Ca*. P. pyri' in pear samples and in *C. pyri*; '*Ca*. P. prunorum' in apricot and peach samples and in *C. pruni*.

'*Candidatus* Phytoplasma mali' subtypes

The subtypes AP, AT-1 and AT-2, as defined by Jarausch *et al*. (2000), were identified in the apple samples which had tested positive for apple proliferation. Following the method proposed by Martini *et al*. (2005), two subtypes (rpX-A and rpX-B) were identified in the same apple proliferation infected trees. Combining the results obtained using the two procedures, we identified in the 23 trees infected by apple proliferation the following subtypes: 14 (61%) AP/rpX-A; 6 (26%) AT-2/rpX-A; 2 (9%) AT-1/rpX-A, 1 (4%) AT-1/rpX-B. Regarding the positive batches of vectors, *C. picta* and *C. melanoneura*, positive results were only obtained with the method based on ribosomal protein gene sequences while it was not possible to amplify phytoplasma DNA with the procedure by Jarausch *et al*. (2000). In both vectors, the subtype rpX-A was found.

Discussion

Regarding '*Ca*. P. mali', PCR was found to be more sensitive than ELISA. However, serological tests can be used for large-scale diagnosis, and PCR as a confirmation in the case of ELISA-negative or doubtful results. The high correlation between the presence of symptoms on collected plant material and the presence of the disease agent (more than 90% of the tested samples were found infected) as well as the presence of apple proliferation-infected vectors (*C. picta* and *C. melanoneura*), strongly indicate that the disease is epidemic in the country. The absence of strict phytosanitary controls in nurseries and fruit orchards and a widespread occurrence of psyllid vectors are facilitating the spread of apple proliferation in Bosnia and Herzegovina. The distribution pattern of '*Ca*. Phytoplasma

mali' subtypes was found to be similar to that described in Germany (Jarausch *et al*., 2004) where AP subtype was predominant, followed by AT-2 and AT-1 but completely different from the situation in the Trentino Alto Adige region (Italy), where the AP subtype was sporadically found (Cainelli *et al*., 2004). Results obtained by using the typing method proposed by Martini *et al*. (2005) which showed rpX-A was the predominant subtype, should be considered as preliminary and need to be confirmed by extended surveys in different apple-growing areas.

Although all 30 pear samples were collected from trees showing pear decline symptoms, only 11 (37%) were positive for the presence of '*Ca*. P. pyri'. This confirms that symptoms of pear decline are not highly specific and can be confused with other disorders such as graft-incompatibility, chlorosis, fungal, viral or bacterial diseases. Although only 37% of the symptomatic samples were positive in laboratory analyses, pear decline was detected in almost all locations inspected. In north-western Bosnia and Herzegovina, *C. pyri* was found to be infected by the disease agent. In terms of single insects, the minimum rate of psyllids carrying phytoplasmas was 1.4%. Vector control appears to be of fundamental importance for reducing the spread of pear decline.

The infection rate of '*Ca*. P. prunorum' in symptomatic samples was 25%, and in symptomless samples 10%. In routine diagnosis and in order to obtain reliable results on the presence of the causal agent in *Prunus* species, the optimal time for sampling leaves is from July to September (Jarausch *et al*., 1999). The rather late period chosen for our survey probably influenced the low-rate detection of the phytoplasma in symptomatic apricot samples. Symptom severity of European stone fruit yellows is influenced by the rootstock. The main apricot rootstocks in Bosnia and Herzegovina are European plum and myrobolan which show intermediate susceptibility and sensitivity (Carraro *et al*., 2004). This could be the cause of the occurrence of '*Ca*. P. prunorum' in some symptomless trees. European stone fruit yellows is a serious problem in the Mediterranean area (France, Italy, Spain), where the cultivation of susceptible and sensitive *Prunus* species (apricot and Japanese plum) is widespread. Consequently, the low incidence of '*Ca*. P. prunorum' in *Prunus* species in Bosnia and Herzegovina can be correlated to the limited cultivation of these highly susceptible species. Despite its low incidence, '*Ca*. P. prunorum' was recorded in northern and southern parts of Bosnia and Herzegovina.

Infected *C. pruni* were collected in an apricot orchard where European stone fruit yellows was diagnosed. Adults were more numerous on wild *Prunus* species and rootstocks than on apricots. This is in agreement with the hypothesis that wild *Prunus* species, especially *Prunus spinosa,* are the preferred host plants of the vector (Carraro *et al*., 2002). Our results desmonstrate that both the disease and the vector are present in Bosnia and Herzegovina even if the disease incidence and the level of infectivity of *C. pruni* seem to be low.

In conclusion, we determined the presence and the incidence of three dangerous phytoplasma diseases and their respective vectors in Bosnia and Herzegovina which represent a serious threat to the local fruit tree industry.

Identification de phytoplasmes pathogènes des arbres fruitiers et de leurs vecteurs en Bosnie-Herzégovine

Des prospections ont été menées à l'automne 2004 et au printemps 2005 dans les zones traditionnellement dédiées à la culture des fruits à pépins et à noyaux en Bosnie-Herzégovine pour évaluer la présence, la distribution et l'incidence des maladies à phytoplasme dans les arbres fruitiers. La présence de psylles vecteurs a également été étudiée. La détection de phytoplasmes dans les échantillons de végétaux et d'insectes et leur identification ont été menées par l'observation de symptômes au champ, et des analyses DAS-ELISA (double antibody sandwich-enzyme linked immunosorbent assay), nested-PCR (nested polymerase chain reaction) et RFLP (restriction fragment length polymorphism). Les analyses de laboratoire ont montré la présence de phytoplasmes appartenant au : i) groupe 16SrX, sous-groupe A ('*Candidatus* Phytoplasma mali') dans 23 des 25 échantillons de pommiers, dans 4 groupes sur 18 de *Cacopsylla picta* (synonyme *Cacopsylla costalis*) et dans 2 groupes sur 9 de *Cacopsylla melanoneura* ; ii) groupe 16SrX, sous-groupe C ('*Candidatus* Phytoplasma pyri') dans 11 des 30 échantillons de poiriers et dans 2 groupes sur 9 de *Cacopsylla pyri* ; iii) groupe 16SrX, sous-groupe B ('*Candidatus* Phytoplasma prunorum') dans 4 échantillons d'abricotiers, 2 de pêchers sur les 42 échantillons d'arbres fruitiers à noyaux et dans 1 groupe sur 14 de *Cacopsylla pruni*. La présence de différents sous-groupes de *Candidatus* Phytoplasma mali a ainsi été prouvée à la fois dans les pommiers et les insectes.

Идентификация фитоплазм плодовых деревьев и их переносчиков в Боснии и Герцеговине

Осенью 2004 г. и весной 2005 г. в традиционных районах производства семечковых и косточковых плодов Боснии и Герцеговины производились обследования для оценки наличия и воздействия фитоплазменных заболеваний на плодовые деревья. Принималось во внимание также наличие переносчиков-листоблошек и медяниц. Выявление фитоплазм в образцах растений и насекомых и их идентификация проводились путем наблюдения симптомов в полевых условиях, двойного связанного иммуносорбентного анализа антител сэндвич-энзим (DAS-ELISA), гнездовых анализов полимеразной цепной реакции (гнездовые-ПЦР) и анализа полиморфизма длины фрагмента рестрикции (RFLP). Лабораторные анализы показали наличие фитоплазм, принадлежащих к: i) группе 16SrX, подгруппе A («*Candidatus* Phytoplasma mali») в 23 из 25 образцов яблонь, в 4 из 18 групп *Cacopsylla picta* (синоним *Cacopsylla costalis*) и в 2 из 9 групп *Cacopsylla melanoneura*; ii) группе 16SrX, подгруппе C («*Candidatus* Phytoplasma pyri») в 11 из 30 образцов груш и в 2 из 9

групп *Cacopsylla pyri*; iii) группе 16SrX, подгруппе B («*Candidatus* Phytoplasma prunorum») в 4 абрикосах, в 2 персиках из 42 образцов косточковых и в 1 из 14 групп *Cacopsylla pruni*. Было доказано наличие различных подтипов *Candidatus* Phytoplasma mali как в яблоне, так и в насекомых.

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