PHYTOPLASMA DISEASES OF FRUIT TREES IN GERMPLASM AND COMMERCIAL ORCHARDS IN TURKEY

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SUMMARY

A survey was carried out in germplasm nurseries and commercial orchards from ten provinces in Turkey during 2003-2005. Samples were collected from trees showing European stone fruit yellows (ESFY) and Pear decline (PD) symptoms. A total of 270 stone fruit and six pear samples were tested by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Universal phytoplasma primers P1/P7 and R16F2n/R2 were used for one-step and nested PCR assays, respectively. Nested-PCR products were digested with *Rsa*I, *Ssp*I and *Mse*I restriction enzymes. Twenty-eight of 270 samples including plum, apricot, peach and almond were infected by *Candidatus* Phytoplasma prunorum. Infection rate by the same phytoplasma species of germplasm nurseries containing mostly foreign cultivars was 54.8%, whereas it was only 3.2% in commercial orchards. *Ca.* Phytoplasma prunorum was detected for the first time in Antalya, Gaziantep, Içel, Isparta, and Yalova provinces in local apricot cultivars as well as Japanese plum, apricot, and almond cultivars imported from Japan. PCR and RFLP tests of pear samples collected from commercial orchards in the Bursa province showed that the local pear cultivar 'Deveci' was infected by *Candidatus* Phytoplasma pyri, which was also found in the pear psyllid *Cacopsylla pyri* L. To our knowledge, this is the first report of detection of *Ca.* Phytoplasma pyri in *C. pyri* in Turkey.

Key words: *Cacopsylla pyri, Candidatus* Phytoplasma prunorum, *Candidatus* Phytoplasma pyri, PCR, RFLP.

INTRODUCTION

Phytoplasmas are wall-less, non-culturable prokaryotes of the class *Mollicutes*, pathogenic to fruit trees. The most common phytoplasmas in pome fruit trees are *Candidatus* Phytoplasma mali, *Candidatus* Phytoplasma pyri, whereas a number of diseases of *Prunus* species are elicited by *Candidatus* Phytoplasma prunorum (Lorenz *et al.*, 1994; Lee *et al.*, 1995; Jarausch *et al.*, 1998). These phytoplasmas are phylogenetically related and belong to the 'Apple proliferation' phytoplasma group, subgroups 16SrX-B and 16SrX-C (Lee *et al.*, 1998, Firrao *et al.*, 2005), but each is considered to be specific for its respective host genus. Recently, a new phytoplasma, almond witches' broom, infecting peach and nectarine in Lebanon was classified as a new member of the pigeon pea witches' broom phytoplasma group (Abou-Jawdah *et al.*, 2002; 2003). Phytoplasmas are transmitted in the persistent mode by psyllid species. In particular, *Ca.* Phytoplasma prunorum is transmitted by *Cacopsylla pruni* Scopoli (Carraro *et al.*, 1998c; Jarausch *et al.*, 2001) and *Ca.* Phytoplasma pyri is transmitted by *Cacopsylla pyri* L. (Carraro *et al.*, 1998a).

Stone fruits are important fruit crops in Turkey, where local land-races and new varieties are cultivated. Severe decline of plum, apricot, and pear trees growing in Turkish nurseries and commercial orchards has recently reached alarming levels. For instance, in foreign (e.g. 'Precoce de Tyrinthe') and local (e.g. 'Sakit') apricot cultivars symptoms were seen, i.e. deformation and rolling of the leaves, reduced yield, and decline, recalling those typically induced by phytoplasma infections. These symptoms were also observed by Çaglayan and Gazel (1999) in apricot, peach and cherry trees in the east Mediterranean region of Turkey, and in pome and stone fruit orchards by Cali (1992) and Nogay *et al.* (2001).

Recently, Jarausch *et al.* (2000) found a Japanese plum tree infected by *Ca.* Phytoplasma prunorum at Izmir, on the Aegean coast. On the other hand, Çaglayan *et al.* (2004) identified by PCR and RFLP *Ca.* Phytoplasma prunorum infections in apricot trees in the provinces of Içel and Adana, and Sertkaya *et al.* (2005) reported the presence of *Ca.* Phytoplasma prunorum in one almond, two apricots, one peach and one myrobalan, and of *Ca.* Phytoplasma pyri in one pear tree in the east Mediterranean region.

Despite of these records, no information was secured on the spread of phytoplasma diseases by vectors and their prevalence in Turkish orchards. Therefore, the

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main objective of this paper was to test local and imported cultivars of fruit trees in germplasm and commercial orchards for the presence of *Ca.* Phytoplasma prunorum and *Ca.* Phytoplasma pyri by PCR and RFLP analysis, and to collect and test psyllids for gathering information on the possible source of infection of local pear cultivars.

MATERIALS AND METHODS

Sampling. Surveys were carried out in ten provinces of Turkey (Fig. 1) from February 2003 to September 2005, in commercial orchards of Bursa, Içel, Hatay, Kahramanmaraş and Malatya provinces and germplasm orchards of Adana, Antalya, Gaziantep, Isparta, Içel, and Yalova provinces. A total of 270 stone fruit (apricot, plum, peach, nectarine, almond, wild apricot) and six pear trees showing typical symptoms of phytoplasma infection were sampled, collecting one- or two-year-old cuttings (Table 1). In winter, the trees were again inspected for off-season growth, which is the most common symptom of *Ca.* Phytoplasma prunorum in the east Mediterranean region. In early autumn of 2005, pear psyllids (*C. pyri*) were collected from symptomatic pear trees using an insect net.

Phytoplasma reference strains. DNA and dried leaf materials infected with *Ca.* Phytoplasma prunorum peach isolate (Nemeth *et al*., 2001), *Ca.* Phytoplasma pyri and *Ca.* Phytoplasma mali isolates (Ember *et al*., 2004) to be used as reference, were obtained from the Plant Health and Soil Conservation Station (Budapest, Hungary). Healthy GF-305 leaves were used as a negative control.

Nucleic acid extraction. Total DNAs from all stone fruit plant samples including healthy control were extracted according to Doyle and Doyle (1990). Approximately 0.1 g of leaf midribs and/or cortical scrapings was ground with liquid nitrogen. The supernatant was clarified by phenol: chloroform: isoamyl alcohol and resuspended in TE buffer.

Fig. 1. Turkish provinces surveyed for the presence of phytoplasmas in fruit trees.

For pear samples, approximately 1 g of leaf midribs fresh or stored in glycerol at -20°C was used to extract nucleic acid by chloroform: phenol (Prince *et al*., 1993). Eighty adult insects were used as one sample and tested by PCR to verify whether they contained *Ca*. phytoplasma pyri. Insect DNA was extracted according to Prince *et al.* (1993).

Primers and PCR amplification. Phytoplasma specific primers P1 (Deng and Hiruki, 1991) and P7 (Smart *et al*., 1996) amplifying approximately a 1800 bp fragment that extends from the 5' end of the 16S rRNA gene to the 5' end of the 23S rRNA, were used for general identification of phytoplasmas in one-step PCR. The universal primer pair R16F2n/R2 designed to amplify a 1200 bp portion of the 16S rRNA gene was used for nested-PCR (Lee *et al*., 1993).

Nucleic acid samples were diluted in sterile deionized water to obtain a final concentration of 20 ng/µl.

Table 1. Number of stone fruit samples collected from different provinces and phytoplasma infected samples in total.

Provinces	Number of ESFY infected samples/total						
	Almond	Apricot	Nectarine	Peach	Plum	Pear	Total
Adana	0/6	1/7	0/2	-[a]	0/4	-	1/19
Antalya	1/1	0/2	-	-	2/3	-	3/6
Bursa	-	-	-	-	-	2/6	2/6
Gaziantep	-	-	-	-	1/6	-	1/6
Içel	-	3/24	-	0/3	3/10	-	6/37
Isparta	-	2/3	-	-	1/3	-	3/6
Hatay	1/23	0/43	0/3	0/34	0/23	-	1/126
Malatya	-	0/27	-	-	-	-	0/27
Yalova	0/5	7/13	-	0/5	6/10	-	13/33
Kahramanmaraş	-	0/9	-	0/1	-	-	0/10
Total	2/35	13/128	0/5	0/43	13/59	2/6	28/276

[a] No samples were collected.

One-step and nested-PCRs were done in mixtures of 50 µl (final volume) containing 5 µl 10xPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 0.01% gelatin) 1.5 mM $MgCl_2$, 250 µM each dNTPs, 20 pmol $µl^{-1}$ each primers, 2 U *Taq* DNA polymerase and 1 µl DNA (1: 50 diluted). One-step PCR products were diluted to 1:50 and 1 µl DNA was used for nested-PCR. The following amplification conditions were used: for the first cycle 94°C for 2 min; 35 cycles 94°C for 1 min (30 sec for nested-PCR) denaturation, 55°C for 2 min (30 sec for nested-PCR) annealing, 72°C for 3 min (1 min for nested-PCR) extension and the final cycle 72°C for 5 min.

PCR products were analyzed by electrophoresis in 1xTAE buffer using 1.5% agarose gel and DNA bands were visualized with a UV transilluminator after staining the gels with ethidium bromide.

RFLP analysis. Nested PCR products including reference isolates (1200 bp) of phytoplasma 16S rDNA sequence were subjected to RFLP analysis. Five µl aliquots of each PCR products were separately digested overnight at 37°C with restriction endonucleases *Rsa*I, *Ssp*I; at 65°C with restriction endonuclease *Mse*I (MBI Fermentas, GmbH, Germany). The digested products were analyzed by electrophoresis using 2% agarose gel and the products were visualized with a UV transilluminator after gels were stained with ethidium bromide.

RESULTS

Symptoms on diseased plants. In February of 2003, in apricot trees of commercial orchards at Mut (Içel) and of germplasm orchards at Pozantı (Adana), symptoms were observed consisting of early bud break followed by yellowing, leaf rolling, and decline. The yield of symptomatic trees was reduced. In the following years, chlorosis, dwarfing, proliferation and die-back symptoms were observed in other germplasm orchards located in different provinces. Most of the plants declined and died in the course of two years after the first symptom appearance. In 2005, early reddening of the leaves and decline were observed on most of the pear trees of 'Deveci' in commercial orchards in the Bursa province. All orchards exhibiting rapid decline suffered severe attacks of *C. pyri* in both 2005 and in the previous year.

Phytoplasma detection by PCR. All 270 samples of apricot, peach, plum, almond and nectarine were tested by one-step and nested PCR, along with six pear trees and a group of 80 psyllids. No amplification was obtained from the reaction mixture without nucleic acid template (blank) or containing DNAs from healthy GF-305. By contrast, phytoplasma-specific 1200 bp amplicons were obtained by nested PCR from leaves and/or phloem tissue and from insect extracts (Fig. 2, 3).

RFLP analysis. Identical patterns were observed in RFLP analysis of *Ca.* Phytoplasma prunorum ribosomal DNAs from 28 diseased apricots, Japanese plum and almond trees (Fig. 4). RFLP analysis confirmed results of group-specific nested PCR, when R16F2n/R2 nested PCR products were digested by *Rsa*I. The digestion led to the production of three fragments while the digestion of nested PCR products with *Mse*I resulted in four frag-

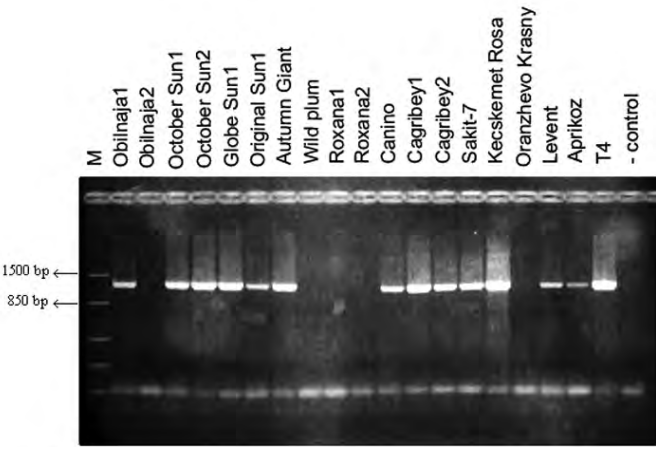

Fig. 2. Results of nested PCR for detection of phytoplasmas in phloem tissues of plums (first 8 lanes) and apricots (following 10 lanes) with universal primer pair P1/P7, followed by amplification with primers R16F2n/R2. M: Marker (MBI Fermentas); T4: *Ca.* Phytoplasma prunorum positive control (Hungarian isolate); control: without DNA.

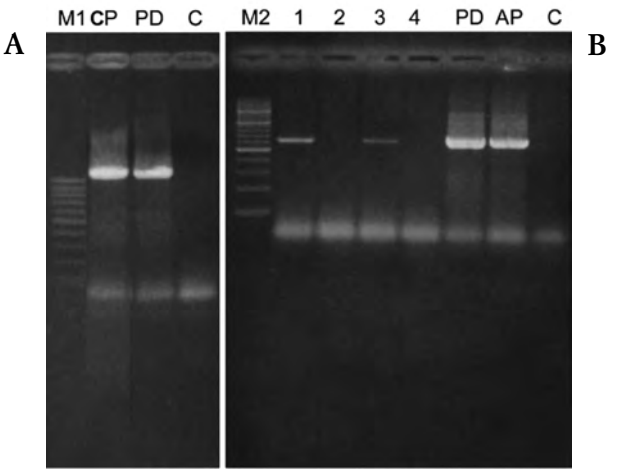

Fig. 3. Results of nested PCR for detection of pear decline phytoplasma in insect bodies (**A**) and in pear phloem tissues (**B**) using universal primer pair P1/P7 followed by amplification with primers R16F2n/R2. M1: Gene Ruler 100 bp DNA ladder; M2: O'Range Ruler 200 bp DNA ladder (MBI Fermentas). CP: *Cacopsylla pyri* L. PD: *Ca.* Phytoplasma pyri positive control. C: Control without DNA. Lanes 1, 3: Pear 'Deveci' samples. Lanes 2, 4: Pear 'Santa Maria' samples. AP: *Ca.* Phytoplasma mali positive control.

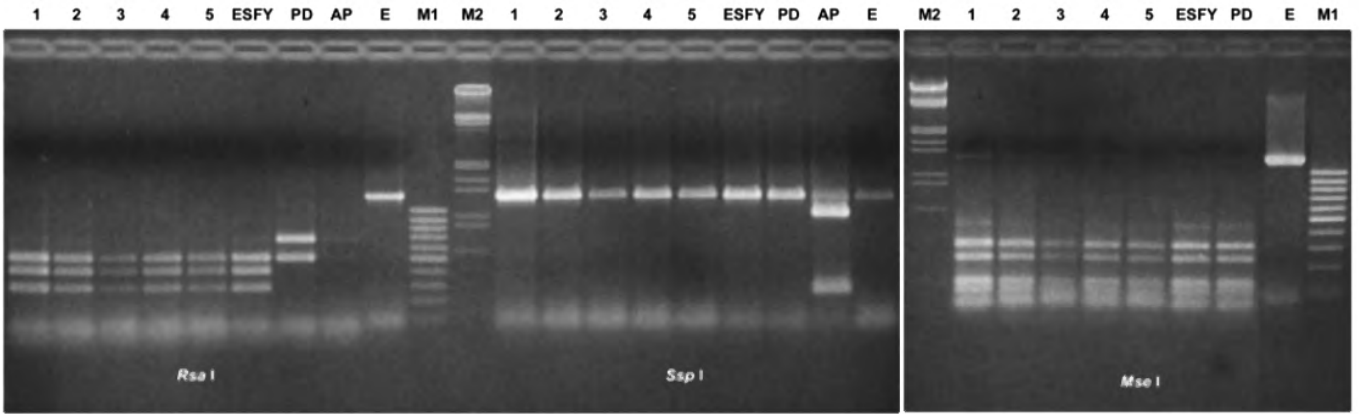

Fig. 4. Results of RFLP analyses of R16F2n/R2 nested PCR products obtained from five apricot samples using *RsaI*, *SspI* and *MseI* restriction enzymes. M1: Gene Ruler 100 bp DNA ladder and M2: l DNA *Eco*RI/*Hind*III ladder (MBI Fermentas); lanes 1-5: Apricot samples infected by *Ca*. Phytoplasma prunorum, ESFY: *Ca.* Phytoplasma prunorum, PD: *Ca*. Phytoplasma pyri, AP: *Ca*. Phytoplasma mali positive controls; E: control without enzyme.

ments. No fragments were generated by digestion with *Ssp*I. RFLP profiles of phytoplasma reference isolates were very similar to those previously published by Lee *et al*. (1998). All 28 apricots, Japanese plum and almond trees had restriction patterns identical to those of reference isolates of *Ca.* Phytoplasma prunorum. Similarly, infected pear samples gave the same banding pattern as that of the *Ca.* Phytoplasma pyri positive control in digestion with *Ssp*I, which differed from that of the *Ca.* Phytoplasma mali isolate (Fig. 5).

Phytoplasma incidence. The overall incidence of phytoplasma infection in tested samples was 10.2%. However, 54.8% of samples form germplasm nurseries was infected by *Ca.* Phytoplasma prunorum whereas disease incidence was only 3.2% in commercial orchards. This phytoplasma species was detected in all provinces under study, except for Malatya and Kahramanmaraş. Since the appearance of early reddening of pear trees was very common in Bursa, *Ca.* Phytoplasma pyri was the only pathogen tested for in this province.

Ca. Phytoplasma prunorum-infected trees were found in the local apricot cultivars 'Sakit', 'Çagribey', 'Levent', 'Aprikoz', and 'Şekerpare', and in the foreign cultivars 'Canino' and 'Kecskemet Rosa' (Table 2). In germplasm orchards, except for 'Çöloglu', 'Mektep' and 'Aprikoz-

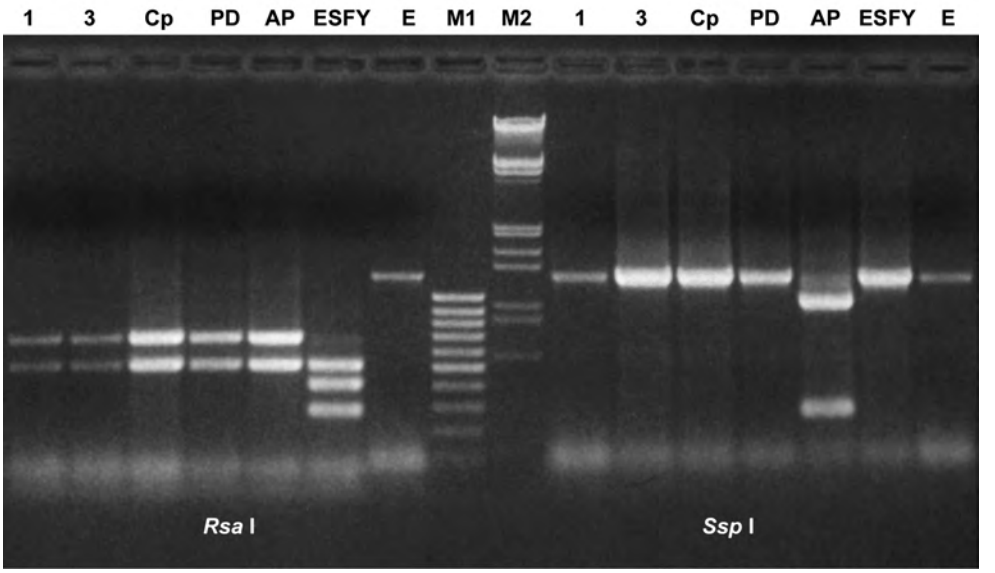

Fig. 5. Results of RFLP analyses of R16F2n/R2 nested PCR products obtained from pear decline phytoplasma in pear phloem tissues and in insect bodies using *RsaI* and *SspI* restriction enzymes. Lane M1: Gene Ruler 100 bp DNA ladder and M2: l DNA *Eco*RI/*Hind*III ladder (MBI Fermentas). Lanes 1, 3: Pear 'Deveci' samples. Cp: *Cacopsylla pyri* L. PD: *Ca*. Phytoplasma pyri, AP: *Ca*. Phytoplasma mali and ESFY: *Ca.* Phytoplasma prunorum positive controls. E: control without enzyme.

302', all other local apricot cultivars were infected by this phytoplasma. All *Ca.* Phytoplasma prunorum-infected plums were Japanese plums, including 'Obilnaja', 'October Sun', 'Globe Sun', 'Original Sun', 'TC Sun', 'Queen Rosa', and 'Autumn Giant' (Table 2). Except for 'Almond-19', all almond cultivars in germplasm orchards were free from *Ca.* Phytoplasma prunorum. In commercial orchards, *Ca.* Phytoplasma prunorum-infected trees were found in the local apricot cv. Sakit and the foreign cv Precoce de Thyrinte.

In commercial orchards of Bursa, most pear trees of the local 'Deveci', but not the imported 'Santa Maria', were infected by *Ca.* Phytoplasma pyri. Finally, *Ca.* Phytoplasma pyri was found in *C. pyri* individuals collected from symptomatic pear trees examined by PCR/RFLP.

DISCUSSION

Since 1999, widespread symptoms of phytoplasma infections have been observed in some Turkish apricot and plum orchards (Çaglayan and Gazel, 1999). Samples collected from germplasm and commercial orchards showed that typical symptoms are strongly associated with the presence of *Ca.* Phytoplasma prunorum phytoplasmas as reported previously (Jaraush *et al.*, 1998).

Table 2. Stone and pome fruit cultivars tested for phytoplasma infections in Turkish germplasm orchards.

Tree Species	Cultivars	Number of positives/Total
Japanese plum	Obilnaja (*P. salicina* x *P. cerasifera*)	2/3
(*Prunus salicina* Lindl.)	October Sun	4/6
	Globe Sun	2/3
	Original Sun	2/3
	TC Sun	1/4
	Queen Rosa	1/3
	Autumn Giant	1/1
	Black Diamond	0/1
	Larry Ann	0/1
	Santa Rosa	0/1
European plum	Firenze-90	0/1
(*Prunus domestica* L.)		
Myrobalan	Wild plum	0/1
(*Prunus cerasifera* Ehrh.)		
Apricot	Çagribey[a]	2/2
(*Prunus armeniaca* L.)	Şekerpare[a]	1/2
	Sakit-2[a]	1/2
	Sakit-7[a]	1/1
	Canino	1/1
	Kecskemet Rosa	1/1
	Levent[a]	1/1
	Aprikoz[a]	1/1
	Roxana	0/2
	Oranzhevo Krasny	0/1
	Çöloglu[a]	0/1
	Apricot-302[a]	0/1
	Mektep[a]	0/1
	Beliana	0/1
Almond	Almond-19	1/1
(*Prunus amygdalus* L.)	Texas	0/1
	Cristomorto	0/1
	Tuono	0/1
	Ferrastar	0/1
	Genco	0/1
Peach	Nemaguard	0/2
(*Prunus persica* L. Batsch)	GF-305	0/2
Pear	Deveci[a]	2/4
(*Pyrus communis* L.)	Santa Maria	0/2

[a]Turkish cultivars.

Similarly, early reddening of pear leaves in autumn was correlated with the presence of *Ca.* Phytoplasma pyri.

*Rsa*I proved to be a *Ca.* Phytoplasma prunorum specific restriction enzyme (Lee *et al*., 1995) and its fragment profile was similar to that previously published (Lee *et al*., 1998). DNA from *Ca.* Phytoplasma pyri-infected pear digested with *Ssp*I (Lorenz *et al*., 1995) did not give any profile, thus differentiating pear isolates and reference *Ca.* Phytoplasma pyri from reference *Ca.* Phytoplasma mali isolates which, following digestions with *Ssp*I, yielded a banding pattern specific for *Ca.* Phytoplasma mali.

The high incidence of *Ca.* Phytoplasma prunorum in germplasm orchards (54.8%) can be taken as an indication that imported material is largely responsible for its introduction in the country. Among plum cultivars, only Japanese plums were infected by *Ca.* Phytoplasma prunorum and, in this group, only 'Black Diamond', 'Larry Ann' and 'Santa Rosa' were free from this pathogen. This is in line with the notion that Japanese plums (*Prunus salicina* L.) are susceptible to ESFY depending on the variety, whereas European plums (*Prunus domestica* L.) are symptomless carriers and therefore are regarded as tolerant to *Ca.* Phytoplasma prunorum (Desvignes and Cornaggia, 1982; Carraro *et al*., 1998b). In our investigation, the European plum 'Firenze-90' was apparently healthy. Since the samples were mainly collected from symptomatic trees, some of the infected but symptomless European plum trees might have escaped our inspection.

In this study, tests concentrated primarily on local cultivars from commercial orchards given that they are more widely grown. However, adaptation assays of foreign cultivars have recently been established in germplasm orchards to widen the range of available cultivars. Thus, the lower incidence of *Ca.* Phytoplasma prunorum in commercial orchards (3.2%) might be due to the still limited commercial use of foreign cultivars.

Ca. Phytoplasma pyri is transmitted by *C. pyri* and through propagation material (Carraro *et al*., 1998a; Jaraush *et al*., 2001). *C. pyri*, the major and best known vector of pear decline, has been heavily present for years in Bursa (Gencer and Kovanci, 2000, Kovanci *et al*., 2000). However, no special management practices were adopted by growers to prevent transmission of *Ca.* Phytoplasma pyri, except for treatments for reducing direct damage from insect feeding. In the present work, we have found that *C. pyri* can acquire this phytoplasma from infected trees, but its transmission from pear to pear by this psyllid is yet to be determined. On the other hand, the observation that there is a high degree of correlation between incidence of pear decline symptoms and high populations of *C. pyri,* suggests its possible implication in the dispersal of the *Ca.* Phytoplasma pyri.

Since there is no established certification program for fruit trees in Turkey, the propagation of the infected trees might cause increased infection in the future. Further investigations are therefore needed to determine the reaction of local and foreign cultivars of stone and pome fruit trees to phytoplasma infection, and the epidemiology of these pathogens. Studies addressing these problems and the molecular biology of *Ca.* Phytoplasma prunorum and *Ca.* Phytoplasma pyri are now underway.

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