Occurrence of potential vectors of phytoplasma in pear orchards with different plantation management

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Abstract

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During 2009–2011, regular captures of *Cacopsylla pyri, C. pyrisuga* and *C. pyricola* in four pear orchards (one conventional orchard, one integrated pest management (IPM) orchard, two organic orchards) were conducted in the Czech Republic. Polymerase chain reaction (PCR) tests were performed on psyllids captured from the orchards with the highest occurrence of these insects. The DNA extracted from pairs of psyllid individuals was analysed by nested PCR (R16F2n/R2-fU5/rU3) and restriction fragment length polymorphism method (RFLP) (*Rsa*I, *Bfm*I). Two *C. pyri* individuals captured on 11/05/2011 in the IPM orchard tested positive for *Ca*. P. pyri. *Ca*. P. pyri presence was confirmed in 8 out of the 9 *Cacopsylla* samples tested by subsequent nested PCR (P1/P7- f01/r01). In 2010 a higher amount of *C. pyri* was captured in the conventional orchard during June/July and in the IPM orchard during March, May, June and July. In 2010 and 2011 no or lower psyllids presence was detected in the organic orchard No. 1 and in the organic orchard No. 2. It is important to control pear decline by controlling the vector of the disease.

Keywords: *Cacopsylla pyri*; *Cacopsylla pyrisuga*; nested PCR; RFLP; *Candidatus* Phytoplasma pyri

Pear decline is an economically important plant disease caused by the phytoplasma *Candidatus* Phytoplasma pyri (Seemüller, Schneider 2004), which belongs to the subgroup 16SrX-C of the apple proliferation (AP) group of phytoplasmas. Pear decline was first reported in North America (McLarty 1948; Woodbridge et al. 1957). The disease had long been known as 'moria' in northern Italy (Refatti 1948). The first report of it in the Czech Republic was by Blattný and Váňa (1974). Pear decline phytoplasma is routinely detected by PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism) techniques in the Czech Republic (Navrátil et al. 2001; Fránová et al. 2008, 2011; Fránová 2011; Ludvíková et al. 2011) and worldwide (Salehi et al. 2008; Hunter et al. 2010; Etropolska et al. 2011). Nowadays real-time PCR (quantitative PCR) is successfully used for detection and quantification of pear decline phytoplasma (Nikolić et al. 2010; Lee, Lin 2011). *C. pyri, C. pyricola* and *C. pyrisuga* are psyllids of the *Cacopsylla* genus (Hemiptera, Psylloidea) considered as important vectors of the *Ca*. P. pyri phytoplasma (Carraro et al. 2001; Garcia-Chapa et al. 2005; Sanchez, Ortín-Angulo 2011). Nested PCR followed by RFLP are often used for investigation of phytoplasma presence in *Cacopsylla* species (Delić et al. 2008; Cieślińska, Morgaś 2011).

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The main aim of this study is the determination of phytoplasma in different psyllids captured in pear trees of orchards with different pest control management. Molecular PCR and RFLP methods were applied for determination of phytoplasma presence in captured *Cacopsylla* psyllids.

MATERIALS AND METHODS

Collection of psyllid species. The occurrence of potential psyllid vectors of *Ca*. P. pyri was examined in pear orchards in the Czech Republic. In the conventional orchard (7 years old) located in Holovousy, the cvs David, Amfora, Bohemica, Dicolor, Electra, Konference, Lucasova and Erika were grown. In the IPM orchard (10 years old) located in Dobrá Voda, the cvs Lucasova and David were grown. In the organic orchard No. 1 (10 years old) located in Bílsko u Hořic as well as in the organic orchard No. 2 (25 years old) located in Holovousy the cv. Beurre Hardy was grown. During 2009–2011, regular psyllids captures were conducted in one conventional orchard, two organic orchards and one IPM orchard. Firstly, captures of psyllid species on *Prunus spinosa* trees were performed in March, because the presence of *C. pruni* is informative for other psyllids presence (Navrátil, personal communication). Then, the *C. pyri, C. pyricola* and *C. pyrisuga* insects were captured in pear orchards by beating tray method, when the insects were shaken from trees onto a sheet and collected by aspirator. The captures were performed in two-week intervals between March and end of August (during the season of vegetation). Collected insects were sent for further species specification and numbering. Psyllids were first numbered and then specifically determined by microscope watching (screening) by Dr. P. Lauterer (Moravian Museum, Department of Entomology, Brno, Czech Republic). The determined insects were then stored at –20°C in absolute ethanol. The PCR analysis of phytoplasma presence in psyllids was performed only on the individuals collected from the orchards with higher abundance of psyllids when more than 30 psyllids was captured. In this case, higher infection pressure is confirmed.

DNA extraction. The DNA extraction was performed from a pair of psyllid individuals. The extraction procedure followed the protocol of the commercial kit (Wizard Genomic DNA Purification Kit, Promega, Madison, USA). The extracts of total DNA were diluted (1:10) with sterile distilled water.

PCR analysis. The extracted DNA from psyllid samples was examined with nested PCR using R16F2n/R2-fU5/rU3 primer pairs (Lorenz et al. 1995; Gundersen, Lee 1996). Samples showing positive signals were submitted to RFLP analysis

Table 1. Number of *Cacopsylla* species captured in 2009 in pear orchards with different pest management

Organic orchard 1							
	1.4.	16.4.	28.4.	12.5.	21.5.	5.6.	17.6.
C. pyri	2	1	–	6 n.g.	3 n.g.	2 n.g.	–
C. pyrisuga	1	19	14 n.g.	11	7	1 n.g.	7 n.g.
C. pyricola	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Organic orchard 2							
	1.4.	17.4.	28.4.	12.5.	21.5.	5.6.	17.6.
C. pyri	n.c.	n.c.	n.c.	1 n.g.	3 n.g.	1 n.g.	2 n.g.
C. pyrisuga	4	18	10	1	4 ow.g., 10 n.g.	3 n.g.	2 n.g.
C. pyricola	n.c.	1	1 n.g.	2 n.g.	1 ow.g., 16 n.g.	13 n.g.	7 n.g.
IPM orchard							
	31.3.	16.4.		12.5.	21.5.	5.6.	17.6.
C. pyri	35	1		2 ow.g., 34 n.g.	33 n.g.	3 n.g.	1 n.g.
C. pyrisuga	n.c.	2		n.c.	n.c.	n.c.	n.c.
C. pyricola	n.c.	n.c.		n.c.	n.c.	n.c.	n.c.

IPM – integrated pest management; n.c. – no captures; n.g. – new generation; ow.g. – overwintered generation; the psyllids captures from mid-July until September were not performed in 2009

Table 2. Number of *Cacopsylla* species captured in 2010 in pear orchards with different pest management

Conventional orchard											
	24.3.	7.4.	20.4.	5.5.	17.5.	4.6.		7.7.	19.7.	4.8.	19.8.
C. pyri	81	19	2	n.c.	n.c.	35		214	494	380	79
C. pyrisuga	1	30	2	n.c.	n.c.	n.c.		n.c.	n.c.	n.c.	2
C. pyricola	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.		n.c.	n.c.	n.c.	1
IPM orchard											
	25.3.	6.4.	20.4.	4.5.	21.5.	1.6.	23.6.	8.7.	19.7.	4.8.	20.8.
C. pyri	140	33	3	1	144	158	80	26	103	37	22
C. pyrisuga	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
C. pyricola	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Organic orchard 1											
	25.3.	6.4.	20.4.	4.5.	21.5.	4.6.	23.6.	8.7.	19.7.	20.8.	
C. pyri	1	3	1	n.c.	9	7	10	90	15	9	
C. pyrisuga	3	33	10	25	5	1	2	n.c.	n.c.	n.c.	
C. pyricola	n.c.	n.c.	n.c.	n.c.	n.c.	1	n.c.	n.c.	n.c.	n.c.	
Organic orchard 2											
	25.3.	6.4.	20.4.	4.5.	21.5.	1.6.	23.6.	8.7.	19.7.	20.8.	
C. pyri	2	n.c.	n.c.	n.c.	2	1	2	9	33	13	
C. pyrisuga	8	47	11	8	6	3	4	1	n.c.	n.c.	
C. pyricola	12	8	1	n.c.	n.c.	19	33	44	51	6	

n.c. – no captures; IPM – integrated pest management

using *Rsa*I and *Bfm*I for 16 h at 37°C. After RFLP analysis, the samples were analysed by electrophoresis on 1.5% agarose gels (80 V, 30 min) in 1× TAE buffer using SYBRGreen I (Lonza Rockland, Inc., Rockland, USA) for visualization under UV light. Identification of phytoplasma presence in *Cacopsylla* species was based on the RFLP of R16F2n/R2 PCR products. Moreover, nine psyllid samples that tested positive in previous PCR-nested analysis were then analysed by nested PCR using P1/P7- f01/r01 group specific primers (Deng, Hiruki 1991; Lorenz et al. 1995; Schneider et al. 1995) and sent for the sequencing analyses.

RESULTS AND DISCUSSION

In 2009**,** a low amount of psyllids (max. 36 of *C. pyri* individuals on 12/05/2009) was captured within all the pear orchards (Table 1). The psyllids captures from mid-July until end of August were not performed. In 2010, a significantly higher amount of *C. pyri* was captured in the conventional management orchard during June and July (214, 494 and 380 individuals) and in the IPM orchard during March (140 individuals), May/June (144 and 158 individuals) and July (262 and 103 individuals), (Table 2). In 2010, an absence of psyllids or lower number of psyllids was detected in the organic orchard 1 (max. of 90 individuals on 08/07/2010) and in the organic orchard 2 (max. 51 on 19/07/2010) (Table 2), similarly low psyllids captures were monitored in 2011 in organic orchard 1 and organic orchard 2 (max. of 62 *C. pyrisuga* individuals captured on 12/04/2011) (Table 3). It is interesting to compare and discuss differences in the size populations of the three psyllid species linked to the control management used. In 2009, interestingly higher amount of psyllids (especially of *C. pyri*) was detected in IPM orchard, as compared with the organic orchards Nos 1 and 2 (Table 1). Similar observation were reported during 2010 and 2011, when significantly higher number of psyllids (*C. pyri*) were detected in conventional and IPM orchard, in comparison to the organic orchard (Tables 2 and 3). This fact could be explained by organic protection system applied in organic orchards, which supports the growth and existence of natu-

Table 3. Number of *Cacopsylla* species captured in 2011 in pear orchards with different pest management

Conventional orchard							
	24.3.	12.4.	11.5.	6.6.	28.6.	27.7.	30.8.
C. pyri	11	3	19	9	95	133	7
C. pyrisuga	n.c.	5	n.c.	n.c.	1	n.c.	n.c.
C. pyricola	1	7	n.c.	n.c.	n.c.	n.c.	n.c.
IPM orchard							
	24.3.	12.4.	11.5.	6.6.	28.6.	27.7.	30.8.
C. pyri	108	16	79	122	785	372	13
C. pyrisuga	3	8	n.c.	n.c.	n.c.	n.c.	n.c.
C. pyricola	n.c.	n.c.	n.c.	n.c.	n.c.	1	n.c.
Organic orchard 1							
	24.3.	12.4.	11.5.	6.6.	28.6.	27.7.	30.8.
C. pyri	1	n.c.	4	1	1	4	1
C. pyrisuga	n.c.	62	20	n.c.	2	n.c.	n.c.
C. pyricola	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Organic orchard 2							
	24.3.	12.4.	11.5.	6.6.	28.6.	27.7.	30.8.
C. pyri	n.c.	n.c.	2	1	2	n.c.	n.c.
C. pyrisuga	2	16	14	n.c.	n.c.	n.c.	n.c.
C. pyricola	2	n.c.	1	23	16	9	1

IPM – integrated pest management; n.c. – no captures

ral enemies of psyllids. In case of the conventional and IPM orchards the psyllids population as well as their natural enemies are destroyed, moreover the psyllids resistance to prophylactics is reported (Buès et al. 2000, 2003; Kocourek, Stará 2006).

Nested PCR using the phytoplasma-universal primer pairs R16F2n/R2-fU5/rU3 with following RFLP analysis using *Rsa*I and *Bfm*I restriction enzymes were conducted on psyllid samples (Table 4). Two *C. pyri* individuals captured on 11/05/2011 in the IPM orchard tested positive for *Ca*. P. pyri (Table 4). In the following analysis the total of 9 psyllid samples (7 samples of *C. pyri* from the conventional orchard, one sample of *C. pyri* from the IPM orchard, one sample of *C. pyrisuga* from an organic orchard) that were phytoplasma positive in previous PCR-nested analysis, were re-analysed by nested PCR using P1/P7-f01/r01 group specific primers (Deng, Hiruki 1991; Lorenz et al. 1995; Schneider et al. 1995) for specific detection of apple proliferation group or 16Sr-X ribosomal group. Nested PCR products, obtained by P1/P7-f01/r01 group specific primers, were sent for sequencing and the *Ca*. P. pyri presence was confirmed in 8 out of the 9 totally tested samples (Fig. 1). The total number of psyllids tested and the positives obtained over total tested for each of the *Cacopsylla* species is presented (Table 5). Successful detection of pear decline phytoplasma in *Cacopsylla* species was confirmed in many studies. Lethmayer et al. (2011) showed few individuals of *C. pyricola*, *C. pyri* and *C. pyrisuga* as carriers of *Ca.* P. pyri. According to Jenser et al. (2009) about 40% of the overwintered *C. pyri* adults are able to harbour the phytoplasma and the application of effective insecticides before budding is particularly important. Sertkaya et al. (2008) indicated that *C. pyri* could transmit pear decline (PD) disease in the region of Turkey. Delić et al. (2008) detected the *Ca*. P. pyri in 2 groups out of 9 of *C. pyri* in Bosnia and Herzegovina. In Hungary, Süle et al. (2007) proved that it was possible to control pear decline by using oil and Vertimec chemicals, which killed *C. pyri* psyllids very efficiently. Serçe et al. (2006) detected *Ca*. P. pyri in *C. pyri* individuals in Turkey. The transmission of *Ca*. P. pyri phytoplasma by *C. pyri* was demonstrated by Lethmayer et al. (2011), moreover the transmission by this vector was also demonstrated

Table 4. Results of PCR/RFLP analysis of PD phytoplasma in captures of *C. pyri*, *C. pyrisuga* and *C. pyricola* conducted in four pear orchards with different pest management regime between 2009–2011 (tested samples/PCR nested positive individuals/RFLP positive individuals)

	2009			2010						2011				
IPM orchard														
	2.7.	14.8.	9.10.	25.3.	21.5.	1.6.	23.6.	8.7.	30.7.	24.3.	11.5.	6.6.	28.6.	27.7.
Cacopsylla pyri	48/0/0	24/0/0	24/0/0	70/1/0	74/2/0	45/0/0	12/0/0	12/2/0	35/0/0	12/5/0	12/3/2	12/7/0	36/2/0	36/15/0
Cacopsylla pyrisuga	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Cacopsylla pyricola	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Conventional orchard														
					24.3.	7.7.	19.7.	4.8.				28.6.	27.7.	
Cacopsylla pyri		n.t.			20/1/0	6/0/0	24/0/0	95/6/0				12/3/0	12/7/0	
Cacopsylla pyrisuga		n.t.			n.t.	n.t.	n.t.	n.t.				n.t.	n.t.	
Cacopsylla pyricola		n.t.			n.t.	n.t.	17/1/0	n.t.				n.t.	n.t.	
Organic orchard 1														
						8.7.						12.4.		
Cacopsylla pyri		n.t.				12/0/0						n.t.		
Cacopsylla pyrisuga		n.t.				n.t.						12/2/0		
Cacopsylla pyricola		n.t.				n.t.						n.t.		
Organic orchard 2														
						6.4.	19.7.					6.6.		
Cacopsylla pyri		n.t.				n.t.	n.t.					n.t.		
Cacopsylla pyrisuga		n.t.				12/1/0	n.t.					n.t.		
Cacopsylla pyricola		n.t.				n.t.	17/1/0					6/2/0		

one sample consisted of DNA extracted from 2 individuals; n.t. – not tested samples; PCR/RFLP – polymerase chain reaction/restrictkon fragment length polymorphism; PD – pear decline

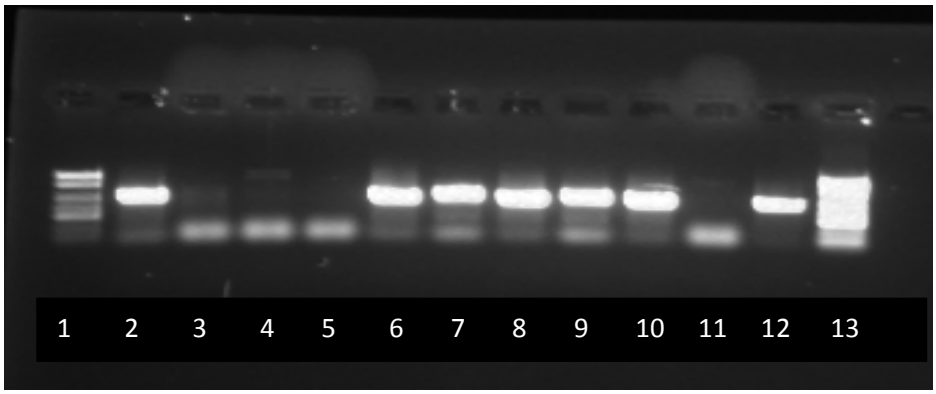

Fig. 1. Agarose gel electrophoresis of polymerase of PCR-nested obtained products (1,050 bp) with P1/P7-f01/r01 primer pairs (Deng, Hiruki 1991; Lorenz et al. 1995; Schneider et al. 1995) of 9 *Cacopsylla* samples; lane 1: Fast Runner DNA Ladder 50–2,000 bp, lane 2: pear decline (PD) positive *C. pyri* collected from conventional orchard; lane 3: PD positive *C. pyri* collected from IPM orchard (confirmed by sequence analysis); lane 4: PD positive *C. pyri* collected from conventional orchard (confirmed by sequence analysis); lane 5: PD negative *Cacopsylla pyri* collected from conventional orchard; lanes 6–10: PD positive *C. pyri* collected from conventional orchard; lane 11: negative control; lane 12: positive control; lane 13: Fast Runner DNA Ladder 50–2,000 bp

Table 5. Positive psyllid samples obtained over total tested for each of the *Cacopsylla* species

	Total No. of tested psyllids	No. of *Ca*. P. pyri positive psyllids	Rate of positive psyllids (%)
Cacopsylla pyri	866	8	0.92
Cacopsylla pyrisuga	24	0	0
Cacopsylla pyricola	40	0	0

in Spain by Garcia-Chapa et al. (2005). Actually the real-time with specific primers for pear decline detection (Nikolić et al. 2010) is more suitable than the nested PCR-RFLP approach. However, in our study, the presence of *Ca*. P. pyri was confirmed by sequencing of nested PCR products, obtained by P1/P7-f01/r01 group specific primers.

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