

Occurrence and detection of lesser known viruses and phytoplasmas in stone fruit orchards in Poland

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ABSTRACT

A survey was carried out on 38 commercial and experimental stone fruit orchards located in major growing areas of stone fruit trees in Poland to determine the incidence of lesser known viruses and phytoplasmas. Leaf samples from 145 sweet cherry and 102 sour cherry trees were tested for *Little cherry virus 1* (LChV-1), *Little cherry virus 2* (LChV-2), *Cherry green ring mottle virus* (CGRMV), *Cherry mottle leaf virus* (CMLV), and *Cherry necrotic rusty mottle virus* (CNRMV) using RT-PCR. Sixty samples collected from peach and 20 apricot trees were also tested for CGRMV. Eleven out of 145 sweet cherry and three out of 102 sour cherry trees were infected by LChV-1. CGRMV was detected in 10 sweet cherry, four sour cherry, 14 peach and two apricot trees. No LChV-2, CMLV and CNRMV were detected in any of the tested trees. Phloem tissue from samples of shoots collected from 145 sweet cherry, 102 sour cherry, 128 peach, 37 apricot, five nectarine and 20 European as well as Japanese plum trees were tested for phytoplasmas. The nested PCR of the extracted DNA with universal and specific primer pairs showed the presence of phytoplasmas in six sweet cherry, three sour cherry, nine peach, four apricot, one nectarine and three Japanese plum trees. The RFLP patterns of 16S rDNA fragments after digestion with *RsaI*, *MseI*, *AluI*, and *SspI* endonucleases indicated that selected stone fruit trees were infected by two distinct phytoplasmas belonging to the apple proliferation group. The stone fruit trees infected by LChV-1, CGRMV and phytoplasmas were grown in orchards localised in all seven regions.

Key words: CGRMV, CMLV, CNRMV, LChV-1, LChV-2, PCR/RFLP, phytoplasmas

Abbreviations:

RT-PCR – reverse transcription-polymerase chain reaction; RFLP – restriction fragment length polymorphism; PNRSV – *Prunus necrotic ring spot virus*; PDV – *Prune dwarf virus*; LChD – Little cherry disease; LChV – *Little cherry virus*; CGRMV – *Cherry green ring mottle virus*; CMLV – *Cherry mottle leaf virus*; CNRMV – *Cherry necrotic rusty mottle virus*; ‘*Ca. P. prunorum*’ – ‘*Candidatus Phytoplasma prunorum*’; ‘*Ca. P. mali*’ – ‘*Candidatus Phytoplasma mali*’; ‘*Ca. P. pyri*’ – ‘*Candidatus Phytoplasma pyri*’; DNA – deoxyribonucleic acid; cDNA – complementary deoxyribonucleic acid; AP – apple proliferation; rRNA – ribosomal ribonucleic acid

INTRODUCTION

Stone fruit trees are the hosts of more than 30 viruses and virus-like pathogens. Among them, *Prunus necrotic ring spot virus* (PNRSV) and *Prune dwarf virus* (PDV) are the most common worldwide and also widely present in Polish orchards. One of the

lesser known pathogens in Poland are *Little cherry virus 1*, *2* or *3* (LChV-1, LChV-2 or LChV-3) causing Little cherry disease (LChD). Sweet cherry fruits of some cultivars affected by LChV are small, pointed, imperfectly coloured, and tasteless, and leaves show a red-brown colouration of the surface (Welsh and Cheney 1976). *Cherry green ring*

mottle virus (CGRMV) infects sweet cherry, sour cherry, peach, apricot and other *Prunus* species, causing yellowing and dark mottling of the sour cherry trees leaves, whereas sweet cherry trees are latently infected (Desvignes 1999). *Cherry mottle leaf virus* (CMLV) induces irregular and chlorotic mottling, leaf distortion and puckering, reduced yield, and smaller and tasteless fruits (James and Mukerji 1993). Trees infected with *Cherry necrotic rusty mottle virus* (CNRMV) show brown, angular necrotic spots, shot holes on the leaves, gum blisters and necrosis of the bark (Wadley and Nyland 1976).

Several phytoplasmas may infect stone fruit trees, but the most common in Europe is '*Candidatus Phytoplasma prunorum*' ('*Ca. P. prunorum*'), associated with European stone fruit yellows disease. This phytoplasma is closely related to '*Candidatus Phytoplasma mali*' ('*Ca. P. mali*') and '*Candidatus Phytoplasma pyri*' ('*Ca. P. pyri*'), which cause apple proliferation and pear decline diseases, respectively (Seemüller and Schneider 2004).

The aim of this study was to determine the incidence of phytoplasmas and lesser known viruses in stone fruit trees grown in commercial and experimental orchards in several regions of Poland as well as the detection of cDNA fragments of the viruses using RT-PCR and the identification of 16S rRNA gene fragments of the phytoplasmas using the PCR/RFLP method.

MATERIAL AND METHODS

Surveys were conducted in June and July 2007-2009 in 38 commercial and experimental stone fruit orchards from seven geographical locations within Poland (Poznan, Wroclaw, Warsaw, Lodz, Lublin, Kielce, and Zielona Gora regions). The orchards' area ranged from 0.5 to 3 ha and the age of the trees ranged from 6 to 15 years. To test the trees for virus presence, leaf samples were collected separately from 4-22 symptomatic trees from each orchard dependent on its area. There were 145 sweet cherry and 102 sour cherry trees tested for *Cherry green ring mottle virus* (CGRMV) and *Cherry necrotic rusty mottle virus* (CNRMV), both unassigned species in the *Flexiviridae* family, *Little cherry virus 1* (LChV-1), an unassigned species in the *Closteroviridae* family, *Little cherry virus 2* (LChV-2) classified as *Ampelovirus* in the *Closteroviridae* family, and *Cherry mottle leaf virus* (CMLV), a *Trichovirus* species in the *Flexiviridae* family (Fauquet et al. 2005). Sixty peach and 20 apricot trees showing disease symptoms were also tested for CGRMV. Samples of shoots from 145 sweet

cherry, 102 sour cherry, 128 peach, 37 apricot, five nectarine and 20 European as well as Japanese plum trees were collected to be tested for phytoplasmas (Table 1).

Nucleic acids extracted from leaves using a RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) were subjected to a reverse transcription-polymerase chain reaction (RT-PCR). Primer pairs GRMV7956/GRMV8316 (Rott and Jelkmann 2001 a), LCV1U16390/LCV1L16808 (Rott and Jelkmann 2001 b), LCVUP2/LCVLO2 (Rott and Jelkmann 2001 b), CML13A/CML4A (James et al. 1999) and NRM48U/NRM48L (Rott and Jelkmann 2001 a) were used for the amplification of CGRMV, LChV-1, LChV-2, CMLV, and CNRMV genome fragments, respectively.

Nucleic acids extracted from phloem tissue using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) were subjected to PCR with universal primers P1/P7 (Deng and Hiruki 1991, Schneider et al. 1995). The products of P1/P7 amplifications were diluted 1:40 with sterile, deionized water and used as templates in the nested PCR with the universal primers R16F2n/R16R2 (Gundersen and Lee 1996), as well as primers specific for the 16SrI, 16SrIII, 16SrV, and 16SrX groups (Kirkpatrick et al. 1994, Lee et al. 1994, 1995). For the analysis of the amplification products, 8 l of the post nested PCR mixtures were evaluated by electrophoresis in a 1.5% agarose gel. The DNA was visualised under UV light after staining with ethidium bromide. The molecular weight of the PCR products was estimated by comparison with 100 bp DNA ladder (Fermentas, Vilnius, Lithuania).

Products of the nested PCR primed by R16F2n/R16R2 from 10 tree samples were subjected to restriction fragment length polymorphism analyses (RFLP) using *RsaI*, *MseI*, *AluI*, and *SspI* endonucleases (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. DNA of phytoplasmas belonging to the apple proliferation group 16SrX subgroups A, B, and C were applied as positive controls. The generated restriction patterns were analysed by electrophoresis in 8% polyacrylamide gels. The resulting RFLP patterns were compared with profiles for positive controls and those obtained for the reference strains (Lee et al. 1998, Seemüller and Schneider 2004).

RESULTS AND DISCUSSION

Only two sweet cherry trees showed reddish and brown discoloration of the leaves and small, pale, pointed fruits characteristic for little cherry disease

(Welsh and Cheney 1976). Symptoms of other virus diseases were not observed. Some of the surveyed trees showed chlorotic leaf roll, reduction of growth and dieback. Similar symptoms were described as characteristic for phytoplasma diseases (Lederer and Seemüller 1992). Previous studies showed the presence of LChV-1, LChV-2, CGRMV and phytoplasmas in Poland mostly in trees grown in experimental orchards or germplasm collections (Cieślińska et al. 2004, Komorowska and Cieślińska 2004, 2005, 2008).

Eleven out of 145 sweet cherry and three out of 102 sour cherry trees were infected by LChV-1 (Tab. 1, Fig. 1). CGRMV was detected in 10 sweet cherry, four sour cherry, 14 peach and two apricot trees assayed during this study (Fig. 2). LChV-2, CMLV or CNRMV was not detected in any of the tested trees. A high incidence of lesser known viruses was reported in other countries. In the Mediterranean, reports occasionally included CGRMV (Zhang et al. 2000, Gentit et al. 2002) and CNRMV (Gentit et al. 2002) but a relatively high incidence of both viruses was noted in Serbia (Mandic et al. 2007). In Japan, LChV-1, LChV-2, CGRMV and CNRMV were detected depending on the virus in 14-92% of tested sweet cherry trees grown in several provinces (Isogai et al. 2004). LChV-1, CGRMV and CNRMV were also detected in California, USA (Sabanadzovic et al. 2005).

The results of nested PCR with R16F2n/R16R2 universal primers indicated that a total of 26 out of the 437 tested stone fruit trees were infected with

phytoplasmas (mean infection rate 5.9%), among them: six sweet cherry, three sour cherry, nine peach, four apricot, one nectarine and three Japanese plum trees. No PCR product was obtained with DNA from a healthy tree. The result of nested PCR with R16(X)F1/R1 primers indicated that positive tested trees were infected by phytoplasmas belonging to the apple proliferation (AP) group (16SrX). No positive results were obtained in reaction with primer pairs specific for any of the 16SrI, 16SrIII and 16SrV phytoplasma groups.

RFLP results confirmed the infection of sweet cherry (3), sour cherry (1), peach (3), apricot (1) and plum (1) trees by phytoplasmas classified to the apple proliferation group (Fig. 3). The digestion profiles of phytoplasma 16S rRNA gene fragments from nine tested trees were indistinguishable from restriction patterns for the reference strain of '*Candidatus* Phytoplasma prunorum' classified to the subgroup 16SrX-B (Lee et al. 1998, Seemüller and Schneider 2004). During our study, '*Ca. P. prunorum*' was detected for the first time in naturally infected stone fruit trees in Poland.

Favourable climate conditions for the spreading of phytoplasmas and the possibility of the occurrence of its vector may lead to an increased epidemiological risk of '*Ca. P. prunorum*' in Poland. European stone fruit yellows caused by '*Candidatus* Phytoplasma prunorum' has been reported in many countries but its incidence in apricot, peach and plum orchards is relatively high in southern Europe. This phytoplasma was detected in sweet cherry, peach,

Table 1. Relative incidence of LChV-1, CGRMV and phytoplasmas detected in stone fruit trees in seven regions of Poland based on RT-PCR and PCR results

Pathogen/ Species	Surveyed orchards/Positive samples/Tested samples (No.)							
	Region							Total
	Poznan	Wroclaw	Warsaw	Lodz	Lublin	Kielce	Z. Gora	
LChV-1								
Sweet cherry	1/1/12	2/1/23	1/1/22	2/2/23	2/3/22	2/3/23	2/0/20	12/11/145
Sour cherry	2/2/16	2/0/17	2/0/16	2/0/16	2/0/17	2/1/12	1/0/8	13/3/102
CGRMV								
Sweet cherry	1/2/12	2/0/23	1/0/22	2/3/23	2/3/22	2/0/23	2/2/20	12/10/145
Sour cherry	2/1/16	2/0/17	2/1/16	2/1/16	2/0/17	2/1/12	1/0/8	13/4/102
Peach	1/2/8	2/3/18	NT*	NT	2/5/20	2/4/14	NT	7/14/60
Apricot	NT	1/1/4	1/0/4	1/0/4	1/1/5	1/0/3	NT	5/2/20
Phytoplasma								
Sweet cherry	1/0/12	2/2/23	1/0/22	2/3/23	2/1/22	2/0/23	2/0/20	12/6/145
Sour cherry	2/2/16	2/0/17	2/0/16	2/1/16	2/0/17	2/0/12	1/0/8	13/3/102
Peach	2/1/14	3/2/26	1/1/8	1/1/11	3/2/29	3/1/28	1/1/12	14/9/128
Apricot	1/1/4	1/0/4	1/0/4	1/0/4	2/1/9	3/2/12	NT	9/4/37
Plum	1/0/5	NT	NT	1/2/8	NT	NT	1/1/7	3/3/20
Nectarine	1/0/1	NT	NT	1/0/1	NT	1/1/3	NT	3/1/5

*NT – not tested

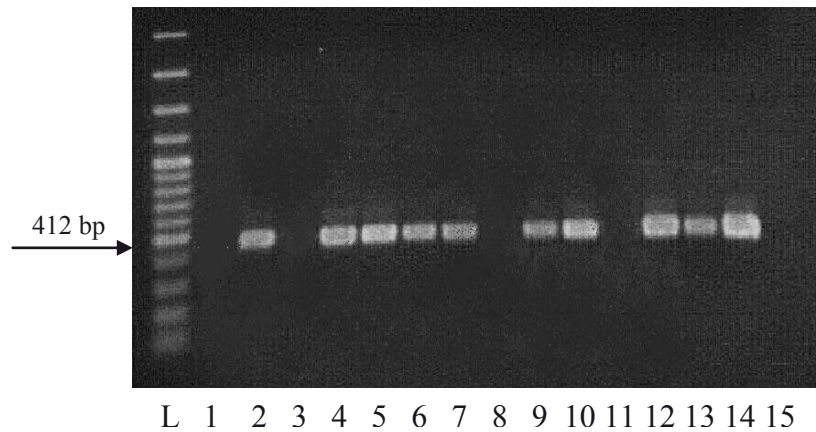


Figure 1. Agarose gel electrophoresis of RT-PCR products obtained with primers LCV1U16390/LCV1L16808 specific for LChV-1. L – 100 bp DNA ladder; 1 – sample of healthy cherry; 2 – positive control; 3, 5, 7, 9, 10, 12, 13, 14 – sweet cherry trees; 4, 6, 8, 11, 15 – sour cherry trees

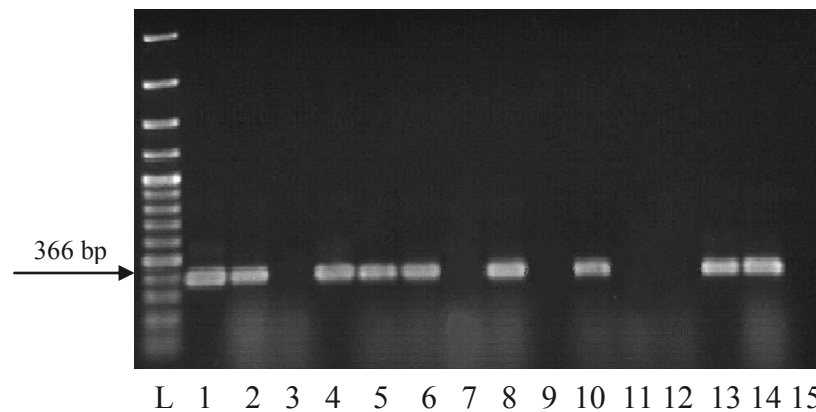


Figure 2. Agarose gel electrophoresis of RT-PCR products obtained with primers GRMV7956/GRMV8316 specific for CGRMV. L – 100 bp DNA ladder; 1, 2, 6, 11, 13 – sweet cherry; 3, 4, 5, 12 – peach; 7, 8 – sour cherry; 9, 10 – apricot; 14 – positive control; 15 – sample of healthy cherry

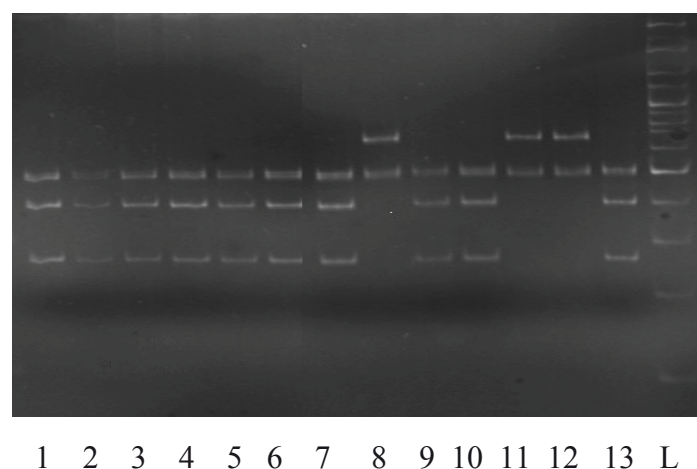


Figure 3. RFLP profiles with *RsaI* enzyme after digestion of 1.2 kbp fragments of 16S rDNA amplified by PCR with R16F2n/R16R2 primers from phytoplasmas infecting stone fruit trees. L – 100 bp DNA ladder, 1, 2, 4 – peach; 3 – sour cherry; 6, 10 – sweet cherry; 7 – Japanese plum; 8 – sweet cherry 'Kordia I/8'; 9 – apricot; 11 – 16SrX-A reference strain; 12 – 16SrX-C reference strain; 13 – 16SrX-B reference strain

apricot and Japanese plum in Italy (Paltrinieri et al. 2001, Poggi Pollini et al. 2001), Spain (Laviña et al. 2004) and the Czech Republic (Navratil et al. 2001, Fialova et al. 2004), in apricot, peach, plum and amygdalus in Germany (Lederer and Seemüller 1992, Jarausch et al. 2008), in apricot and Japanese plum in Albania (Myrta et al. 2003) and in southern England (Davies and Adams 2000), as well as in apricot in France (Morvan et al. 1988) and Austria (Laimer da Camara Machado et al. 2001). The incidence of '*Candidatus Phytoplasma prunorum*' depends on the region and species of the tested tree. Its relatively high incidence was noted in apricot (about 80% of infected trees) in some regions of Germany (Jarausch et al. 2008) and in plum (25-78% of infected trees) grown in several orchards in the Catalonia region of Spain (Laviña et al. 2004).

In our study, the profile for phytoplasma from 'Kordia I/8' sweet cherry from one of the orchards localised in the Lodz region was indistinguishable from the restriction pattern for '*Candidatus Phytoplasma pyri*' (pear decline phytoplasma, subgroup 16SrX-C) after using the *RsaI* enzyme. It is the first evidence of the occurrence of this phytoplasma in Poland. Our findings support a postulation that pear decline and apple proliferation phytoplasmas do not seem to be limited only to pome fruit trees. Nested PCR/RFLP analyses showed the presence of '*Candidatus Phytoplasma pyri*' in sweet cherry in Italy (Paltrinieri et al. 2001) and peach in the Czech Republic (Navratil et al. 2001, Fialova et al. 2004). In turn, RFLP profiles corresponding to the '*Candidatus Phytoplasma mali*' reference strain were found in Japanese plum (Lee et al. 1995) and in peach (Paltrinieri et al. 2001) in Italy as well as in sweet cherry in the Czech Republic (Navratil et al. 2001). It is known that stone fruit species can be affected also by phytoplasmas belonging to other groups.

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WYSTĘPOWANIE I WYKRYWANIE WYBRANYCH MNIEJ ZNANYCH WIRUSÓW I FITOPLAZM W SADACH DRZEW PESTKOWYCH W POLSCE

Streszczenie: W 38 sadach drzew pestkowych zlokalizowanych w siedmiu różnych rejonach kraju dokonywano lustracji pod kątem występowania chorób wywoływanych przez mało znane w Polsce wirusy i fitoplazmy. Pobrano liście ze 145 drzew czereśni i 102 drzew wiśni do badań na obecność wirusów drobnienia owoców czereśni (LChV-1 i LChV-2), wirusa zielonej pierścieniowej pstrości czereśni (CGRMV), wirusa cętkowanej plamistości liści czereśni (CMLV) i wirusa nekrotyczno-rdzawej plamistości czereśni (CNRMV) przy użyciu metody RT-PCR. Ponadto, testom na CGRMV poddano również 60 drzew brzoskwini i 20 drzew moreli. LChV-1 wykryto w jedenastu drzewach czereśni i trzech drzewach wiśni, zaś CGRMV w dziesięciu drzewach czereśni, czterech wiśni, czternastu brzoskwini i dwóch moreli. W żadnym z badanych drzew nie stwierdzono obecności LChV-2, CMLV i CNRMV. Stosując PCR z użyciem dwóch par starterów

uniwersalnych oraz starterów specyficznych na kilka grup fitoplazm, przeprowadzono testy na obecność fitoplazm w 145 drzewach czereśni, 102 drzewach wiśni, 128 drzewach brzoskwini, 37 drzewach moreli, 20 drzewach śliwy i pięciu drzewach nektaryny. Wykazano, że spośród wszystkich badanych, drzewa sześciu czereśni, trzech wiśni, 9 brzoskwini, czterech moreli, trzy drzewa japońskiej śliwy i jedno drzewo

nektaryny były porażone przez fitoplazmy. Analiza polimorfizmu długości fragmentów restrykcyjnych (RFLP) po trawieniu enzymami *RsaI*, *MseI*, *AluI*, *SspI* fragmentów 16S rDNA fitoplazm wykazała, że wybrane do badań drzewa były porażone przez dwie różne fitoplazmy zaliczane do grupy fitoplazmy proliferacji jabłoni.

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