The occurrence of *Ilarviruses* in Latvian fruit orchards

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Abstract

In order to study the occurrence of ilarviruses in fruit orchards in Latvia the samples from apple, pear and *Prunus* trees from commercial orchards and varietal collections were collected during spring 2007 and 2008. Polyclonal antibodies were used for DAS ELISA test for large-scale screening. In Total 890 samples from apple, 252 samples from pear and 655 samples from *Prunus* spp. were tested for the occurrence of fruit tree ilarviruses – ApMV, PDV and PNRSV. The screening results showed that all tested ilarviruses were present in the fruit orchards. ApMV was detected in 2.1% of the tested apple samples and in 1.8% of the plum samples, but it was not detected in pears. In *Prunus* spp. PNRSV was detected in 13.6% and PDV in 11.6% of samples. Mixed infections of ilarviruses were detected in 4% of the tested *Prunus* spp. samples of which 2% was PNRSV in combination with PDV. The plant samples from apple and pear trees were tested for ApMV infection also by RT-PCR and compared with data obtained by DAS ELISA. The RT-PCR results showed that 22% and 20.2% of tested apple and pear trees are infected with ApMV, respectively. The occurrence of ilarviruses in the tested plants varied greatly among the cultivars. The commonly grown cultivars such as 'Ausma', 'Rubin', 'Kursa', 'Bere Kievskaya', 'Perdrigon', 'Mirabelle de Nancy' and 'Skoroplodnaya' were highly infected. Since a certification system for planting material is not established in the country, there is very high risk for continuous spread of these viruses in the orchards. Similarly, as showed for ApMV, also PNRSV and PDV possibly are more widely spread than detected with preliminary screening by DAS ELISA. The study should be continued and other test methods, such as RT-PCR, used. The obtained data indicate a great need for the establishment of a certification system for fruit tree propagation.

Keywords: ApMV, apple, ELISA, PDV, pear, PNRSV, *Prunus*, RT-PCR

Introduction

Apples, pears and plums are important horticultural crops in Latvia, occupying around 85% of commercial fruit orchard area. Stone and pome fruit trees are affected by a large number of viruses, which cause significant economic losses (Saade et al., 2000). *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple mosaic virus* (ApMV) are worldwide pathogens of stone and pome fruit trees and they often occur in mixed infections (Vaškova et al., 2001). Symptoms of infection may not be visible or can appear as leaf chlorosis and reduction in growth (Scott et al., 2001). PDV and PNRSV may also cause necrotic spots and shot-holes in some *Prunus* species. On plums, PDV causes rosetting of the internodes, stunting and malformation of leaves (Ogawa et al., 1995). PNRSV on plums induces chlorotic rings or line patterns on the foliage, which later become necrotic and drop off, developing the shot-hole effect (Diekmann & Putter, 1996). ApMV is named after the disease it causes in apple, the first host in which it was described, although at least 65 other plant species are susceptible hosts of ApMV, including pears and plums (Petrzik & Lenz, 2002). ApMV induces pale yellow to cream-colored areas on leaves of infected apple trees and line patterns or bands along major veins on plums, but pears are mostly symptomless (Jones et al., 1990; Petrzik, 2005). All three viruses are transmitted by graft, pollen and seed, except ApMV for which only graft transmission is known. They also have similar biological properties that contribute to their wide distribution (Vaškova et al., 2000).

The use of healthy plant material is a requirement to prevent virus spread in woody crops. In this context certification schemes worldwide are being established with the objective of identifying healthy sources for propagation (Massart et al., 2008). The certification program for planting material has not been established in Latvia yet. Therefore the risk that viruses have spread uncontrolled in fruit orchards with infected planting material and by natural transmission is very high. Previous studies on occurrence of viruses in pome fruits in Latvia were carried out in 1980s and were based only on visual observations and biological indexing. The occurrence of viruses in stone fruits has not been studied before in Latvia. During the last decade new commercial orchards have been widely planted, and the assortment of cultivars and rootstocks have changed greatly. Nowadays data are not available about the spread of viral diseases in fruit orchards, including such important pathogens as ApMV, PNRSV and PDV. The aim of this research was to determine the occurrence of ApMV, PNRSV and PDV in stone and pome fruit orchards in Latvia.

Materials and methods

In total 50 apple, 36 pear and 28 plum commercial orchards and varietal collections were surveyed during spring 2007 and 2008. In total 890 samples from different apple (*Malus domestica* Borkh.), 252 samples from different European pear (*Pyrus communis* L.) and 655 samples from several European plum (*Prunus domestica* L.), cherry plum (*Prunus cerasifera* Ehrh.), *Prunus* hybrids and other *Prunus* spp. genotypes were collected. Leaves were sampled randomly from symptomless and symptomatic trees from all cultivars, which were present in the surveyed orchard. Ten fully expanded leaves were collected around the canopy of each individual tree from the middle of each scaffold branch, according to EPPO standards (EPPO, 2004). The samples were transported to the laboratory in an ice bag, immediately used for analyses or frozen in liquid nitrogen and stored at -80°C.

For the large-scale screening and detection of ApMV, PDV and PNRSV in plant material, commercially available double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) kit (Bioreba AG, Switzerland) was used according to the manufacturer's instructions with some modifications. The coating and conjugate conditions were changed from the manufacturer's standard procedure of a 4 h incubation at 30°C to an overnight incubation in the refrigerator at 4 – 6 °C. The absorbance was read at 405/492 nm with dual filter microplate reader Asys Expert 96 (Hitech, Austria) after 30 min, 1 h and 2 h of incubation. A "cut-off" value was calculated according to the manufacturer's technical information (Bioreba AG, Switzerland).

The samples from apple and pear trees were tested for ApMV infection with reverse transcription polymerase chain reaction (RT-PCR). The same leaf samples were used for RT-PCR and DAS ELISA. For total RNA isolation frozen leaf tissues were ground into a fine powder in liquid nitrogen. The extraction of RNA was carried out with the RNeasy Plant Mini kit (Qiagen AG, Germany) following the manufacturer's recommendations. Lysis buffer RLT was used for apple and RLC buffer for pear leaf tissues. The quantity and quality of the RNA was measured using a spectrophotometer NanoDropR ND-1000 (Thermo Scientific, USA). RNA was stored at -20°C and for long-term storage at -80°C.

RT-PCR assays were carried out with OneStep RT-PCR kit (Qiagen AG, Germany) following the manufacturer instructions. ApMV coat protein specific primers and *nad5* primers as an internal control were used (Hassan et al., 2006; Menzel et al., 2002) at concentrations 0.4 µM for each ApMV and 0.25 µM for each *nad5* primer. The primers' sequences are shown in Table 1. RT-PCR was carried out in a thermocycler Mastercycler® ep (Eppendorf AG, Germany) at the following cycling conditions: reverse transcription step 30 min at 50ºC, activation of the hotstart Taq polymerase at 95ºC for 15 min, followed by 40 cycles of: 30 s at 94ºC, 45 s at 55ºC, 2 min at 72ºC and a final extension step at 72ºC for 10 min (Hassan *et al*., 2006). PCR products were separated by electrophoresis in 2% agarose gels in TAE buffer, stained with ethidium bromide, and visualized under UV light. The occurrence of viruses was calculated as a percentage of positive samples from totally tested samples.

Tab. 1 Primer sequences for detection of ApMV and internal control

Primer	Primer sequences	Product size	References
ApMV	F 5' CGTAGAGGAGGACAGCTTGG3'	450 bp	Hassan et al., 2006
	R 5' CCGGTGGTAACTCACTCGTT 3'		
nad5	F 5' GATGCTTCTTGGGGCTTCTTGTT 3'	181 bp	Menzel et al., 2002
	R 5' CTCCAGTCACCAACATTGGCATAA 3'		

Results

The occurrence of ApMV in apple and pear orchards: In the orchard surveys no obvious symptoms were observed on fruit trees except some leaf mosaic on apple trees. With the DAS ELISA test 2.1% of samples from apple trees were positive for ApMV, but all samples from pears were negative. Duplex RT-PCR resulted in the amplification of a viral DNA fragment of 450 bp in length and and internal control of 181 bp (Figure 1). RT-PCR results showed high rates of ApMV infection of pome fruit trees while DAS ELISA gave fewer positive results (Table 2). Among orchards the occurrence of ApMV varied from 0% to 75%. Among different apple and pear cultivars a high variability in the occurrence of the virus was observed. Almost all commonly grown cultivars were infected with ApMV. Among those, the Latvian apple cultivar 'Ausma' and the cultivar 'Rubin' (originating from Kazakhstan) were highly infected;62.5 % and 77.1 % of plant samples, respectively. On these two cultivars obvious symptoms of ApMV, such as, pale yellow to cream-colored areas on leaves, were also observed in the orchards. In other apple cultivars the number of infected samples with ApMV ranged from 0% to 38.5 %. Pear cultivars 'Kursa' and 'Bere Kievskaya' had the highest number of infected trees; 40 % and 50 % of plant samples, respectively. In other pear cultivars the occurrence of ApMV ranged

from 0% to 33.3%. All tested samples from the common apple cultivars ‘Orlik’ and ‘Alro’ and common pear cultivars ‘Clapp’s Favourite’, ‘Conference’ and ’Talsu Skaistule’ were negative for ApMV.

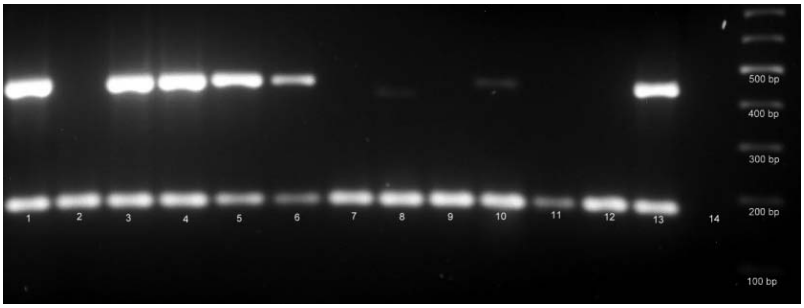

Fig. 1 Gel electrophoresis showing RT-PCR results: apple samples in lane 1-6; pear samples in lane 7-12; positive control in lane 13; water control in lane 14

Tab. 2 The occurrence of ilarviruses in fruit trees and orchards (%)

Fruit trees	ApMV DAS ELISA		ApMV RT-PCR		PNRSV DAS ELISA		PDV DAS ELISA	
	Samples	Orchards	Samples	Orchards	Samples	Orchards	Samples	Orchards
Apples	2.1	14	22.0	90	nt*	nt	nt	nt
Pears	0	0	20.2	55.6	nt	nt	nt	nt
Plums	1.8	28.6	nt	nt	13.6	67.9	11.6	78.6

* nt – not tested.

The occurrence of ApMV, PNRSV and PDV in plum orchards: In the orchard surveys no obvious symptoms were observed on plum trees, except some necrotic spots and shot-holes on plum leaves. The obtained data showed that ilarviruses are widespread:in only two plum orchards were no viruses detected. The occurrence of ilarviruses varied greatly among fruit tree species and orchards (Table 2). The occurrence with ApMV was rather low in all inspected orchards. It ranged from 0% to 16.7 % while occurrence of PNRSV and PDV was from 0% to 85.7% in individual plum orchards. Mixed infections with two or three viruses were detected in 4% of tested plum leaf samples. Mostly mixed infections with PDV and PNRSV were detected and only a few samples were infected with all three *Ilarviruses*.

Among different *Prunus* spp. genotypes a high variability in the occurrence of the viruses was observed (Table 3). The occurrence of ApMV was rather low in all common *Prunus* cultivars. It ranged from 0% to 15%. The *Prunus* hybrid 'Skoroplodnaya' showed a high occurrence of PDV (67.7%) but in other *Prunus* cultivars it ranged from 0% to 30.8 %. A high occurrence PNRSV was observed in several *Prunus* cultivars, such as ‘Perdrigon’ (46.7%), ‘Mirabelle de Nancy’ (44.4%), and ‘Reine-Claude d'Oullins’ (45%). In other *Prunus* cultivars the occurrence of PNRSV ranged from 0% to 25%. All samples of cultivars ‘Jubileum’, ‘Tragedy’, and ‘Stanley’ were negative to all tested *Ilarviruses*.

Tab. 3 Occurrence of ilarviruses in common *Prunus* cultivar (%)

***Prunus* cultivars**	**ApMV**	**PDV**	**PNRSV**
‘Experimentalfältets Sviskon’	0	25	13
‘Jubileum’	0	0	0
‘Julius’	0	11	0
‘Kubanskaja Kometa’	5	31	6
‘Latvijas Dzeltenā Olplūme’	4	7	11
‘Lāse’	0	0	7
‘Mirabelle de Nancy’	0	0	44
‘Perdrigon’	7	7	47
‘Prince of Wales’	0	19	25
‘Reine-Claude d' Oullins’	15	10	45
‘Reine-Claude Verte’	14	0	14
‘Skoroplodnaya'	3	68	13
‘Stanley’	0	0	0
‘Tragedy’	0	0	0
‘Victoria’	0	4	9

Discussion

The research presented here demonstrated that ApMV, PNRSV and PDV are widespread in pome and stone fruit tree orchards in Latvia and most of the commonly grown cultivars are infected. In individual orchards the high occurrence of *Ilarviruses* could be due to the preference of cultivars for commercial growing. The most common apple, pear and *Prunus* cultivars are highly infected with ApMV, PDV and PNRSV, which indicates that infected planting material was used in propagation, because only graft-transmission for ApMV is known, and the natural spread of PNRSV and PDV is usually slow (Diekmann & Putter, 1996).

ApMV was detected in apple and plum trees, but not in pear samples with the DAS ELISA test indicating possible false negatives also for other viruses tested in this study. Low sensitivity of the ELISA test and ApMV high sensitivity to temperature fluctuation result in false negatives as has been demonstrated in other studies (Kobylko et al., 2005). In other studies it has been shown that ApMV occurrence in pears is about 77% (Petrzik, 2005). In this research we found that the occurrence of ApMV at 20%. As weak DNA bands were obtained from pear sample PCR productsit might indicate that ApMV is present in pear trees at very low concentrations and therefore was not detectable with the ELISA test. Although the high sequence correlation among apple and pear ApMV isolates has been reported (Petrzik, 2005), the obtained weak DNA bands from pear samples may also indicate that the primers, which were used in this study might be less specific for Latvian pear isolates. Furthermore, in other research where the same primers were used, ApMV was not detected in pears (Hassan et al., 2006). To test this hypothesis and confirm ApMV occurrence in pears the study should be continued with another primers and comparisons of sequences from PCR products should be done.

The obtained results confirmed the presence of ApMV, PNRSV and PDV in Latvia and showed that these viruses are widespread in plum orchards. Although in other countries in Europe ApMV is more common on *Prunu*s than on *Malus* (Desvignes, 1999), in this research ApMV was more widespread on *Malus* than on *Prunus*. To exactly determine ApMV occurrence in plum orchards, it would be necessary to test plum samples with a more sensitive method such as RT-PCR or immunocapture RT-PCR. In other studies in Europe the occurrence of PDV has been demonstrated to range from 0.4% to 47% and PNRSV from 5.6% to 46% (Massart et al., 2008), which corresponds to the data obtained in this study. Out of the three ilarviruses, PNRSV, due to its modes of transmission, is more widespread in plum orchards than PDV and ApMV, as has also been demonstrated in this study. Some experimental evidence suggest that thrips are also involved in PNRSV natural transmission (Shiel & Berger, 2000). Although in other studies PNRSV, PDV and ApMV have been demonstrated to occur often in mixed infections (Scott et al., 2001; Petrzik & Lenz, 2002), in this study mixed infections in plum trees were detected only in a few cases. Desvignes (1999) reported that PNRSV and PDV concentrations are high and constant in plant tissues and various virus strains can be easily detected by ELISA. However, false negatives may occur with ELISA and are less likely when using molecular techniques.

The obtained results showed that ApMV, PDV and PNRSV are spread in fruit orchards and most of the commonly grown cultivars are infected. Since a certification system for planting material is not established in the country, there is very high risk for continuous spread of these viruses in the orchards. Similarly, as shown for ApMV, it is possible thatPNRSV and PDV are more widespread than detected with preliminary screening with DAS ELISA. This study should be continued and other test methods, such as RT-PCR, used. The obtained data indicate a great need for the establishment of a certification system for fruit tree propagation.

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