The Occurrence of *Apple Stem Pitting Virus* and *Apple Stem Grooving Virus* within Field-Grown Apple Cultivars Evaluated by RT-PCR

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Abstract

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The reverse transcription polymerase chain reaction (RT-PCR) was successfully used to determine the occurrence of *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV) in field-grown apple cultivars. Both viruses were detected frequently in all 16 tested apple cultivars. As many as 27.86% ASPV-infected and 44% ASGV-infected trees were recorded among a total of 420 tested trees from 15 different orchards. Mixed infection with ASGV and ASPV was recorded in 16.7% of the trees.

Keywords: *Apple stem pitting virus*; *Apple stem grooving virus*; apple cultivars; RT-PCR

Apple stem pitting virus (ASPV) and *Apple stem grooving virus* (ASGV) are widely distributed in apple and pear orchards (Németh 1986). ASPV *(Foveavirus)* (Martelli & Jelkmann 1998) and ASGV *(Capillovirus)* (Yoshikawa *et al.* 1992) cause a disease associated with the tree decline, stem pitting, and vein yellows (ASPV), and graft union necrosis (ASGV) in apples and pears (Desvignes *et al.* 1990). Both viruses are mostly symptomless in apple and pear cultivars (Németh 1986), and often occur in mixed infection in apple orchards (Leone *et al.* 1998; Kundu 2002).

Bioassays and laboratory assays are available to detect of ASPV and ASGV. The former being less effective (Stouffer & Fridlund 1989). ELISA based techniques are very useful for routine detection of ASGV (Fuchs 1981; Polák & Zieglerová 2001) but the virus may escape detection because of low concentration (Kinard *et al.* 1996). Detection of ASPV by ELISA is so far not possible due to the absence of a good-quality antiserum. The molecular amplification-based assay, reverse transcription polymerase chain reaction (RT-PCR) has been widely used for the detection of ASPV (Schwarz & Jelkmann 1998; Kummert *et al.* 1998) and ASGV (Kinard *et al.* 1996; MacKenzie *et al.* 1997).

This paper reports the frequency of ASPV and ASGV occurrence and of the mixed infection by these viruses in field-grown apple cultivars in 15 orchards in intensive apple production areas of the Czech Republic.

MATERIALS AND METHODS

Virus isolates. ASPV apple isolate (Plant Research International, Wageningen, The Netherlands) was maintained and propagated in *Nicotiana occidentalis* 37B, and the ASGV apple isolate (University of Halle, Wittenberg, Germany) in *Chenopodium quinoa*.

Sampling. Trees from thirteen apple orchards and two apple nurseries located in intensive ap-

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ple growing areas throughout the Czech Republic (Table 1) were tested. As positive control of ASPV and ASGV isolates were used .

Extraction of total RNA. Extraction of total RNA from apple leaves (*Malus domestica* Borkh.*)* or herbaceous hosts (*Nicotiana occidentalis* 37B for ASPV or *Chenopodium quinoa* for ASGV) was described by Kundu (2002) with modification. Leaf times were ground in a mortar and pestle in repeance of extraction buffer (1:20, 20mM Tris-HCl pH 7.8 containing 200mM LiCl, 20mM EDTA and 1% SDS). The homogenate was transferred to sterile centrifuge tubes and centrifuged at 10 000 g for 10 min. The supernatant (500 µl) was transformed to sterile centrifuge tubes, 5 µl of proteinase K (20 mg/ml) were added and incubated at 37°C for 1 h at 10 000 g for 10 min. The supernatant (500 µl) was mixed with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) for 10 min by vortexing and centrifuged at 10 000 g for 10 min. The aqueous phase (400 µl) was replaced into a new sterile centrifuge tube and the process was repeated twice. The aqueous phase (400 µl) was then mixed with a triple volume of absolute ice-cold ethanol (99.8% v/v) and 80 µl 10M LiCl and the mixture was kept at –20°C for 1 h to precipitate nucleic acids. The precipitate was collected by centrifugation at 20 000 g for 10 min and washed twice for 30 min in 70% ethanol (v/v) at room temperature. Then it was centrifuged again at 20 000 g for 8 min, ethanol was removed, the pellet was dried under vacuum, and re-suspended in 40 µl of sterile deionised DEPC-treated water and stored at –20°C until used.

Reverse transcription polymerase chain reaction (RT-PCR)

Synthesis of cDNA. Synthesis of cDNA was performed in microtubes as follows: 1µl RNA was diluted with 10 µl DEPC treated water and 10 pmol of downstream primer (ASP-A or ASGV-2) was added. Incubation at 70°C for 5 min and chilling on ice followed. Then, RT-mixture consisting of 6.25 µl RNAase-free water, 300 units of M-MLV reverse transcriptase, 5 µl M-MLV reverse transcriptase buffer, 20 units of RNasin and 0.2mM of each dNTP (all from Promega) was added to a final volume of 25 µl. The reaction mixture was then incubated at 37°C for 1 h and boiled for 5 min.

Amplification of cDNA. The PCR reaction mixture consisting of 2.5 µl buffer for *Taq* polymerase, 2.5 units of *Taq* polymerase, 10 pmol of upstream and downstream primers (ASP-A/ASP-C for ASPV – Jelkmann & Keim-Konrad 1997 and ASGV-U per ASGV-2 for ASGV – James 1999) (Table 1), 0.2mM dNTPs and 1.25mM $MgCl_2$ (all from Promega) was prepared in microtubes. The mixture was adjusted to 24 µl with sterile deionised water and 1µl of cDNA was added into each reaction tube.

The reaction was carried out in a thermocycler (MJ research) as follows:

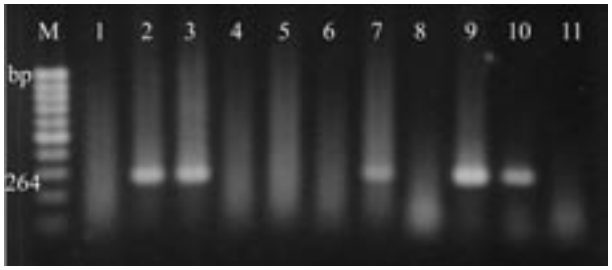
ASPV – 40 cycles at 94°C for 45 s (denaturation) then 55°C for 1 min (annealing) and 72°C for 2 min (polymerisation), and after the last cycle a final 5 min elongation step at 72°C.

ASGV – 35 cycles at 94°C for 30 s (denaturation) then 55°C for 45 s (annealing) and 72°C for 1 min (polymerisation), and after the last cycle a final 10 min elongation step at 72°C.

Analysis of amplified products. Aliquots of the PCR products were analysed by electrophoresis on agarose gel (1.5%) in TBE buffer (90mM Tris-borate, 2mM EDTA) at 120 V for 45 min. The amplified DNA fragments were stained with ethidum bromide (0.5 µg/ml), visualised under a UV transilluminator, and photographed using Polaroid system. The size of fragments was determined by comparison with DNA molecular markers 100 bp (MBI Farmentas).

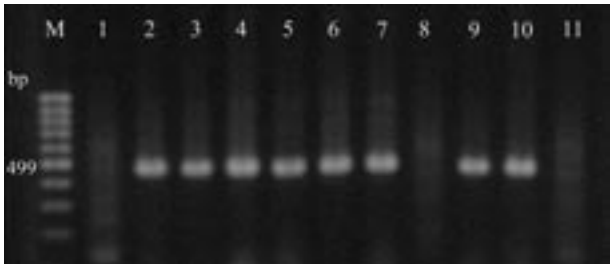
Table 1. List of primers

Virus	Primer	Sequence (5'–3')	Nucleotides	Product size
ASPV	ASP-C (sense)	CTCTTGAACCAGCTGATGGC	8993–9012	264 bp
	ASP-A (anti-sense)	ATAGCCGCCCCGGTTAGGTT	9237–9256	
ASGV	ASGV-U (sense)	CCCGCTGTTGGATTTGATACACCTC	5873–5897	499 bp
	ASGV-2 (anti-sense)	GGAATTTCACACGACTCCTAACCCTCC	6345–6371	



Lane M – molecular marker 100 bp (MBI Fermentas)
Lane 1–9 – tree 2–10
Lane 10 – ASPV infected *Nicotiana occidentalis* 37B (positive control)
Lane 11 – healthy control

Figure 1. Agarose gel electrophoresis of RT-PCR products of ASPV of cultivar Šampion from the orchard Roudnice n/L[1]



Lane M – molecular marker 100 bp (MBI Fermentas)
Lane 1–9 – tree 2–10
Lane 10 – ASGV infected *Chenopodium quinoa* (positive control)
Lane 11 – healthy control

Figure 2. Agarose gel electrophoresis of RT-PCR products of ASGV of cultivar Šampion from orchard Roudnice n/L[1]

RESULTS AND DISCUSSION

The RT-PCR may described here proved highly specific for the detection ASPV and ASGV. The selected primers were specific for the detection of the viruses in apple trees as well as in the herbaceous hosts (Kundu 2002). The present protocol suitable for determining ASPV and ASGV incidence in field-grown apple trees. The 264 bp PCR fragment for primer pairs ASP-A/ASP-C, specific for ASPV, and the 499 bp PCR fragment for primer pairs ASGV-U/ASGV-2, specific for ASGV were amplified from all tested cultivars from all orchards, except from the healthy controls. RT-PCR products of ASPV and of ASGV from cv. Šampion from the orchard Roudnice n/L[1] (Table 2) is shown in Figures 1 and 2, respectively, as an example of the detection of these viruses in the orchards. The incidence of ASPV and ASGV in the tested apple trees of all orchards are reported in Table 2. The presence of these two main pome fruit viruses was detected frequently in the apple cultivars tested by RT-PCR (though the plants were symptomless). Of the total 420 tested trees from 13 intensive apple orchards and 2 apple nurseries, 27.86% were infected with ASPV and 44% with ASGV. These viruses were not detected only in the orchard Králova, established with certified virus-free materials. The distribution of these viruses was recorded as more or less frequent in all other orchards. A mixed infection by both ASGV and ASPV was recorded in 16.7% trees in 16 tested cultivars. Such mixed infection by pome fruit tree viruses seems to be very common in apple orchards (Leone *et al*. 1998). The detection of these viruses in nurseries (Table 2) agrees with our previous observations on ASPV incidence in apple nurseries (Kundu 2001). The present results showed a high frequency of the occurrence of both viruses tested in field-grown apple orchards regardless the age of orchards. ASGV was more frequent than ASPV in the tested orchards. A similar level of incidence of ASGV, based on detection by ELISA, was previously reported in some apple orchards in the Czech Republic (Janečková & Pluhař 1987; Polák & Zieglerová 2001). Although ASPV and ASGV were detected in all trees of a certain cultivar (ASPV in cv. Idared and ASGV in cv. Spartan, orchard Slaný), no indications were obtained on the sesceptibility of these cultivars.

The high incidence of these viruses in the tested orchards calls for an evaluation of the health status of nursery proliferious before their cultivation in field. The RT-PCR protocol described here is an effective tool to detect these viruses in the field-grown apple trees as well as in nurseries.

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Table 2. The incidence of ASPV and ASGV in tested apple cultivars

Orchards	Cultivars	Number of trees			
		tested	ASPV positive	ASGV positive	mixed infection
Horoměřice[1]	Idared	10	8	6	5
	Spartan	10	8	9	8
	Vista Bella	10	7	2	2
	Stark Earliest	10	8	10	8
Slaný[1]	Idared	10	4	1	0
	Spartan	10	4	10	4
	Golden Delicious	10	5	5	3
	James Grieve	10	0	7	0
Slaný[2]	Rubín	10	6	7	4
	Gold Star	10	7	9	6
Český Brod[1]	Gloster	10	3	5	2
	Idared	10	3	5	1
Český Brod[2]	Elstar	10	0	5	0
	Rubín	10	3	5	2
Kolín[1]	Gala	10	2	5	0
	Topaz	10	5	3	2
	Jonagold	10	4	4	1
Roudnice n/L[1]	Golden Delicious	10	4	3	2
	Spartan	10	3	6	3
	Šampion	10	4	7	3
Roudnice n/L[2]	Šampion	10	8	8	6
	Melrose	10	0	1	0
	Idared	10	0	1	0
Vilémov[1]	Rubín	10	0	8	0
	Idared	10	0	4	0
Vilémov[2]	Šampion	10	0	0	0
	Golden Delicious	10	3	7	2
Kozov[1]	Spartan	10	3	8	2
	Idared	10	8	1	0
Kozov[2]	Idared	10	1	1	0
	Šampion	10	2	7	2
	Janagold	10	0	4	0
Králová[2]	Gala	10	0	0	0
	Elstar	10	0	0	0
	Golden Delicious	10	0	0	0
	Idared	10	0	0	0
Veltím[3]	Golden Delicious	10	0	3	0
	Šampion	10	0	4	0
Mcely[3]	Goldstar	10	2	2	1
	Golden Delicious	10	2	3	1
	Idared	10	0	0	0
	Klára	10	0	9	0
Total		420	117 (27.86%)	185 (44%)	70 (16.7%)

[1]orchards older than 25 years; [2]orchards younger than 10 years; [3]nursery

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Souhrn

Kundu J.K. (2003): **Výskyt viru vrásčitosti kmene jabloně (*Apple stem pitting virus)* a viru žlábkovitosti kmene jabloně (*Apple stem grooving virus)* v sadech jabloní pomocí RT-PCR.** Plant Protect. Sci., **39**: 88–92.

Metoda RT-PCR (reverse transcription polymerase chain reaction) byla úspěšně použita pro hodnocení výskytu viru vrásčitosti kmene jabloně (*Apple stem pitting virus*-ASPV) a viru žlábkovitosti kmene jabloně (*Apple stem grooving virus*-ASGV) v 15 sadech jabloní v ČR. Při testování jednotlivých odrůd jabloní byl detekován vysoký podíl infekčních stromů. Mezi 420 testovanými stromy z 15 sadů bylo detekováno 27,86 % ASPV pozitivních a 44 % ASGV pozitivních stromů. Byla rovněž zjištěna směsná infekce těchto dvou virů u 16,7 % stromů.

Klíčová slova: *Apple stem pitting virus*; *Apple stem grooving virus*; odrůdy jabloně; RT-PCR

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