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RESEARCH PAPERS

Quantitative detection of four pome fruit viruses in apple trees throughout the year

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Summary. A one-step real-time RT-PCR assay (RT-qPCR) with melting curve analysis, using the green fluorescence dye SYBR Green I, was developed to detect and quantify RNA targets from *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple chlorotic leaf spot virus* (ACLSV) in infected apple trees. Single PCR products of 87 bp (ApMV), 70 bp (ASGV), 104 bp (ASPV) and 148 bp (ACLSV) were obtained, and melting curve analyses revealed distinct melting temperature peaks for each virus. A dilution series using *in vitro* synthesized transcripts containing the target sequences as standards yielded a reproducible quantitative assay, with a wide dynamic range of detection and low coefficients of variance. The content of selected viruses in apple plant tissues was stable throughout the year, and their accumulation did not significantly change between different plant tissues. The only minor exceptions were for ApMV and ACLSV, in which noticeable differences in their concentrations in various biological material were observed within the year. This divergence did not influence their year-round detectability. This one-step RT-qPCR assay is a valuable tool for year-round diagnostics, and molecular studies of the biology of ApMV, ASGV, ASPV and ACLSV.

Key words: apple viruses, real-time PCR, SYBR Green I, ELISA, RT-PCR.

Introduction

Of the many viruses affecting apple trees, *Apple mosaic virus* (ApMV, genus *Ilarvirus*), *Apple stem grooving virus* (ASGV, genus *Capillovirus*), *Apple stem pitting virus* (ASPV, genus *Foveavirus*) and *Apple chlorotic leaf spot virus* (ACLSV, genus *Trichovirus*) are economically the most important viral pathogens (Desvignes *et al*., 1999). These viruses can cause significant yield reductions of up to 60% (Campbell, 1963; Posnette, 1963; Schmidt, 1972; Zahn, 1996), particularly when co-infection occurs, which is frequent (Posnette, 1989). All of these pathogens are distributed worldwide wherever apple species (*Malus* × *domestica* Borkh.) are cultivated (EPPO, 2007). Apple cultivars infected with these viruses, except ApMV, remain mostly symptomless (Nemeth, 1986).

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Vegetative propagation of infected planting material is considered the main transmission pathway of all these four viruses. The major control strategies (pathogen detection, exclusion by crop certification or quarantine, eradication from infected cultivars and rootstocks in infected orchards and geographical regions, elimination in planting material, and selection of tolerant or resistant crop cultivars) are applicable to all systemic pathogens that infect pome fruit trees. These control strategies rely heavily on accurate and sensitive detection methods. Therefore, a prerequisite for efficient control of these viruses is the availability of biological, immunological, and molecular detection methods, which make it possible to identify the pathogens in infected plant tissues (Hadidi *et al*., 2011).

For the certification of plant material, apple plants have to be tested for all four of these distinct viruses, along with other pathogens (EPPO, 1999). Many diagnostic methods for virus detec-

tion have been developed and adapted, and they are used worldwide for routine apple testing; these methods include indexing on indicator plants, serological techniques and molecular methods based on polymerase chain reaction (PCR) (Mackenzie *et al*., 1997; James, 1999; Menzel *et al*., 2002; Menzel *et al*., 2003; Spiegel *et al*., 2005, 2006; Hassan *et al*., 2006; Jarošová and Kundu, 2010a). However, it is unknown whether these various detection assays are reliable for routine diagnostics of various tree tissues throughout the year. According to many authors, viruses exist at different concentrations in different hosts. However, it is not easy to determine the reasons for these divergences, specifically whether they are caused by true changes in virus concentrations, or the reliability of detection methods is influenced by inhibitors (Kundu, 2003; Arntjen and Jelkmann, 2010; Svoboda and Polák, 2010). A well-known challenge in RNA extraction from woody hosts is that trees and shrubs contain high amounts of polyphenols and polysaccharides (Mitra and Kootstra, 1993), which are believed to interfere with the sensitivity of the detection assays. It is likely that the amounts of inhibitors differ between individual tissues at different periods of the year (Fuchs, 1982; Stewart and Nassuth, 2001).

Although all of the techniques described above are capable of detecting viruses at low concentrations, only serological techniques can roughly quantify pathogens. Real-time PCR (qPCR), which is one of the most important advances in quantitative technology, with a number of significant advantages over conventional PCR (Ratti et al., 2004), could also be used for virus quantification. This provides a means of detecting and quantifying DNA targets by monitoring PCR product accumulation during cycling as indicated by increasing fluorescence (Mackay, 2004). It has become the most accurate and sensitive method for detection and quantification of plant pathogens since the first commercial qPCR instrument became available (Heid *et al*., 1996).

Real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) assays became the most reliable technologies for the detection and the quantification of plant viruses infecting field crops (Korimbocus *et al*., 2002; Balaji *et al*., 2003; Mumford *et al*., 2004; Ratti *et al*., 2004; Clark *et al*., 2012; Wosula *et al*., 2013), horticultural crops (Roberts *et al*., 2000; Marbot *et al*., 2003; Lunello *et al*., 2004; Schneider *et al*., 2004; Picó *et al*., 2005; Osman *et al*., 2007; Jarošová and Kundu, 2010b) and ornamental plants (Eun *et al*., 2000; Nicolaisen, 2003). In addition, the technique has successfully been applied to viruses infecting horticultural woody plants, including detection of ASPV using molecular beacons (Klerks *et al*., 2001) or fluorogenic 3´minor groove binder (MGB) DNA probes (Roussel et al,. 2005), ASGV detection using TaqMan-MGB probes (Guo *et al*., 2006), and ACLSV detection using fluorogenic 3´-MGB DNA probes (Salmon *et al*., 2002; Roussel *et al*., 2005). Roussel *et al*. (2005) also attempted use of real-time RT-PCR for ApMV detection in bark tissues of dormant wood, but the technique failed to find all viruses with a single specific probe.

The aim of the present study was to develop a consistent procedure based on one-step real-time RT-PCR using SYBR Green I to reliably detect ApMV, ASGV, ASPV and ACLSV in apple trees throughout the year, and to roughly determine their absolute accumulation in infected tissues. The results from RT-qPCR were compared with those obtained using standard serological and molecular methods (DAS/DASI ELISA, RT-PCR) recommended in the EPPO certification schemes.

Materials and methods

Plant material

Plant samples from four apple (*Malus* × *domestica* Borkh.) cultivars (Rubin, Topaz, Bohemia and Angold), co-infected with ApMV, ASGV, ASPV and ACLSV, were collected randomly from a private orchard in the region of Central Bohemia (Czech Republic) in all four seasons from 2014 to 2015. The presence of all four viruses in tested apples was confirmed by conventional RT-PCR tests (detailed protocols are described below). The virus co-infection was of benefit since only one source of biological material for all four viruses was used in the study. However, the interaction of the four pathogens in one host cannot be excluded.

Multiple *Malus* sp. tissues were used for testing, including leaf buds, flower petals, leaves and inner bark. Buds and petals were tested in the period of their appearance on the trees; leaves were collected every 3 weeks during vegetation seasons from March to October, and inner bark was collected in only March, June, September and December.

Oligonucleotide primer design

We designed specific primers for quantification of the selected viruses. *In silico* PCR was performed for all of the primer-primer combinations using BLASTn against the NCBI GenBank database, to ensure their specificity prior to primer synthesis (Sigma-Aldrich Co.). Their specificity was evaluated by conventional RT-PCR and RT-qPCR using melting curve analysis**.** Positive samples from infected apple tree and negative samples from healthy pear, apricot, peach, plum, cherry and hop plants were included in the analysis. Primer sequences are given in Table 1.

According to Jarošová and Kundu (2010c), an internal control gene, elongation factor-1 alpha (ELF1A; accession numbers AF479046), was used to evaluate the RT-qPCR assays.

One-step RT-qPCR assays with melting curve analysis using SYBR Green I

Total RNA was extracted from 50 mg host tissue using a Spectrum™ Plant Total RNA kit (Sigma-Aldrich Co.). One-step RT-qPCR was performed using a *Power* SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems Inc.) with approx. 100 ng of total RNA in a 10 μL mixture containing 0.3 μM of both primers (Table 1). A CFX96 Touch™ cycler (Biorad) was used for the thermal cycling. The RT-qPCR profile consisted of cDNA synthesis (30 min at 40°C), activation of the Hot Start Taq polymerase (10 min at 95°C), and 40 amplification cycles (30 s at 95°C, 60 s at 60°C with optics on). Melting curves were determined at the end of every cycle. Reactions were performed in duplicate, and each run contained negative controls. The CFX Manager™ software (Biorad) was used for analysis of all amplified PCR products,. Briefly, a threshold was assigned to the log phase of product accumulation. The point at which the amplification curve crossed the threshold was defined as the cycle threshold value (Ct). With increasing target quantity in the PCRs, the Ct value decreased linearly, so Ct values could be used as quantitative measurements of the input target amount (Heid *et al.*, 1996). The samples amplified in each plate were quantified using their corresponding regression lines. Thus, the amount of starting template in any PCR could be accurately determined, expressed as the copy number of the target ApMV, ASGV, ASPV or ACLSV cDNAs per nanogram of total RNAs in the original plant extract.

Table 1. Forward (F) and reverse (R) primers used for quantitative real-time RT-PCR detection of the genomic RNA of four pome fruit viruses.

Virus	Primer	Type	Nucleotide sequence (5' → 3')	Position (nt)	Expected PCR product (bp)
ApMV	ApMVXF1	F	CAAGCGAACCCGAATAAGG	1231 – 1318 (NC_003480)	87 bp
	ApMVXR1	R	CTAACCTCCCAAGCTGTCC	1300 – 1318 (NC_003480)	
ASGV	ASGVF1	F	GGATTTAGGTCCCTCTCAGC	5600 – 5619 (NC_001749)	70 bp
	ASGVR1	R	CTTGTTGAAGCACGTCTTCC	5651 – 5670 (NC_001749)	
ASPV	ASPVF3	F	GAACTGCYGCAGAGGAAG	77 – 94 (NC_003462)	104 bp
	ASPVR3	R	CATGYTTGTCCTTCTCYAC	163 – 181 (NC_003462)	
ACLSV	ACLSVF2	F	GAGGCTCTATTCACATCTTG	5617 – 5636 (NC_001409)	148 bp
	ACLSVR2	R	CATGYTTGTCCTTCTCYAC	5747 – 5765 (NC_001409)	

Preparation of RNA transcripts for standard curves

To determine the absolute number of gRNA copies in RNA extracts, RNA transcripts of the selected gRNA region were synthesised *in vitro*, and serial dilutions were used in real-time RT-PCR assays to generate external standard curves. Recombinant pSC A amp/kan plasmids (Agilent Technologies) carrying the cloned ApMV, ASGV, ASPV and ACLSV PCR target sequences were linearised with Bam HI (Fermentas), and the restriction digest was terminated by adding 1/20 volume of 0.5M EDTA, 1/10 volume of 3M Na acetate, and 2 volumes of absolute ethanol. After incubation at -20°C for 1 h, the DNA was pelleted by centrifugation (10,000 g, 15 min) and resuspended in 50 μL ddH_2O. The linearized plasmid DNAs were then purified using the QIAquick gel extraction kit (Qiagen).

RNA transcripts were generated using the T7 RNA enzyme mix from a Megascript® Kit - High Yield Transcription (Ambion, Life Technologies). Plasmid DNAs were digested with a DNase-free™ Kit (Ambion, Life Technologies). The RNA concentrations were determined using a Nanodrop 2000 (ThermoScientific). Conversion of mg L^{-1} of single stranded RNA to molar concentrations was calculated using the average molecular weight of a ribonucleotide (321.45 Da) and the number of bases of the transcript (Nb). The following mathematical formula was applied: mol of ssRNA = mg L^{-1} (of ssRNA) × (321.45 Da) × (Nb). The Avogadro constant (6.023×10^{23}) was used to estimate the number of transcripts. The initial concentration of transcripts (gRNA μL^{-1}) was 1.34×10^{17} for ApMV, 1.59×10^{16} for ASGV, 1.12×10^{17} for ASPV, and 9.33×10^{16} for ACLSV.

A 12-fold serial dilution of the transcripts was prepared from 10^5 to 10^{16}, and aliquots were subsequently prepared and stored at −80°C until used. Dilutions employed to generate the standard curves were from 10^{10} to 10^{15} for ApMV, from 10^{10} to 10^{16} for ASGV, from 10^7 to 10^{15} for ASPV, and from 10^6 to 10^{14} for ACLSV. The slopes of the calibration curves of the standards and field samples were used to calculate efficiencies using the mathematical formula: slope = -1 / log (efficiency).

DAS/DASI-ELISA assays

Double and triple antibody sandwich ELISA was performed as described by Clark and Adams (1977) and Clark and Joseph Bar (1984), using the commercially available ApMV (Agritest), ASGV (Bioreba), ASPV (Bioreba) and ACLSV (Loewe) antibodies, according to the manufacturer's instructions.

To estimate the reliability of the DAS/DASI-ELISA assays, 200 mg of each sample from various plant tissues were ground in ten volumes (w/v) of PBST buffer and used as starting material. The samples had been regularly taken and analyzed from four ApMV, ASGV, ASPV and ACLSV co-infected apple cultivars during 2014 and 2015.

The ELISA reactions were read at 405 nm in a microtitre plate reader (Tecan Sunrise™) and evaluated using the Magellan™ data analysis software (Tecan). ELISA readings were considered positive when the absorbance of sample wells was at least two times greater than the mean absorbance reading of three healthy controls. The DAS/DASI-ELISA was evaluated as reliable for the pathogen detection (in the particular plant tissue and during the particular period) since all four tested samples from four apple varieties gave positive responses. In cases of least one giving negative response, the technique was considered as unreliable.

To estimate relative virus concentration using DAS/DASI-ELISA assays in various biological tissues throughout the year, dilution series (five-fold serial dilution down to 10^6) of each tested sample was prepared and used as starting material. The samples were regularly taken and analyzed from four ApMV, ASGV, ASPV an ACLSV co-infected apple cultivars every trimester (in March, June, September and December) during 2014. The relative concentrations in plant tissues were estimated when all four tested samples gave positive response in a particular dilution.

Conventional RT-PCR assays

Total RNA was extracted using a Spectrum™ Plant Total RNA kit (Sigma-Aldrich Co.). To estimate the reliability of the RT-PCR assay, 100 mg of each sample were ground in ten volumes (w/v) of lysis buffer, and used as starting material. The samples were regularly taken and analyzed from four ApMV, ASGV, ASPV an ACLSV co-infected apple cultivars during 2014 and 2015.

Reverse transcription was performed using approx. 750 ng of total RNA in a 50 μL mixture containing 1× First-Strand Buffer (Invitrogen™), 0.5 μg random hexamers (Roche Diagnostics), 0.5 mM dNTP,

40 U RiboLock™ RNase Inhibitor (Fermentas), 4 mM DTT (Invitrogen™) and 40 U M-MLV Reverse Transcriptase (Invitrogen™) for 55 min at 42°C, with a final incubation at 70°C for 10 min.

PCR was performed using specific primers, namely ApMV16178 (5´TCCTGAGCAGTCGAGAAGTG3´)/ ApMV16179 (5´CGTTATCACGTACAAATCCCT3´), for detection of ApMV (Petrzik, unpublished), ASGVUs/ASGV2as for ASGV (James, 1999), ASPCs/ASPAas for ASPV (Jelkmann and Keim-Konrad, 1997), and ACLSV1F/ACLSV1R for ACLSV (Watpade *et al*., 2012). The cycling parameters were as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for ApMV, 66°C for ASGV, 60°C for ASPV and 57°C for ACLSV, for 30 s and elongation at 72°C for 45 s, with a final extension at 72°C for 5 min. The PCR-amplified fragments were visualised after electrophoresis on ethidium bromide-stained 1% agarose gels.

The RT-PCR was evaluated as reliable for pathogen detection (in the particular plant tissue and during the particular period) since all four tested samples from four apple varieties gave positive responses. In cases of at least one giving negative response, the technique was considered as unreliable.

To estimate relative virus concentrations using RT-PCR assay in various biological tissues throughout the year, dilution series (a five-fold serial dilution down to 10^6) of each tested sample was prepared and used as starting material. The samples were regularly taken and analyzed from four ApMV, ASGV, ASPV and ACLSV co-infected apple cultivars every trimester (in March, June, September and December) during 2014. The relative concentrations in plant tissues were estimated when all four tested samples gave positive responses in particular dilutions.

Results

One-step RT-qPCR assays

All newly designed specific oligonucleotide primers amplified the expected targets (namely 87 bp fragments for ApMV, 70 bp for ASGV, 104 bp for ASPV, and 148 bp for ACLSV). The specificity of primers was initially confirmed by visualizing the qPCR fragments after electrophoresis on ethidium bromide-stained 1% agarose gels (Figure 1). The presence of a single fluorescent peak in the melting curve analysis of all samples further supported primer specificity and the absence of potential interference from non-specific fluorescent signals (data not shown). Melting curve analyses of the amplicons associated with the various viruses allowed their accurate identification. ApMV had a T_m of approx. 78.5°C, ASGV had a T_m of 73.5°C, ASPV had a T_m of 77°C and ACLSV had a T_m of 76°C (Figure 2).

The standard curves were optimized for individual viruses, and their slopes confirmed a high PCR amplification efficiency (E = 98–100% for ApMV, 99-

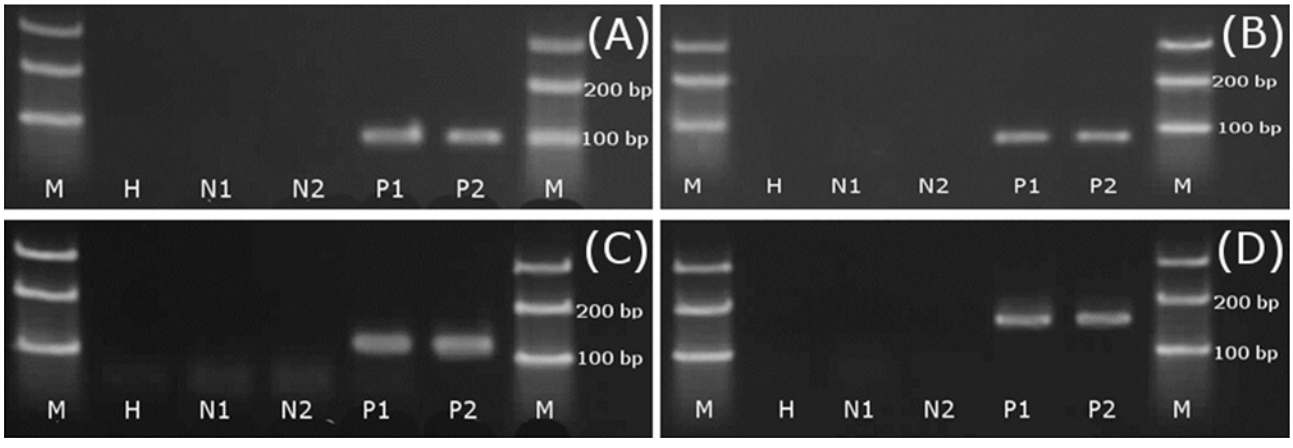

Figure 1. Electrophoreograms of PCR products obtained from RT-PCR using the specific primers designed for RT-qPCR purposes. (A): ApMVXF1/ApMVXR1 (87 bp); (B): ASGVF1/ASGVR1 (70 bp); (C): ASPVF3/ASPVR3 (104 bp); and (D): ACLSVF2/ACLSVR2 (148 bp). [(M): DNA marker (O'RangeRuler 20 bp DNA Ladder, Fermentas); (H): RT-PCR product of water control; (N1): RT-PCR product of healthy control obtained from apple; (N2): RT-PCR product of healthy control obtained from pear; (P): RT-PCR products of two various infected apple trees. Agarose gels stained by ethidium bromide].

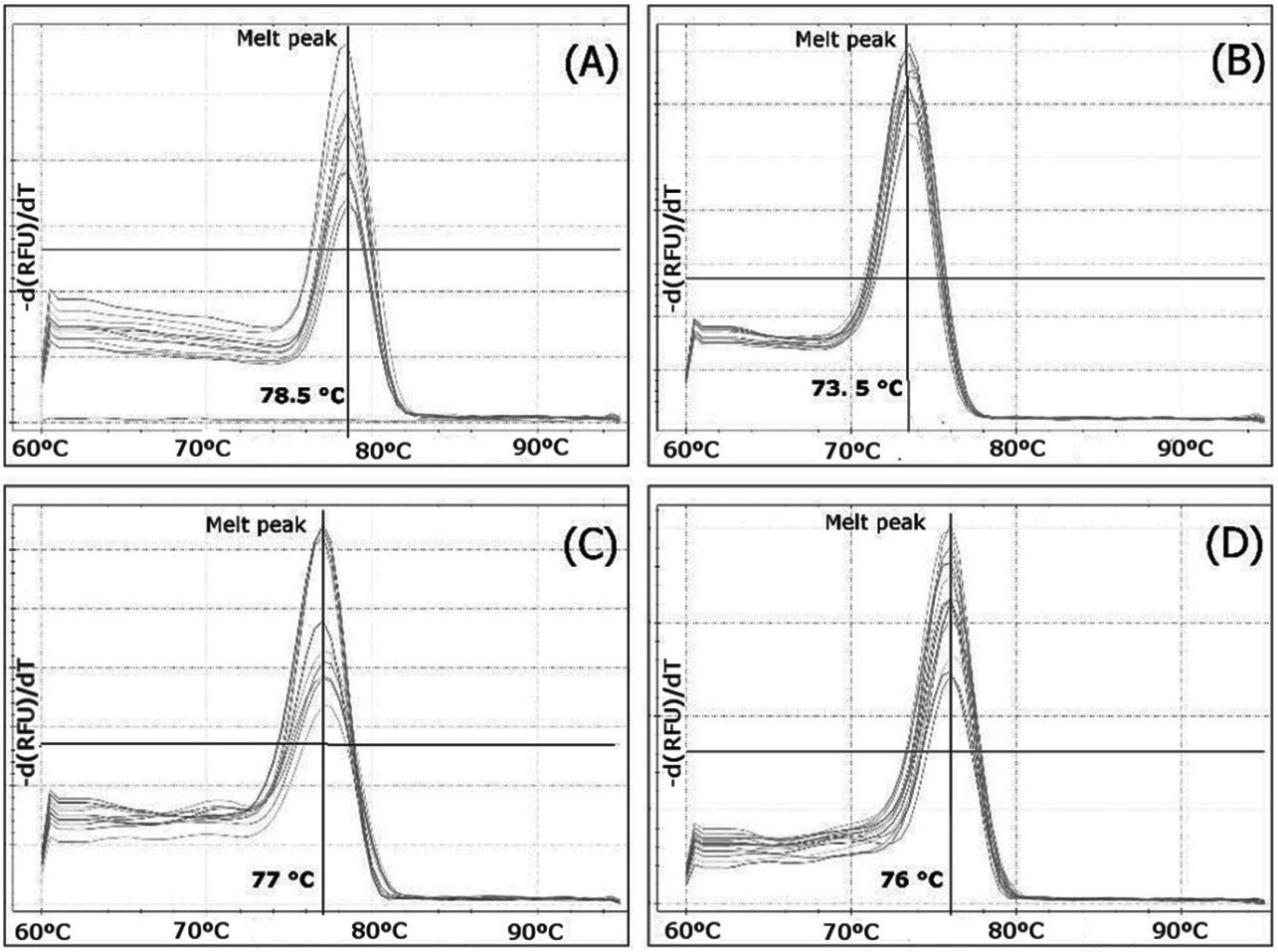

Figure 2. Melting curves using SYBR Green I for the detection of *Apple mosaic virus*, *Apple stem grooving virus*, *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* fragments amplified with specific primers (Table 1), showing virus identification in infected apple trees. (A): ApMV; (B): ASGV; (C): ASPV and (D): ACLSV.

100% for ASGV, 98–100% for ASPV and 98–100% for ACLSV). Strong correlations were observed between the quantities of virus RNA and the corresponding C_T values ($R^2 = 0.98$ for ApMV, $R^2 = 0.98$ for ASGV, $R^2 = 0.99$ for ASPV and $R^2 = 0.95$ for ACLSV). The RT-qPCR assay enabled detection of 10^5 gRNA copies for ApMV and ASGV and 10^4 gRNA copies for ASPV and ACLSV (Figure 3).

The RT-qPCR method reliably detected all selected viruses in all tested plant tissues, including leaves, flower petals, inner bark and buds, throughout the year during which samples were assessed. The quantification demonstrated that the fluctuation of each of the four viruses showed the same pattern in all tested apple cultivars (Tables 2 and 3).

ApMV was quantified at 10^{10} to 10^{15} copies. Comparison of the number of gRNA copies for the leaf and inner bark samples revealed that the virus concentrations fluctuated in both monitored tissues (Table 2). In the leaves, the greatest virus load was observed at the beginning of the vegetation period (from March to June), then slowly decreased in the following months to the least value of approx. 10^{10} at the end of vegetation (in October). In the inner bark, the virus loads reached their peak in June and then decreased in the following months to the lowest value of approx. 10^{10} in December. ApMV accumulation in buds and flower petals was similar to that observed in spring leaves or summer inner bark, approx. 10^{14} to 10^{15} (Table 2).

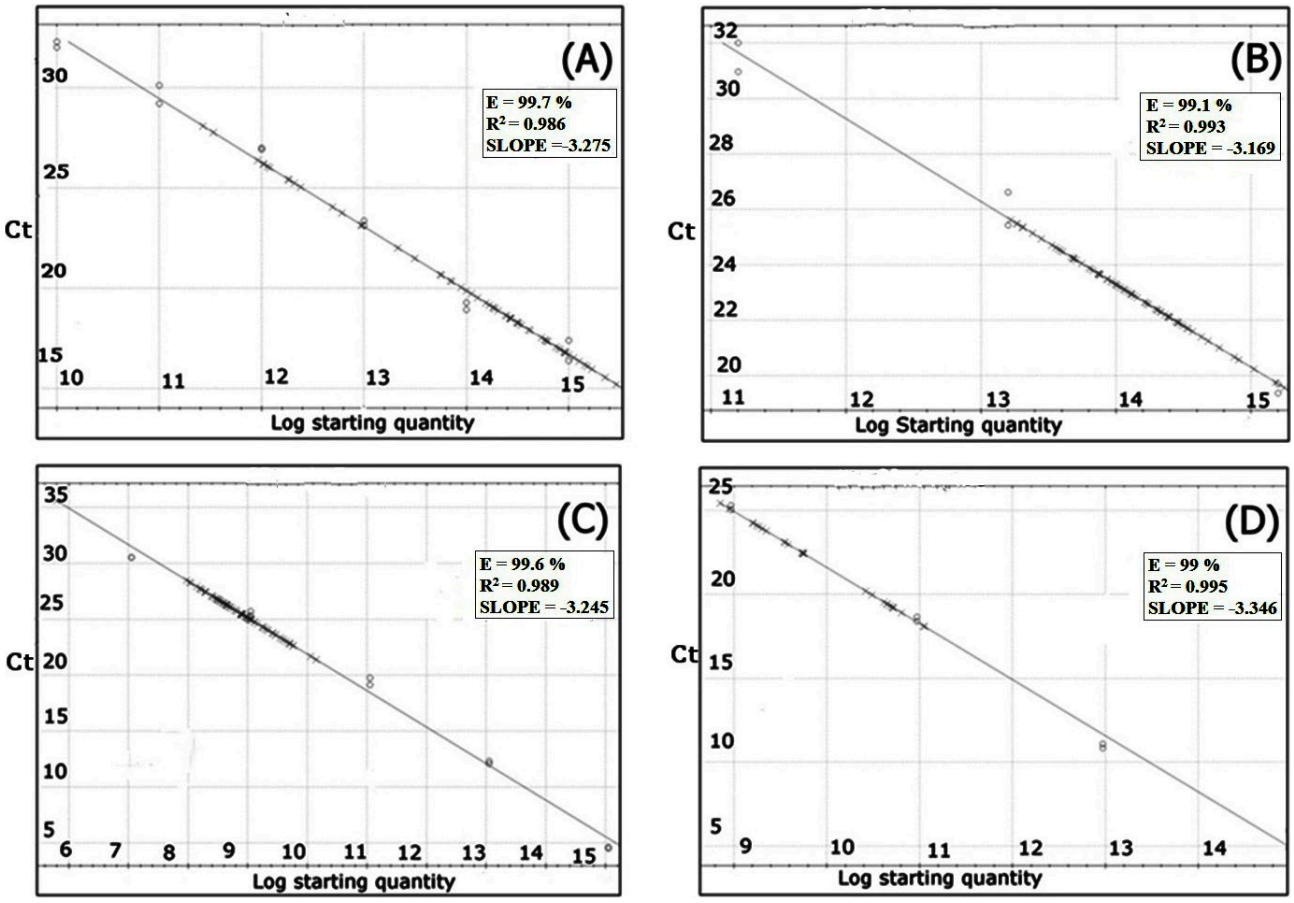

Figure 3. Standard curves obtained from *Apple mosaic virus*, *Apple stem grooving virus*, *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* amplification analyzed using linear regression, plotting the threshold cycle values (Ct) vs. the logarithm of the actual starting RNA copy number. (A): ApMV; (B): ASGV; (C): ASPV and (D): ACLSV. (O): standard; (X): unknown; (-): SYBR.

On the other hand, the amounts of ASGV and ASPV loads were stable in all tested tissues throughout vegetation period. ASGV was measured at 10^{13} to 10^{14} copies (Table 2) and ASPV at 10^{8} to 10^{9} copies (Table 3). In the case of ACLSV, the greatest value of approx. 10^{12} was monitored in flowers in April. ACLSV accumulation in buds and inner bark ranged from 10^{9} to 10^{10} copies and in leaves reached a peak in May and June with 10^{11} copies (Table 3).

Amplification of the ELF1A endogenous target (fragment of 165 bp, T_m of 80°C) was included as a control for assessing the extraction of amplifiable total RNA, as a control for RNA quality and for identifying false negatives. The standard curve was optimised for Ct values between cycles 10 and 32, corresponding to approximately 10^{7}–10^{15} copies. A high level of PCR amplification efficiency (E = 98-100%) and strong correlation (R^2 = 0.98) were confirmed from the standard curves. The Ct values of tested samples for the ELF1A were detectable and stable, corresponding to 10^{9} copies (data not shown). Therefore, total RNAs of tested samples were suitable for quantification of the studied viruses in all plant tissues during the vegetation period.

Detection of pome viruses using DAS/DASI-ELISA and conventional RT-PCR

Comparisons of the reliability of DAS/DASI ELISA, conventional RT-PCR, and real-time RT-PCR are shown in Tables 4 and 5. Overall, the conventional

Table 2. List of absolute quantification of genomic RNA copies, obtained in RT-qPCR with an external standard curve, from different plant tissues of four apple cultivars (during ten months in 2015), that were infected with *Apple mosaic virus* and *Apple stem grooving virus*.

Cultivar	Month	ApMV				ASGV			
		BU	FL	LE	IB	BU	FL	LE	IB
M1	Mar	3.91×10^{14}	-	1.56×10^{14}	1.06×10^{12}	4.02×10^{13}	-	7.41×10^{13}	1.44×10^{13}
	Ap	-	2.22×10^{15}	9.17×10^{14}	-	-	2.15×10^{14}	3.45×10^{14}	-
	May	-	-	4.48×10^{14}	-	-	-	3.18×10^{14}	-
	June	-	-	6.34×10^{13}	2.64×10^{14}	-	-	1.0×10^{14}	1.84×10^{13}
	July	-	-	1.84×10^{12}	-	-	-	9.42×10^{13}	-
	Aug	-	-	6.68×10^{11}	-	-	-	3.75×10^{13}	-
	Sep	-	-	7.01×10^{11}	1.26×10^{12}	-	-	1.76×10^{13}	1.55×10^{13}
	Oct	-	-	1.15×10^{10}	-	-	-	1.6×10^{13}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	4.66×10^{11}	-	-	-	2.02×10^{13}
M2	Mar	2.99×10^{14}	-	2.54×10^{14}	5.17×10^{13}	4.79×10^{13}	-	7.66×10^{13}	1.65×10^{13}
	Ap	-	8.4×10^{14}	1.77×10^{15}	-	-	2.87×10^{14}	5.87×10^{14}	-
	May	-	-	9.67×10^{14}	-	-	-	2.49×10^{14}	-
	June	-	-	2.18×10^{14}	2.88×10^{14}	-	-	1.63×10^{14}	1.31×10^{14}
	July	-	-	3.04×10^{12}	-	-	-	1.09×10^{14}	-
	Aug	-	-	4.84×10^{12}	-	-	-	9.83×10^{13}	-
	Sep	-	-	8.17×10^{11}	7.83×10^{12}	-	-	8.67×10^{13}	2.81×10^{13}
	Oct	-	-	5.94×10^{11}	-	-	-	2.91×10^{13}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	7.06×10^{11}	-	-	-	1.44×10^{13}
M3	Mar	7.67×10^{14}	-	8.42×10^{14}	1.06×10^{12}	2.04×10^{13}	-	8.56×10^{13}	4.8×10^{13}
	Ap	-	3.68×10^{15}	1.17×10^{15}	-	-	3.7×10^{14}	2.9×10^{14}	-
	May	-	-	5.89×10^{14}	-	-	-	2.8×10^{14}	-
	June	-	-	1.28×10^{14}	2.71×10^{14}	-	-	2.04×10^{14}	6.37×10^{13}
	July	-	-	1.04×10^{13}	-	-	-	1.71×10^{14}	-
	Aug	-	-	2.55×10^{12}	-	-	-	1.15×10^{14}	-
	Sep	-	-	1.06×10^{12}	2.14×10^{13}	-	-	5.53×10^{13}	5.53×10^{13}
	Oct	-	-	8.31×10^{11}	-	-	-	1.6×10^{13}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	8.31×10^{11}	-	-	-	2.83×10^{12}
M4	Mar	-	-	9.17×10^{14}	9.37×10^{12}	-	-	7.41×10^{13}	1.84×10^{13}
	Ap	-	-	2.67×10^{14}	-	-	-	9.42×10^{13}	-

(Continued)

Table 2. (Continued).

Cultivar	Month	ApMV				ASGV			
		BU	FL	LE	IB	BU	FL	LE	IB
	May	-	-	7.21×10^{14}	-	-	-	3.38×10^{13}	-
	June	-	-	8.89×10^{13}	6.98×10^{13}	-	-	3.32×10^{13}	5.53×10^{13}
	July	-	-	4.43×10^{12}	-	-	-	3.38×10^{13}	-
	Aug	-	-	2.24×10^{12}	-	-	-	2.81×10^{13}	-
	Sep	-	-	6.09×10^{11}	1.06×10^{12}	-	-	4.59×10^{13}	3.34×10^{12}
	Oct	-	-	2.58×10^{10}	-	-	-	1.6×10^{13}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	2.39×10^{10}	-	-	-	4.8×10^{13}

(M1), cv. Rubin; (M2), cv. Bohemia; (M3), cv. Topaz and (M4), cv. Angold.
(BU), buds; (FL), flowers; (LE), leaves and (IB), inner bark.
(-), data not available.

Table 3. List of absolute quantification of genomic RNA copies, obtained in RT-qPCR with an external standard curve, from different plant tissues of four apple cultivars (during ten months in 2015), infected with *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* in 2015.

Cultivar	Month	ASPV				ACLSV			
		BU	FL	LE	IB	BU	FL	LE	IB
M1	Mar	9.55×10^{8}	-	7.97×10^{8}	7.68×10^{8}	2.87×10^{9}	-	2.48×10^{9}	3.15×10^{9}
	Ap	-	5.78×10^{9}	1.06×10^{8}	-	-	3.89×10^{12}	7.9×10^{10}	-
	May	-	-	4.05×10^{8}	-	-	-	1.48×10^{11}	-
	June	-	-	1.78×10^{9}	1.14×10^{9}	-	-	9.05×10^{10}	2.53×10^{10}
	July	-	-	9.9×10^{8}	-	-	-	2.39×10^{9}	-
	Aug	-	-	4.3×10^{8}	-	-	-	4.27×10^{9}	-
	Sep	-	-	5.66×10^{8}	7.51×10^{8}	-	-	4.91×10^{9}	1.51×10^{9}
	Oct	-	-	5.44×10^{8}	-	-	-	2.01×10^{9}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	1.57×10^{8}	-	-	-	2.48×10^{9}
M2	Mar	1.46×10^{9}	-	4.97×10^{9}	3.02×10^{9}	4.26×10^{9}	-	9.43×10^{10}	4.47×10^{9}
	Ap	-	2.93×10^{9}	8.09×10^{8}	-	-	5.11×10^{12}	4.17×10^{11}	-
	May	-	-	2.22×10^{9}	-	-	-	1.12×10^{11}	-
	June	-	-	1.31×10^{9}	4.11×10^{9}	-	-	9.84×10^{10}	3.31×10^{10}
	July	-	-	9.57×10^{8}	-	-	-	4.15×10^{9}	-
	Aug	-	-	5.53×10^{8}	-	-	-	5.35×10^{9}	-

(Continued)

Table 3. (Continued).

Cultivar	Month	ASPV				ACLSV			
		BU	FL	LE	IB	BU	FL	LE	IB
	Sep	-	-	3.15×10^{8}	1.68×10^{9}	-	-	7.32×10^{9}	2.45×10^{9}
	Oct	-	-	4.33×10^{8}	-	-	-	5.29×10^{9}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	1.82×10^{9}	-	-	-	1.18×10^{9}
M3	Mar	1.31×10^{9}	-	3.82×10^{9}	1.3×10^{9}	8.17×10^{9}	-	5.45×10^{10}	1.98×10^{9}
	Ap	-	5.0×10^{9}	7.87×10^{8}	-	-	7.46×10^{12}	9.72×10^{10}	-
	May	-	-	1.06×10^{9}	-	-	-	3.38×10^{11}	-
	June	-	-	1.14×10^{9}	2.1×10^{9}	-	-	2.59×10^{11}	9.33×10^{10}
	July	-	-	5.22×10^{8}	-	-	-	9.82×10^{9}	-
	Aug	-	-	5.81×10^{8}	-	-	-	4.58×10^{9}	-
	Sep	-	-	2.24×10^{8}	1.18×10^{9}	-	-	1.23×10^{10}	5.39×10^{9}
	Oct	-	-	2.33×10^{8}	-	-	-	2.21×10^{10}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	9.65×10^{8}	-	-	-	4.61×10^{9}
M4	May	-	-	8.04×10^{8}	4.5×10^{9}	-	-	5.81×10^{9}	3.49×10^{9}
	Ap	-	-	1.14×10^{9}	-	-	-	1.58×10^{11}	-
	May	-	-	2.77×10^{9}	-	-	-	9.05×10^{10}	-
	June	-	-	1.41×10^{9}	1.68×10^{9}	-	-	3.06×10^{9}	3.06×10^{9}
	July	-	-	8.24×10^{8}	-	-	-	2.62×10^{9}	-
	Aug	-	-	1.31×10^{9}	-	-	-	3.80×10^{9}	-
	Sep	-	-	6.23×10^{8}	3.02×10^{9}	-	-	6.66×10^{9}	6.66×10^{9}
	Oct	-	-	8.57×10^{8}	-	-	-	7.75×10^{9}	-
	Nov	-	-	-	-	-	-	-	-
	Dec	-	-	-	1.78×10^{9}	-	-	-	2.04×10^{9}

(M1), cv. Rubin; (M2), cv. Bohemia; (M3), cv. Topaz and (M4), cv. Angold.
(BU), buds; (FL), flowers; (LE), leaves and (IB), inner bark.
(-), data not available.

RT-PCR tests provided better results than the serological methods in comparative analysis within the vegetation seasons for ApMV, ASGV and ASPV. In the case of ACLSV, serological methods proved to be as reliable as RT-PCR. However, with the exception of RT-PCR detection of ASPV, neither DAS/DASI ELISA nor conventional RT-PCR was as reliable as the RT-qPCR assay.

Regarding virus presence in various plant tissues, the results obtained from DAS/DASI-ELISA and RT-PCR were very heterogeneous, which is in contrast to real-time RT-PCR (Tables 4 and 5, Figures 4 and 5).

Serological techniques detected all viruses in the leaves. However, no virus was detected from leaf tissues during the whole vegetation period. In inner bark, only the presence of ASPV was confirmed

Table 4. Comparison between the reliability of DAS/DASI ELISA, RT-PCR and RT-qPCR for the detection of *Apple mosaic virus* and *Apple stem grooving virus* in different plant tissues in different months of 2015.

Virus	Detection technique		Mar	Apr	May	June	July	Aug	Sep	Oct	Nov	Dec
ApMV	DASI-ELISA		+									
	RT-PCR	BU	+									
	RT-qPCR		+									
	DASI-ELISA		N/A	+								
	RT-PCR	FL	N/A	+								
	RT-qPCR		N/A	+								
	DASI-ELISA		+	+	+	+	-	-	-	-		
	RT-PCR	LE	+	+	+	+	+	+	+	+		
	RT-qPCR		+	+	+	+	+	+	+	+		
	DASI-ELISA		-	N/A	N/A	+	N/A	N/A	-	N/A	N/A	-
	RT-PCR	IB	+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	-
	RT-qPCR		+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+
			Mar	**Apr**	**May**	**June**	**July**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
ASGV	DAS-ELISA		+									
	RT-PCR	BU	+									
	RT-qPCR		+									
	DASI-ELISA		N/A	-								
	RT-PCR	FL	N/A	+								
	RT-qPCR		N/A	+								
	DASI-ELISA		+	+	+	+	-	-	-	-		
	RT-PCR	LE	+	+	+	+	+	+	+	+		
	RT-qPCR		+	+	+	+	+	+	+	+		
	DASI-ELISA		+	N/A	N/A	-	N/A	N/A	-	N/A	N/A	-
	RT-PCR	IB	+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+
	RT-qPCR		+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+

(BU), buds; (FL), flowers; (LE), leaves; (IB), inner bark.
(+), reliable (all four tested samples taken from four apple varieties gave positive response);
(-), unreliable (at least one of four samples taken from four apple varieties gave negative response);
N/A, data not available.

during all tested seasons (in March, June, September and December). All four apple viruses were serologically detected in buds and flowers of the apple trees, with exception of ASGV, which was not confirmed in flower petals (Tables 4 and 5).

On the basis of DAS/DASI-ELISA results regarding virus accumulation in various plant tissues, the greatest accumulation of ApMV was recorded in leaves at the beginning of the vegetation season, followed by flowers and buds. In comparison with ApMV, ASGV exhibited the greatest accumulation in spring leaves. ASPV exhibited similar virus amounts

Table 5. Comparison between the reliability of DAS/DASI ELISA, RT-PCR and RT-qPCR for the detection of *Apple stem pitting* and *Apple chlorotic leaf spot virus* in different plant tissues in different months of 2015.

Virus	Detection technique		Mar	Apr	May	June	July	Aug	Sep	Oct	Nov	Dec
ASPV	DAS-ELISA		+									
	RT-PCR	BU	+									
	RT-qPCR		+									
	DASI-ELISA		N/A	+								
	RT-PCR	FL	N/A	+								
	RT-qPCR		N/A	+								
	DAS-ELISA		+	+	+	+	+	+	+	-		
	RT-PCR	LE	+	+	+	+	+	+	+	+		
	RT-qPCR		+	+	+	+	+	+	+	+		
	DASI-ELISA		+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+
	RT-PCR	IB	+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+
	RT-qPCR		+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+
			Mar	**Arp**	**May**	**June**	**July**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
ACLSV	DAS-ELISA		+									
	RT-PCR	BU	+									
	RT-qPCR		+									
	DASI-ELISA		N/A	+								
	RT-PCR	FL	N/A	+								
	RT-qPCR		N/A	+								
	DAS-ELISA		+	+	+	+	+	+	-	-		
	RT-PCR	LE	+	+	+	-	+	-	+	+		
	RT-qPCR		+	+	+	+	+	+	+	+		
	DASI-ELISA		-	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+
	RT-PCR	IB	-	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+
	RT-qPCR		+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	+

(BU): buds, flowers (FL), leaves (LE) and inner bark (IB).
(+): reliable (all four tested samples taken from four apple varieties gave positive response);
(-): unreliable (at least one of four samples taken from four apple varieties gave negative response).
N/A: data not available.

in all plant parts within the year (in buds, flowers, inner bark, and leaves in March, in leaves and inner bark in summer and autumn). The greatest concentrations of ACLSV were detected in flowers (Figure 4).

Using the conventional RT-PCR technique, we detected all tested viruses in leaves for the whole vegetation period, with the minor exception of ACLSV which was undetectable in June and September. The year-round detectability was also confirmed for ASGV and ASPV in inner bark. The use of buds and flower petals, as biological material for RT-PCR testing, has been shown to be successful (Tables 4 and 5).

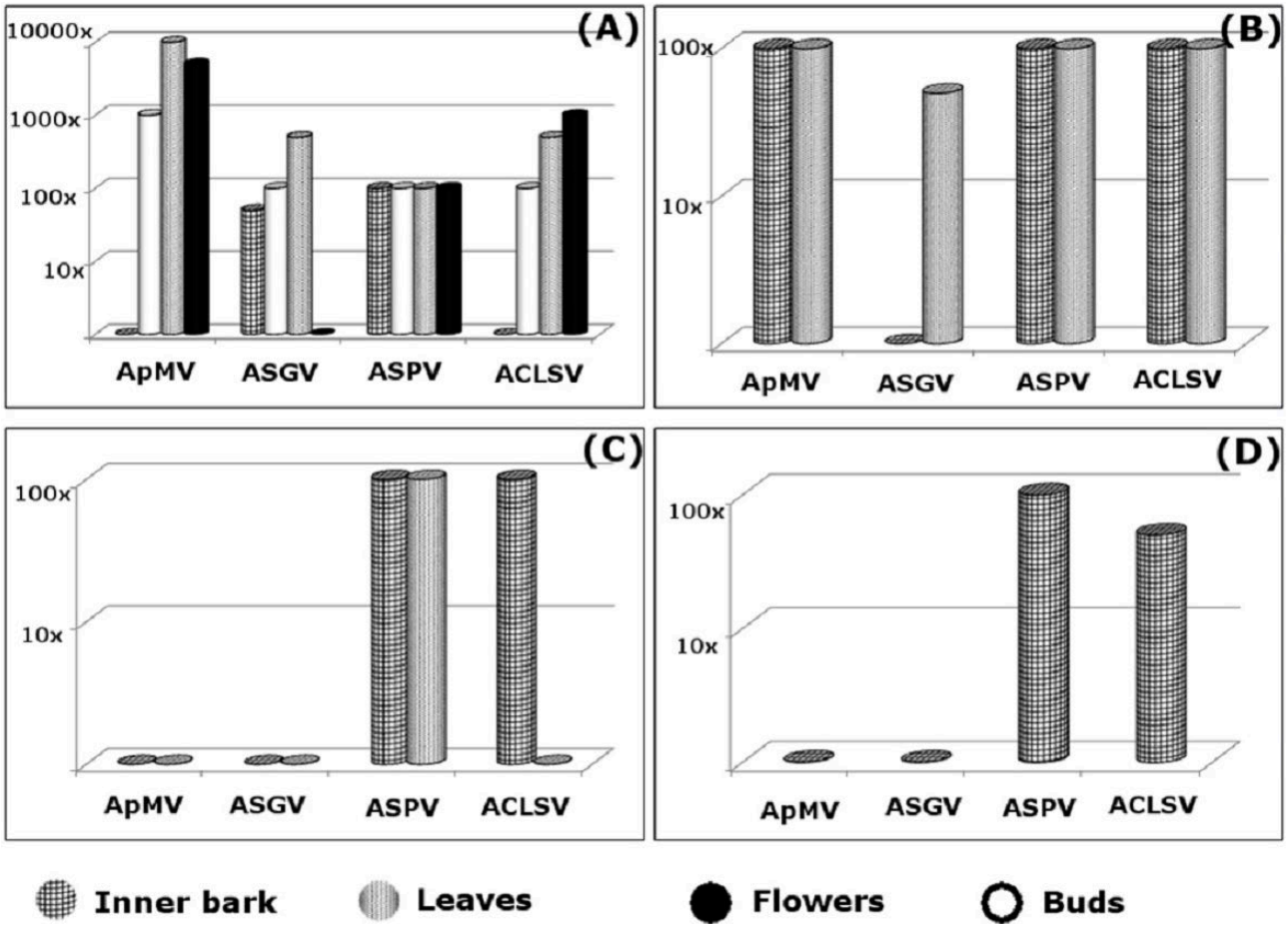

Figure 4. Comparison of the relative concentrations of *Apple mosaic virus*, *Apple stem grooving virus*, *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* in different plant tissues using DAS/I-ELISA throughout 2014. (A): spring; (B): summer; (C): autumn and (D): winter. The relative concentration is symbolized in the y-axis and is expressed by logarithm dilution series (10×, 50×, 100×, 500×, 1000×, 5000× and 10000×).

On the basis of RT-PCR results regarding virus accumulation in various plant tissues, ApMV was found in greatest concentrations in flowers and ASGV in leaves. ASPV exhibited similar virus amounts in all plant parts throughout the year as was demonstrated from the serological methods. ACLSV reached greatest concentrations in flower petals in March (Figure 5).

Discussion

Diagnoses of apple viruses, the causal agents of economically important fruit tree diseases, have been performed by biological indexing, serology, molecular hybridization or various PCR techniques (Mink *et al.*, 1971; Nemeth, 1986; Pasquini and Barba, 1991; Alrefai *et al.*, 1994; Candresse *et al*,. 1995; Jelkmann and Keim-Konrad, 1997; James, 1998; Karešová and Paprstein, 2001; Jelkmann, 2004). However, none of these procedures allows accurate assessment of virus concentrations throughout the year. Here, we outline development of a quantitative real time RT-PCR protocol that reliably measured the number of viral gRNA copies in RNA extracts from infected host tissues.

Based on our results from RT-qPCR quantification, the selected viruses were stable and did not substantially change in concentrations among differ-

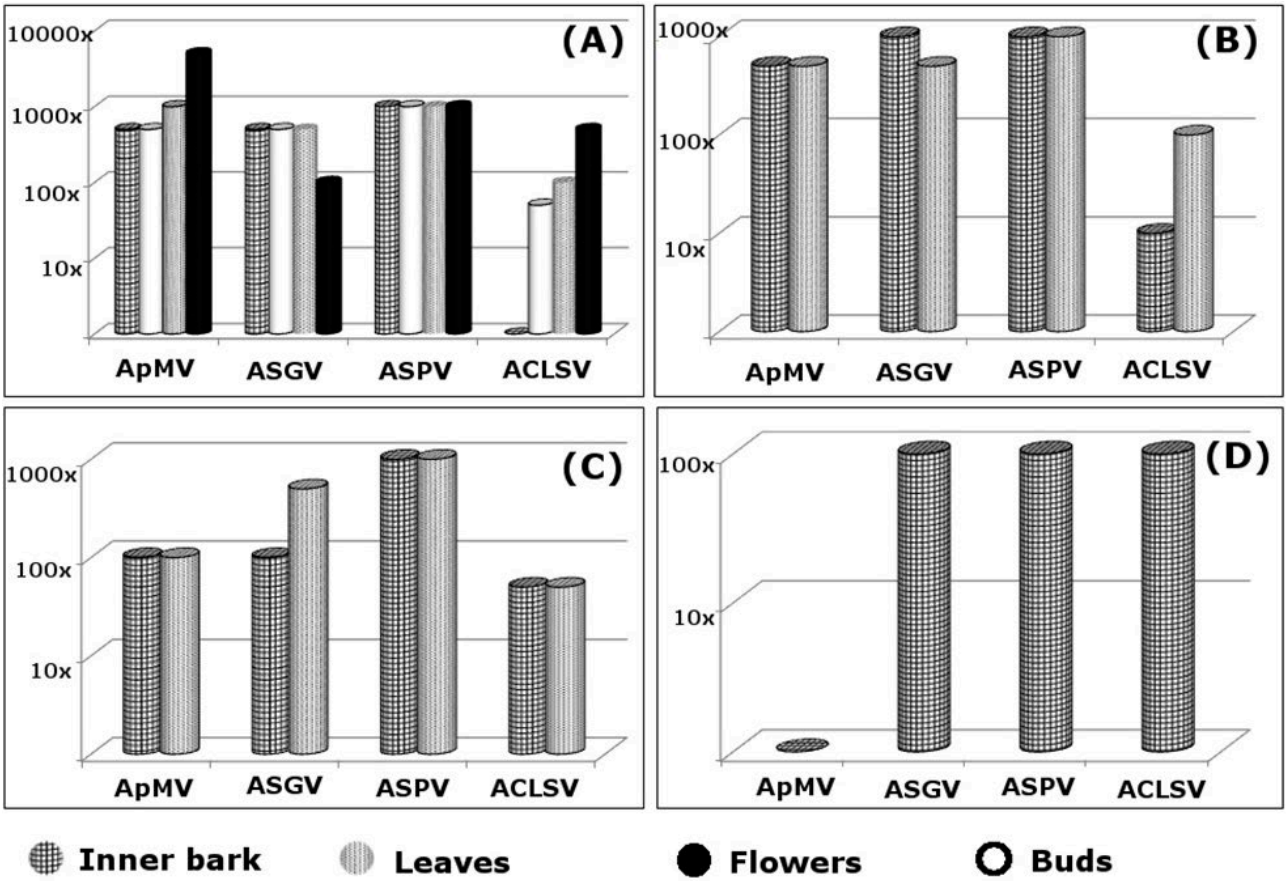

Figure 5. Comparison of the relative concentrations of *Apple mosaic virus*, *Apple stem grooving virus*, *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* in different plant tissues using RT-PCR throughout 2014. (A): spring; (B): summer; (C): autumn and (D): winter. The relative concentration is symbolized in the y-axis and is expressed by logarithm dilution series (10×, 50×, 100×, 500×, 1000×, 5000× and 10000×).

ent plant tissues during the year. Although the virus concentrations fluctuated slightly during vegetation seasons, these changes were not significant in most cases. The only exception was for ApMV and ACLSV, for which some differences in concentrations were observed. In the case of ApMV, noticeable differences in virus concentrations were observed among different plant tissues through the year. However, this divergence did not influence its detectability throughout the year. The concentration of ACLSV was more stable during vegetation; nevertheless, the virus accumulation in flowers was greater than in other plant parts of the trees, and its concentration in leaves was slightly greater during spring months. Fuchs *et al.* (1985) stated that the concentration of *Ilarviruses* and *Trichoviruses* fluctuated during the growing season and decreased in the summer due to high environmental temperatures. However, our results suggest that fluctuation in virus concentration is small, and thus the changes in virus content are unlikely to significantly influence the reliability of diagnostic tests.

Serological tests are reliable only during the short term of vegetation (Torrance and Dolby, 1984; Desvignes *et al.*, 1999; Kirby *et al.*, 2001), and accurate results were obtained only when young leaves and flowers were used as the sampling tissues in our tests. The conventional RT-PCR for virus detection from different plant tissues was more reliable than DAS/DASI ELISA, which is in accordance with the results of Kinard *et al.* (1996). They showed that the ACLSV and ASGV concentrations in the leaf canopy

of actively growing trees were nearly uniform. However, unlike Kinard *et al.* (1996), many authors have demonstrated considerable differences in ApMV, ASGV, ASPV and ACLSV accumulation in different plant tissues using RT-PCR (Kryczynski *et al.*, 1995; Menzel *et al.*, 2002; Ma *et al.*, 2008).

Conventional RT-PCR can be used for a longer period throughout the year than the serological methods (Mackenzie *et al.*, 1997; Kirby *et al.*, 2001; Menzel *et al.*, 2003; Spiegel *et al.*, 2006). From our results, however, reliability of conventional RT-PCR is significantly less than that of RT-qPCR. This discrepancy begs the question of what causes the decrease in sensitivity. Our results from RT-qPCR show that the differences in virus detection using serological methods or standard RT-PCR are due to something other used than changing virus concentrations in host tissues. Changes in pathogen detectability are likely to be influenced by more than one factor, and these factors could operate together. Therefore, the reason for the differences in sensitivity is likely to be complicated. Mackenzie *et al.* (1997) showed that despite the high sensitivity and specificity of PCR assays, many factors, such as RNA purity and primer specificity, influence diagnostic performance and cause false-negative results. Further, Ma *et al.* (2008) stated that inhibitors in plant extracts could interfere with the amplification processes during conventional RT-PCR, and could cause detection failures. Mitra and Kootstra (1993) proposed that one of many causes for detection failures is that woody plants contain many polyphenols and polysaccharides, which can interfere with the sensitivity of virus detection in ELISA tests. The inhibitory effects of these compounds, which are highly concentrated in apple trees (Schmitz and Noga, 2000) and might be still present in total nucleic acid extracts (Menzel *et al.*, 2002), can also be observed in standard RT-PCR where they may affect the reverse transcriptase and/or *Taq* polymerase (Demeke and Adams, 1992; Staub *et al.*, 1995 Pandey *et al.*, 1996). However, anything that decreases reliability of conventional RT-PCR most likely also influences RT-qPCR methods, which are significantly more reliable, probably thanks to their greater sensitivity (Mumford *et al.*, 2000; Weller *et al.*, 2000; Boonham *et al.*, 2002, 2004; Korimbocus *et al.*, 2002).

We anticipate that RT-qPCR will be a useful tool for virus detection in other non-*Malus* hosts, where viruses could be present at low concentrations and therefore have not been previously detectable using conventional methods. One of the chief limiting factors in the application of all PCR techniques in routine diagnostics lies in the preparation of high-quality nucleic acids, free of PCR inhibitors. This is particularly true for woody plants such as tree fruit varieties of the *Malus, Prunus,* and *Pyrus* genera (Korschineck *et al.*, 1991). In the case of apple trees, RNA isolation using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich Co.) was optimal for both PCR techniques, providing quality total RNA at greater concentrations than the silica-capture RNA isolation method (Rott and Jelkmann, 2001), which is normally used in our laboratory. However, commercial extraction kits using the selective binding properties of a silica-based membrane may not be suitable for plants rich in polyphenolic and polysaccharide compounds.

In conclusion, RT-qPCR is a simple and reliable procedure for detecting viral pathogens in apple trees throughout the year. Nevertheless, less sensitive serological and conventional RT-PCR techniques are still favoured over RT-qPCR for routine virus screening by diagnostic laboratories. One of the main reasons for this is because RT-qPCR assays require expensive specialised equipment. On the other hand, a real-time RT-PCR assay can be performed easily within one day, takes place in a single closed tube, does not require any post-PCR detection and therefore reduces the risk of carry-over contamination and misdiagnosis in routine use (Roussel *et al.*, 2005). If DAS/DASI ELISA or conventional RT-PCR are to remain the main diagnostic methods, they should be used in combination for accurate and specific detection of virus pathogens of apple.

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