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Optimization of tissue and time for rapid serological and molecular detection of *Apple stem pitting virus* and *Apple stem grooving virus* in apple'

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Abstract Majority of the apple trees are known to be infected by two latent viruses, *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV). The importance of ASGV and ASPV is due to their non expression of symptoms, worldwide occurrence and wide host range on pome and stone fruits. Due to their latent nature in apple, early and rapid diagnostics plays important role for production of virus free quality planting material. The present investigation was conducted to detect and quantify ASPV & ASGV from different plant parts (spatial) in apple trees during different seasons (temporal) for optimisation of tissue and time for their rapid and early detection. Detection and relative quantification using immuno-molecular diagnostic techniques like, Double Antibody Sandwich-ELISA, Reverse Transcription-PCR and Real Time RT-PCR in various plant parts (leaf, whole flower, sepal, petal, anther, stigma with style, bark, fruit, seed and root) during different seasons was done. The DAS-ELISA based detection revealed infection in all plant parts except root and fruit with ASGV and ASPV, showing more expression in leaves followed by bark and whole flower. Similar results were also observed on RT-PCR based detection. Quantitative real time PCR analysis showed variation in expression of ASGV and ASPV in different parts during different seasons. Results confirmed that the ASGV and ASPV expression is higher in leaves followed by bark and whole flower. Periodic detection of these viruses in different plant parts during all the four seasons revealed varied virus titer from one season to another in the same plant. During all the seasons, both ASPV and ASGV were detected in bark in measurable titer using immuno-molecular detection tools, however via DAS-ELISA, ASGV remained undetected during dormant season. Hence leaves and bark except leaf during fall, can be directly used as detection material for their early and rapid detection leading to production of virus free planting material.

Keywords Apple · ASGV · ASPV · Detection · Latent viruses · Real time PCR

Introduction

Apple (*Malus* × *domestica* Borkh.) is commercially most important, remunerative horticultural crop, widely grown in temperate regions of the world (Ferree and Warrington 2003). It is a member of family *Rosaceae* which includes many well-known genera with economically important fruits and berries. Apple is prone to several diseases caused by fungi, bacteria, viruses, viroids and phytoplasmas (Muneer et al. 2017). Among viruses and virus-like pathogens, the apple trees are known to be infected by *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and viroid, *Apple scar skin viroid* (ASSVd).

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The viruses ASGV and ASPV are two major latent viruses of apple which lead to significant economic damage and top working disease in apple (Campbell 1963; Yanase 1983; Wang et al. 2011; Grimova et al. 2016). Usually these viruses do not express visible symptoms in the infected trees, although the infection gradually leads to considerable reduction in yield and quality of fruits (Plese et al. 1975; Tiziano et al. 2003). Both viruses belong to family *Betaflexiviridae*, with ASGV belonging to genus *Capillovirus* and ASPV to the genus *Foveavirus* (Adams et al. 2012). ASPV infection could cause growth reduction in apple trees up to 10% and the yield loss may reach up to 30%, while being latent, the infection causes loss of 5–10% as the fruits get mature early and deformed by deep invaginations (Ajay et al. 2015). Both viruses are transmitted by grafting (Yanase 1983; Llacer et al. 1985; Katwal et al. 2016); hence the infection remains persistent in apple trees throughout their life (Yanase 1983). Apple trees are perennial and vegetatively propagated, thus diagnosis of viruses at earlier stages of plant growth becomes essential to avoid yield losses and prevent further spread through infected scion material. Major strategy for management of viral diseases in apple is via propagation of virus free planting material, which is highly dependent on quick and reliable detection of these viruses in mother plants. Therefore, reliable detection of viral infections especially latent viruses is of great importance not only in the apple virus diagnostics, but also in indexing plants for producing virus free planting material. Virus titer varies from tissue to tissue and during different seasons (Kundu et al. 2003). Therefore selection of suitable tissue at specific time provides proper diagnosis of these viruses. Based on these background informations, the present study was carried out to optimise the plant tissue and time for specific, quick, reliable detection of latent viruses in apple using immuno-molecular detection techniques.

Materials and methods

Sample collection

Different samples including roots, leaves, bark, whole flower, stigma, anther, fruit, seed etc. (Fig. 1), were collected from three already established virus infected apple plants (cv. Golden delicious) from the apple orchard of ICAR-Central Institute of Temperate Horticulture, Srinagar "during 2016-17 and 2017-18" for detection and quantification of apple latent viruses (ASPV & ASGV) using Double antibody sandwich-enzyme linked immunosorbent assay(DAS-ELISA) and Real Time RT-PCR, in different tissues and during different

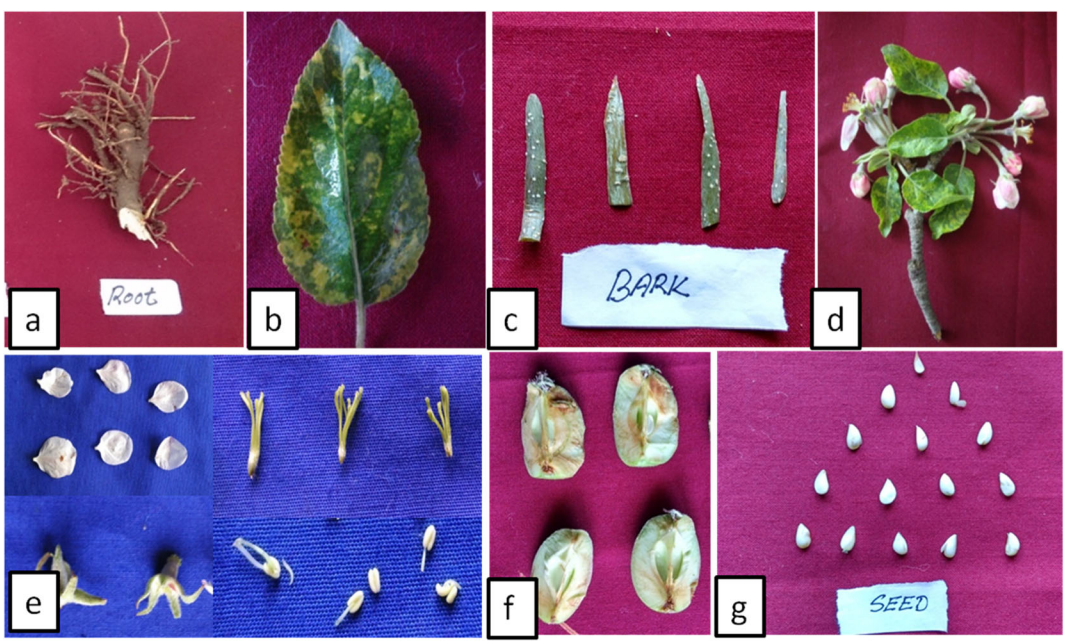

Fig. 1 Different tissues of suspected symptomatic apple tree for latent virus detection., **a** root, **b** leaf, **c** bark, **d** whole flower, **e** floral parts (petal, sepal, anther, stigma), **f** fruit, **g** seed

seasons, depending upon the availability of tissue in a particular season. The samples were collected randomly for ASGV and ASPV, because these are symptomless in most of the apple cultivars.

Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA)

Periodic detection of both viruses in different plant parts was done during the entire year. The samples were tested serologically as described by Clark and Adams (1977) both spatially as well as temporally using DAS ELISA kit (BIOREBA Switzerland). The results were assessed by measuring the absorbance at 405 nm wavelength using ELISA reader (BioTeK-ELX808). The ELISA optical density values served as a measure of relative virus concentration in different parts of apple tree. The ELISA readings were considered positive when the absorbance of sample wells was at least two times greater than the mean absorbance reading of negative control.

Total RNA extraction

Total RNA was extracted from 100 mg tissue, using plant total RNA Mini kit (Roche Life sciences) as described in the manual instruction. Quantitative and qualitative analysis of RNA was checked on Nanodrop (Themo scientific). Furthermore integrity of RNA was ascertained on 1% agarose gel (Kumar et al. 2014). Protocol for RNA isolation was same for all tissues**.**

Reverse transcriptase-PCR (RT-PCR)

RNA was first reverse transcribed into cDNA by using AMVRT cDNA kit using random hexamers, 0.5 mM dNTP, 40 U RNase Inhibitor and 40 U M-MLV Reverse Transcriptase for 55 min at 42 °C, with a final incubation at 70 °C for 10 min (Roche Applied Science, Penzberg, Germany). RT-PCR was performed in 25 µl total volume containing 5.5 µl of nuclease - free water, 4 µl (1 ng) of cDNA, 12.5 µl of one step PCR master mix (Roche Applied Science, Penzberg, Germany), 1.5 µl each of (10 µM) specific forward and reverse primers of Coat protein gene (CP) for ASPV with primer sequence F (5'-ATGTCTGGAACCTCATGCTGCAA-3′) R (5' TTGGGATCAACTTTACTAAAAGCATAA-3′) and replicase gene for ASGV as F (5'CATATGTT CACTGAGGCAAAAGCTG-3′), R (5'CGATCCAG AAACCCATCAAAGACTT-3′) (Kumar et al. 2014). The reactions were carried out in a thermo cycler (Takara Japan) using different programmes for coat protein gene of ASPV and replicase gene of ASGV, which are as follows: ASPV – 30 cycles at 94 °C for 45 s (denaturation) then 55 °C for 1 min (annealing) and 72 °C for 2 min (polymerization), and a final elongation step for 5 min. ASGV – 30 cycles at 94 °C for 30 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 1 min (polymerization), and a final elongation step for 10 min (Kundu et al. 2003). The PCR products were electrophoresed in 1.5% agarose gel in −0.5X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 V. For estimating amplicon size, 100 bp DNA molecular ladder was used (ABgene, UK) and electrophoresis was done for 1 h (Grimova et al. 2016). The fragments were observed under UV lamp in gel-documentation (Bio Rad, Gel Doc XR system 170–8170).

Real time PCR

The Real Time PCR was performed in 96-well plates with a Light Cycler 480 real-time PCR instrument (Roche Diagnostics) using the Light Cycler 480 SYBR Green I Master kit. Reactions were performed in triplicate, with each run having negative and positive controls. The master mix contained 5 µl SYBR Green I Master, 2 µl PCR-grade water, 2 µl cDNA, and 0.5 µl of each of the 10 µM forward and reverse coat protein and replicase gene-specific primer of ASPV & ASGV respectively, in a final volume of 10 µl reactions. The reactions were incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. The tubulin gene was used as the reference gene for both the primers (Radonic et al. 2004). Gene expression in the leaf was taken as a positive calibrator for relative quantification analysis. The Ct values of positive calibrator/control and the samples were normalized to the endogenous housekeeping gene tubulin. Relative gene expressions were determined according to the $\Delta\Delta C_t$ method, using the formula (Livak and Schmittgen 2001)

$2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = [\Delta]\ Ct_{sample} - [\Delta]\ Ct_{reference}$

$[\Delta]C_{t\ sample}$ -C_t value for any sample normalized to the endogenous housekeeping gene.

$[\Delta]C_{t\ reference}$ - C_t value for the reference sample normalized to the endogenous housekeeping gene.

Results

Virus detection using DAS-ELISA

Immunodiagnostic assay using DAS-ELISA confirmed the presence of ASGV and ASPV in all plant parts, except root and fruit. The DAS-ELISA values indicated virus titer was higher in leaves, bark and whole flower as compared to seed, fruit, sepal etc. of the apple tree. The virus titer varied from one season to another in the same plant in different tested tissues, during winter season only ASPV infection was observed in bark, while ASGV was not detected, during spring maximum infection of both the viruses was detected in leaves followed by bark and whole flower. During summer and fall viruses were detectable both in leaves and bark. Based on the DAS-ELISA results regarding virus titer in various plant parts, the highest accumulation of ASPV and ASGV was recorded in leaves at the beginning of the vegetation season, followed by flowers and bark. The presence of these two viruses along with their titer in different parts during different seasons is shown in Table 1.

Molecular detection through RT-PCR

Results obtained through DAS-ELISA were validated through RT-PCR. It was observed that the specific primers of coat protein gene of ASPV amplified 370 bp and replicase gene specific primer of ASGV amplified 200 bp amplicon from all tested samples except roots, fruits and healthy controls. Both the viruses were detected in leaves and bark for the whole vegetation period. The amplified products of ASPV and ASGV from different parts along with positive control are shown in Fig. 2 and Fig. 3.

Relative quantification through real time PCR

The Real Time-PCR method reliably detected ASPV & ASGV in all tested plant tissues, excluding roots throughout the year during which samples were assessed. Both the viruses in leaves, showed the highest titer at the beginning of the vegetation period (March to June), then slowly decreased in the fall, at the end of vegetation (in October). In the inner bark, the virus titer

Table 1 Detection of ASGV and ASPV in different plant parts and seasons using DAS-ELISA

Tissue	Virus	Dormant (December)	Spring (April)	Summer (July)	Fall (September)
Leaf	ASPV	N/A	+++	+++	+
	ASGV	N/A	+++	+++	+
Anther	ASPV	N/A	+	N/A	N/A
	ASGV	N/A	+	N/A	N/A
Flower	ASPV	N/A	++	N/A	N/A
	ASGV	N/A	++	N/A	N/A
Petal	ASPV	N/A	+	N/A	N/A
	ASGV	N/A	+	N/A	N/A
Bark	ASPV	+	++	++	+
	ASGV	–	++	++	+
Fruit	ASPV	N/A	N/A	–	–
	ASGV	N/A	N/A	–	–
Seed	ASPV	N/A	N/A	+	+
	ASGV	N/A	N/A	+	+
Sepal	ASPV	N/A	+	N/A	+
	ASGV	N/A	+	N/A	+
Positive control		+	+	+	+
Negative control		–	–	–	–

+: Low concentration, ++: Medium concentration and +++: High concentration, −: No concentration, N/A-Data not present

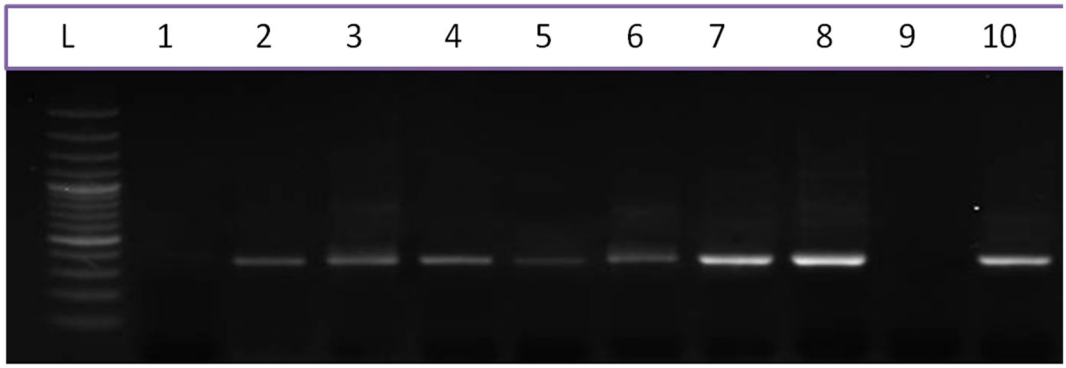

Fig. 2 Amplification of coat protein gene (370 bp) of ASPV in different plant parts during spring season, L-Ladder (100 bp),1-Root, 2-Leaf, 3-Flower, 4-Anther, 5-petal, 6-sepal, 7-seed, 8-Bark, 9-Fruit, 10-Positive Control

reached their peak in June and then decreased in the following months to the lowest value in December. Variable results were observed for relative quantification of ASPV and ASGV in different plant parts namely, sepals, anther, petals, bark, whole flower and seed with respect to leaf as positive calibrator (PC). The relative expression of both the viruses in different tissues is shown in Fig. 4a, b and Fig. 5. The season wise relative expression of ASGV and ASPV in different tissues is shown in Table 2.

Discussions

Apple is susceptible host to a wide range of pathogens including viruses, many of which are present in all apple-growing regions in the world (Way et al. 1990).Viruses in apple "especially latent viruses" can cause significant yield reduction, particularly when co-infection of several viruses occurs (Winkowska 2016). Apple virus detection has been done by various techniques namely, biological indexing, serology, molecular hybridization (Mink et al. 1971; Nemeth 1986; Candresse et al. 1995; Jelkmann 2004). In an attempt to reliably detect the presence of latent viruses in various parts of apple plants and to optimize tissue and season for early and round the year detection, different techniques viz., DAS-ELISA, RT-PCR, qRT-PCR, were used, which proved as valuable tools.

Our DAS-ELISA results confirmed presence of both the viruses in different parts of apple tree except root. As both the viruses were undetected in roots, which could be due to the use of virus free rootstocks like EMLA series (Richard 2008), as plants used for diagnosis in our study, were established on virus free rootstocks. Why the virus could not move from scion to stock, needs to be ascertained. The detection efficiency was observed highest in leaves and bark during all seasons, except for leaves during dormant season. The ASGV was earlier also detected from different plant parts i.e., bark, leaf buds, leaves and petals as reported by Rankovic and Vuksanovic (1983), Fuchs et al. (1979), Machita et al. (1986). Both the viruses were detected in suitable titer in leaves during spring season and are in conformity with results of Fuchs (1982). These findings were also

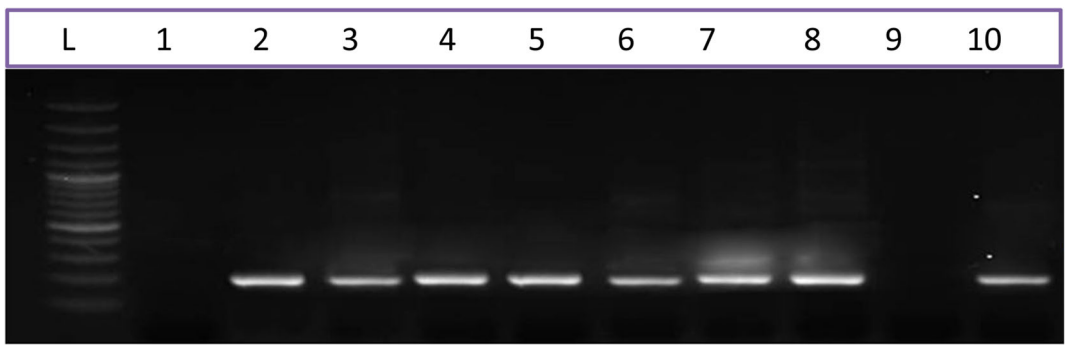

Fig. 3 Amplification of replicase gene (200 bp) of ASGV in different plant parts during spring season, L-Ladder (100 bp), 1-Root, 2-Leaf, 3-Flower, 4-Anther, 5-petal, 6-sepal, 7-seed, 8-Bark, 9-Fruit, 10-Positive Control

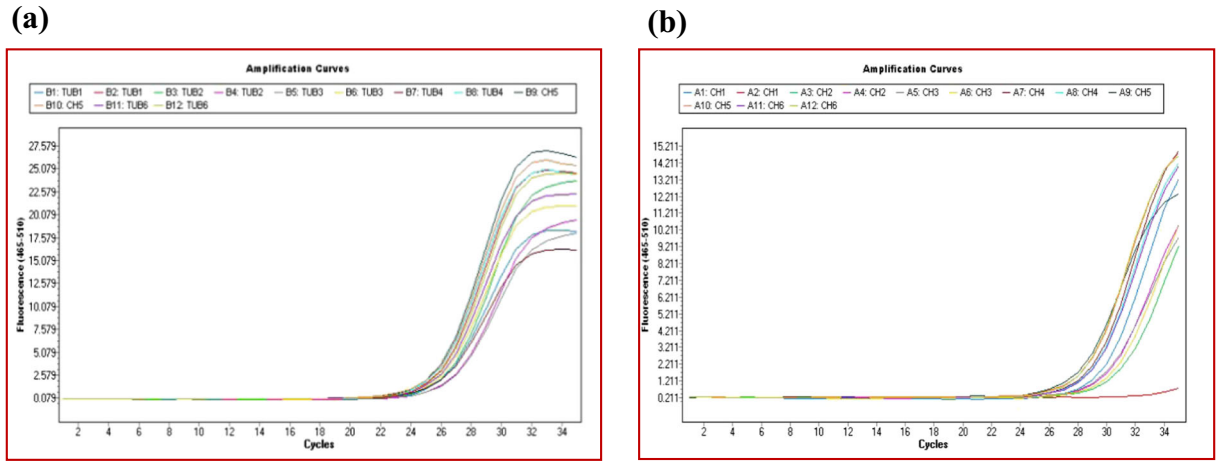

Fig. 4 **a**, **b** Amplification curves of ASPV (**a**) and ASGV (**b**) from different parts of apple tree

supported by Fuchs et al. (1979), Katwal et al. (2016). Corvo and Barros (2001) also confirmed ASGV from tissues like winter bud sticks, flowers, leaves and growing shoots including dormancy. During all the growing seasons, ASPV was detected in bark, but highest detection efficiency was observed during spring season, (April to June). The ASPV was also detected in leaves during three seasons except dormant season with high titer only during spring and summer season. In bark, only ASPV was detected during all tested seasons (Spring, Summer, Autumn and Winter) using DAS-ELISA as earlier also reported by Lucie et al. (2016). Similar studies conducted by, Flegg and Clark (1979), Kundu et al. (2003) and Svoboda and Polak (2010) also reported the suitability for use of different plant parts of an apple tree for detection of these viruses. As DAS-ELISA is more specific in nature, hence it is an effective and reliable detection method for diagnostics of apple latent viruses during all the seasons using bark and leaf as detection material.

The ELISA based techniques often fail, because of low virus titer, during fall and dormant seasons in plant parts like bark and leaves, due to the presence of various inhibitory compounds in the sap of woody plants (Caglayan et al. 2006) hence molecular approaches are most sensitive and reliable means for detection of plant viruses. In our study, the RT-PCR results confirmed both the viruses from all the tissues except root and fruit. Our results are in agreement with Fuchs (1982), where it was found that leaves and bark were more suitable tissues for detection of ASGV from January (bark and dormant buds) to mid-June (leaves) using RT-PCR. As the leaf material was observed most suitable tissue for RNA isolation, hence, provides highest detection efficiency in comparison to bark or any other tissue by using RT-PCR. The RT-PCR proved more effective and reliable due to its higher sensitivity as compared to DAS-ELISA. As ASGV was undetected in bark using DAS-ELISA, but was detected using RT-PCR, during dormant season (data not shown). Since RT-PCR is more

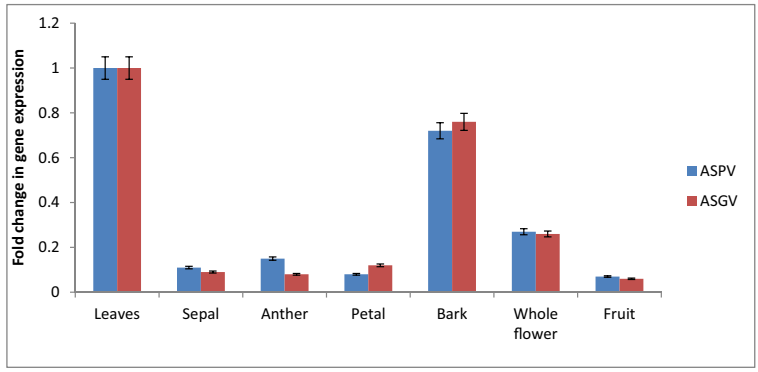

Fig. 5 Bar chart representing the relative expression of *CP* gene in ASPV and *Rep* gene in ASGV over tubulin gene during spring season, bars represent mean for three replications and error bars represent standard error of the mean

Table 2 Relative quantification of ASPV and ASGV in different tissues during different seasons

Tissue	Virus	Percent expression during Dormant Season	Real-time PCR mean C_t values	Percent expression during Spring Season	Real-time PCR mean C_t values	Percent expression during Summer Season	Real-time PCR mean C_t values	Percent expression during Fall Season	Real-time PCR mean C_t values
Leaves	ASPV	–	–	100(PC)	26.19	100(PC)	26.1	100(PC)	26.37
	ASGV	–	–	100(PC)	26.06	100(PC)	26.7	100(PC)	26.23
Anther	ASPV	–	–	15.05	26.43	–	–	–	–
	ASGV	–	–	8.7	25.86	–	–	–	–
Flower	ASPV	–	–	27.7		–	–	–	–
	ASGV	–	–	26.8		–	–	–	–
Sepal	ASPV	–	–	11.1	25.85	–	–	–	–
	ASGV	–	–	6.2	29.58	–	–	–	–
Petal	ASPV	–	–	8.31	26.43	–	–	–	–
	ASGV	–	–	12.6	25.86	–	–	–	–
Bark	ASPV	45.3	24.6	72.9	25.43	61.8	27.45	43.6	29.76
	ASGV	24.23	16.19	76.79	26.20	62.9	28.56	34.9	30.10
Fruit	ASPV	–		7.1	26.44	–	–	7.34	26.54
	ASGV	–		6.2	25.38	–	–	7.12	25.23
Seed	ASPV	–		–		7.3	27.19	7.79	27.45
	ASGV	–		–		11.6	23.04	12.8	22.34

specific, sensitive and reliable compared to serological methods (Lievens et al. 2005). Kundu et al. (2003) also corroborate with our results by detecting ASGV in all tissues (bark, dormant buds, petals and leaves) tested both by ELISA and RT-PCR from January to mid-June. The RT-PCR proved highly specific for the detection of ASPV and ASGV as the selected primers are specific for the detection of these viruses in apple trees (Kundu 2002). In general, the leaf tissues are suggested for the effective ASGV detection by both methods (RT-PCR and DAS-ELISA). While, during winter season bark can be used as detection material for both the viruses (ASPV and ASGV) due to unavailability of leaves. Similar results were also reported by Caglayan et al. (2006), Ajay et al. (2015) and Grimova et al. (2016).

The relative quantification revealed that both viruses were in higher titer in leaves, followed by bark and whole flower. Gadiou and Kundu (2012) found considerable change of temporal and spatial distribution of ASPV by evaluating two reference genes for relative quantification in apple trees and postulated that employing *GAPDH* and *S19* as housekeeping genes for accurate quantification of ASGV in apple leaf samples. Both RT-PCR and quantitative RT-PCR can be used sporadically and always in accordance with available biological material during growing season. From our study results revealed variation in virus detection, as both the viruses (ASPV & ASGV) during spring season were undetected in fruit both by DAS-ELISA as well as RT-PCR, during dormant season, ASGV was undetected from bark using DAS-ELISA, but were detected using Real time RT-PCR in both the seasons. Variation in pathogen detection is likely to be influenced by several factors, and these factors could operate simultaneously. Therefore, the reason for the differences in sensitivity is likely to be complicated. 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. The inhibitory effects of these compounds might be still present in total nucleic acid extracts (Menzel et al. 2002), which may affect the reverse transcriptase during RT-PCR (Demeke and Adams 1992; Pandey and Adams 1996. However, anything that decreases reliability of conventional RT-PCR is most likely to influence RT-qPCR methods, which are significantly more reliable, due to their greater sensitivity (Mumford et al. 2000; Weller et al. 2000). The relative quantification of both the viruses in different tissues during different seasons will

assist in selection of plant tissue for easy and rapid diagnosis.

Conclusion

Apple is susceptible to a number of pathogens including viruses, which can cause significant yield reduction, both quantitatively and qualitatively. Due to latent nature of ASGV and ASPV in apple, it becomes very difficult for nursery growers and orchardists to manage these viruses, hence there arises need for proper and reliable detection of these viruses at earlier stages. During the present investigation, both the viruses were found to be present in almost every part of plant. Among the detection techniques, qRT-PCR followed by RT-PCR and DAS-ELISA were found to be effective and reliable methods for detection during all the seasons. Among the plant parts tested, leaf and bark were found to be the best detection material for both the viruses.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of Interest.

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