Determination of Major Viral and Sub Viral Pathogens Incidence in Apple Orchards in Himachal Pradesh

Surender Kumar · Rahul Mohan Singh ·
Raja Ram · J. Badyal · Vipin Hallan ·
A. A. Zaidi · Anupam Varma

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Abstract Apple is the major commercial horticulture crop in Himachal Pradesh and other hill states of Jammu & Kashmir, Uttarakhand and some parts of Northeastern states of India. In order to gather data on health status and incidence of virus and virus-like pathogens in apple orchards, survey was conducted in the month of June and September, 2010 in Hatkoti, Rohru, Kuthara, Jubbal and Khadapathar areas of major apple producing Shimla district of Himachal Pradesh. A total of 250 samples were collected and analyzed by DAS-ELISA, NASH and RT-PCR. NASH results indicated that a total of 117 samples were infected with *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple scar skin viroid* (ASSVd). Results showed that ASSVd is predominant in these areas with highest infection rate of 27.6% followed by ASPV (17.2%), ACLSV (16.8%), ApMV (15.2%) and ASGV (12%). Mixed infection of these viruses and viroid was frequently detected in apple trees in Himachal Pradesh. The trees, which were positive

for viruses and viroids, showed a variety of fruit deformation and rusting symptoms besides leaf deformation, mosaic and chlorosis.

Keywords ASSVd · ApMV · ACLSV · ASGV · ASPV · NASH · DAS-ELISA

Apple is grown worldwide, with India at seventh position in apple production. Himachal Pradesh, also known as fruit state of India, contributes 88% of the total fruit production in the country (<http://hpmc.gov.in>). The area under apple plantation is 97,423 ha and annual production is 2,80,105 MT (<http://nhb.gov.in/database-2009.pdf>). Annual production (quantity and quality) of perennial fruit crops depends on both biotic and abiotic factors. Among biotic factors, apple is susceptible to infection by bacteria, fungi, phytoplasma, viruses and viroids. Among viruses and virus-like pathogens, majority of the 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 *Apple scar skin viroid* (ASSVd), the major viroid.

Apple chlorotic leaf spot virus (ACLSV), family *Flexiviridae*, genus *Trichovirus*, positive sense ssRNA, approximately 7.5 kb genome with polyA tail [20] infects most rosaceous fruit tree species and is responsible for severe graft incompatibilities in some *Prunus* combinations [4]. *Apple mosaic virus* (ApMV), one of the oldest known and widespread virus belongs to *Ilarvirus* [5], family *Bromoviridae*. ApMV has positive-sense ssRNA genome divided into three components designated as RNA 1, 2 and 3, and also contains a sub-genomic RNA 4 encoding for coat protein [16]. The virus is economically very important and spreads by infected propagating material [12] with infection resulting in reduced production and tree decline

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S. Kumar · R. M. Singh · R. Ram · V. Hallan (✉) · A. A. Zaidi
Plant Virology Lab, CSIR-Institute of Himalayan Bioresource
Technology, Palampur 176061, HP, India
e-mail: rnaivi@gmail.com

J. Badyal
Department of Horticulture, CSK Himachal Pradesh Krishi
Vishvavidyalaya, Palampur 176062, HP, India

A. Varma
Advanced Centre for Plant Virology, Division of Plant
Pathology, IARI, New Delhi, India

[3]. *Apple stem grooving virus* (ASGV), type member of the genus *Capillovirus*, family *Flexiviridae* has flexuous filamentous particles, 600–700 nm long. ASGV genome consists of a single stranded polyadenylated, positive sense RNA of ~6.5 kb [19]. It is generally associated with severe pitting and grooving of the xylem, brown line and graft union abnormalities, reduced vigor of the canopy and an overall decline in susceptible *Malus* species with significant yield losses [18]. Like ASGV, ASPV is also a latent virus which develops symptoms when inoculated on woody indicators and on sensitive rootstocks. ASPV has single stranded, positive sense RNA as the genome which is polyadenylated at 3' end with size ranging from 8.4 to 9.3 kb according to the virus species [7]. ASSVd belongs to family *Pospoviridae* having circular ssRNA of ~330 bp. Symptoms induced by ASSVd on fruits include distortion, color dappling or chlorosis, scarring and cracking [8]. Except ApMV all other pathogens remains latent effecting plant health and production [14]. Symptom severity increases on fruit ripening. Areas which are responsible for planting material and scion wood production were focused in this study. A total of 250 samples were collected randomly from different orchards located in Shimla district and were analyzed for the presence of major apple viruses and viroid by DAS-ELISA, NASH and RT-PCR. Further, the effect of these pathogens on positive plants was observed at the time of fruit maturity. Major aim of this study was to inspect this major apple fruit, root stock and scion producing area systematically.

Samples (250) were collected in June, 2010 from Hatkoti, Rohru, Kuthara, Jubbal and Khadapthar areas of Shimla district, Himachal Pradesh. Samples were collected randomly (with or without symptoms), taking into consideration their rootstock and scion. After testing samples for above mentioned pathogens, another survey was carried out in month of September to observe their effect both on plant and fruit. Nucleic acid hybridization was performed by the procedure as described in Laboratory manual [17]. Samples were crushed in TNE buffer (100 mM Tris; 2.0 M NaCl, 10 mM EDTA; pH 7.4), centrifuged at 6000 rpm/3m and supernatant was used for tissue blot on nitrocellulose membrane. The DNA probes labelled with α -P³²dCTP were synthesized from the cloned coat protein gene of respective viruses and partial genome of ASSVd cloned in TA vector (RBC, Taiwan). Probe extension was carried in robocycler (Stratagene, USA) 37°C for 60 min. with Klenow enzyme. Hybridization was carried out and the membrane was exposed to X-ray film, kept in a cassette and placed in deep freezer for 24 h. It was then developed and photographed using gel documentation system (Alpha Digi DocTM, USA). Whole protocol was followed as described previously [17]. All samples were analyzed by antibody based diagnostic tools for ACLSV, ApMV,

ASGV and ASPV (Bioreba, Switzerland) using DAS-ELISA [2]. Samples were tested in triplicate and OD reading $\geq 3 \times$ the negative control was taken as positive.

All ELISA positive, along with some negative and, suspected viroid samples were analyzed by RT-PCR. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen, Germany). Reverse transcription was carried out in a 25 μ l reaction mixture with 7 μ l total RNA (1–2 μ g), 1.0 μ l of 200 nM reverse primer, 1.0 μ l of 40 mM dNTP mix (Fermentas, Lithuania), 5 μ l of 5 $\times$ RT buffer and 100 units of M-MuLV Reverse Transcriptase (USB Corporation, USA). The reaction mixture was incubated at 37°C for 75 min followed by 80°C for 5 min. PCR was performed in a 50 μ l reaction mix containing 5 μ l of 10 $\times$ Taq Buffer A (Genei, India), 1.0 μ l dNTP mix (10 mM), 1 μ l each of forward and reverse primers, Taq DNA polymerase (1.5 U) (Genei, India) and 5 μ l cDNA. PCR reaction was performed in a thermal cycler (G-Storm GS2, Gene Technologies, UK). PCR products were electrophoreses on 1% Agarose gel with 1 μ g/ml of ethidium bromide in 1 $\times$ TAE buffer. Detail of primers, their target region, annealing temperature and time with references has been described (Table 1). To obtain desired amplification, amplification was carried at 72°C for 40 s for all pathogens. Other PCR conditions remained same as for general protocol as per Table 1. After PCR amplified product was fractionated on 1% agarose gel by electrophoresis, amplicons were cut and DNA was eluted with gel elution kit (Promega, Madison, USA). The eluted product were ligated in TA cloning vector (RBC, Taiwan) and cloned into *Escherichia coli* DH5 α and sequenced using ABI prism Big DyeTM Terminator v3.1 Ready Reaction Cycle sequencing Kit (Applied Biosystems, USA).

Shimla district is one of the largest producers of apple in HP, India and is responsible for rootstock and scion production for distribution to farmers. Orchards were surveyed in this district, 250 samples were collected and analyzed with ELISA, NASH and RT PCR and 46.8% of plants were found infected with viral and subviral pathogens. Various rootstocks and scion combination tested were MM-106 bearing various scions Starkrimson (SC), Silver Spur (SP) on MM-106, Bright and Early (B&E) on MM-106, Vance (VS) on MM-109, Vance (VS) on MM-111, Oregon Spur (OS) on MM-109 and MM-111, Red Chief (RC) on EM-7A, M26 Bright and Early (B&E), and MM-106 Top Red (TR). In all combinations, viral and subviral pathogens were detected, implying that no combination was resistant or free to these pathogens. A total of 117 samples were found infected, of which 37.6% were infected with one, 66.3% infected with two, 22.9% with three, 6.4% with four and 2.7% samples were infected with all mentioned pathogens. Their analysis by ELISA resulted in identification of 50 positive samples showing infection with

Table 1 List of primers, their target region, cycling conditions and product size

Target	Primer name	Primer sequence	Annealing temperature (°C) and time (s)	Product size (bp)	References
Partial genome	ASSVd	PBCV194H: 5'-TGTCCTCGCTAGTCGAGCGGA-3' PBCV100C: 5'-AGACCCCTTCGTCGACGACGA-3'	55/30	214	[13]
CP	ApMV	CpF: 5'-CTCAAGCGAACCCGAATAAGGGTAAGAA-3' CpR: 5'-TCGTCGATAAGTAGAACATTTCGTCGGTATTGTC-3'	57/35	547	This study
CP	ACLSV	Up: 5'-CAGACCCCTTCATGGAAAGACAG-3' Dn: 5'-TGACTCTTTATACTCTTTTCATGGGTTC-3'	58/35	645	This study
Replicase gene	ASGV	F: 5'-CATATGTTCACTGAGGCAAAAGCTG-3' R: 5'-GGATCCAGAAAACCCATCAAAGACTT-3'	58/30	198	This study
CP	ASPV	Sense: 5'-ATGTCTGGAACCTCATGCTGCAA-3' Antisense: 5'-TTGGGATCAACTTTACTAAAAGCATAA-3'	57/30	370	[10]

Fig. 1 **a** Apple stem grooving virus detected in apple samples by slot blot hybridization. Lanes 1–6, 9, 15–16 were positive whereas 17–18 contain positive control and lane 10 contains negative control. **b** ASSVd detection in apple samples by dot blot hybridization. Lanes

15–16, 20, 24–25, 27, 31, 34, 36, 39, 41, 42, 44–45 were found positive. Lanes 1 and 2 contain negative control whereas lanes 47 and 48 contain positive control

ACLSV, ASPV, ApMV and ASGV (13.3%, 8.8%, 7.7% and 5.5% respectively). Maximum samples were found positive for ACLSV while ASGV was the least prevalent (ELISA). Among all the 250 samples, 117 samples were found positive by NASH (Supplementary Table 1, Fig. 1)

for various viruses and viroids tested. ASSVd was predominant with an incidence of 27.6% followed by ASPV (17.6%), ACLSV (16.8%), ApMV (15.2%) and ASGV (12.8%). Comparing results of NASH and ELISA, it was observed that most of these pathogens have low titer and

Fig. 2 Symptoms as observed on the sampled trees during June and September; photos **a–c** symptoms on leaf and fruit during initial developmental stage during June, whereas **d–f** shows observed symptoms on mature fruits during September. **a** Distorted fruits. **b** Distorted leaves. **c** Mosaic like pattern on leaves. **d** Scarring. **e** Distortion. **f** Poor and uneven color development on fully mature

apple fruits. Sample **a** and **d** was found infected with ASPV, ASSVd and ApMV (S. No. 2 in Supplementary Table 1); **b** and **e** were found infected with ASGV, ASSVd, ApMV and ACLSV (S. No. 28 in Supplementary Table 1) whereas samples **c** and **f** were found infected with ASPV, ASSVd and ACLSV

this could be the possible reason for successful detection of ACLSV in higher percentile as compared to ASPV, which were successfully detected by molecular methods (NASH and RT-PCR), also symptom severity depends on many physical and environmental factors.

Results of NASH (Fig. 1) and ELISA were validated by RT-PCR (Supplementary Fig. 1). In addition to 30 ELISA positive samples, 20 samples which were ELISA negative were also tested and of these 16 were found positive reiterating that nucleic acid based methods offer better sensitivity. Expected amplification was obtained for ACLSV (~645 bp), ApMV (~547 bp), ASPV (~370 bp), and ASSVd (~212 bp) and for ASGV (~198 bp) (as shown in Supplementary Fig. 1). Sequenced data was deposited in EMBL database under accession numbers FR750244 for ASPV, FR750245 for ASGV, FR750246 for ApMV, ACLSV under Accession No. 750247 and HE601745 for ASSVd. Submitted sequences showed maximum identity at nucleotide level with Indian isolates for ASPV, ASGV, ApMV up to 99%, FR694922; 98%, FN565167; 99%, FN564150 respectively, whereas ACLSV and ASSVd showed maximum identity with Canadian and Chinese isolates (91%, GQ334190; 98% HM367077). Previously, workers [15] attempted to characterize these pathogens, along with *Prunus necrotic ring spot virus*, *Arabis mosaic virus* (ArMV), and *Tomato ringspot virus* (ToRSV) from Shimla, Solan, Kullu, Mandi, Kangra, Chamba, Kinnaur

(HP) and Srinagar in J&K and reported incidence of 19.7% for ACLSV, 13.9% for ASGV, ASPV (9.3%), ArMV (9.3%), PNRSV (4%), ASSVd (5%) and ApMV only in M9 root stock. They also reported the presence of ToRSV in cv. Royal Delicious in Kangra and Kullu Districts. In another study, workers determined the incidence of *Apple dapple viroid* in Shimla district by return page and further on symptoms observed on fruits, observing only 2% viroid incidence [6]. The results presented in this report reflect a much higher incidence of the viroid. There can be several reasons for the difference in incidence mainly a much more sensitive NASH and RT-PCR methodology and it can also be concluded that the incidence is continuously increasing in the region. The infected trees showed a variety of symptoms on various parts including fruits when surveyed in September. Symptoms included mosaic and chlorotic pattern, necrotic spots, pitting and grooving on stems, mottled and reduced leaves size, various deformities on fruits (Fig. 2). In H.P. this region were known for quality and quantity production of apple, besides main source of root stock and scion wood for other parts of state and other states like J&K and Uttarakhand. As these pathogens are transmitted mechanically or by grafting infected scion or rootstock, therefore incidence study of this region holds great significance so as to identify virus free planting material for scion wood material. Viral and subviral pathogen free planting material is necessity both for quantity

and quality production, because all these were well known agents for crop loss as reported from various parts of world. According to another study [1] ApMV infection alone is able to cause 46% yield reduction in Golden Delicious crop where as yield loss increase in mix infection (ApMV and Rubbery wood disease agent) results in up to 67% losses. Another study reported the effect of ACLSV, ASGV on Golden Delicious cv. and reported 17% reduction in yield and 49% reduction in profit margin [10]. Infection effects not remain limited to yield but also responsible for 15.2–70% tree loss [21]. Study of economic implication of virus free planting material in USA indicated that the total avoidable losses due to various viruses for apple fruit and planting material industries was \$63,406,789 [1]. Outcome of such study helps to emphasize the importance of awareness programs towards good orchards practices and to check viral and subviral pathogens spread from plant to plant and orchard to orchard. In this study molecular methods for viral and sub viral pathogen detection (RT-PCR and NASH) for early diagnostics was optimized, which helps in effective diagnostics both in vitro and in vivo of apple orchards and nurseries. Also results suggests the need for development of reliable, fast and cost effective molecular method which detects these major pathogens in one reaction like multiplex reverse transcription-polymerase chain reaction [11] or the use of polyvalent probe [9]. Three methods were described for detection of viral pathogens and two for subviral pathogen detection. Results showed that RT-PCR and Dot/Slot Blot hybridization and much more sensitive in comparison to ELISA for large number of samples.

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