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# Research Article
# Prevalence and Serological Detection of Apple Viruses in Himachal Pradesh

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## Abstract

Investigations were conducted to record the incidence of viruses in apple orchards of the state along with serological detection by employing ELISA. Seventeen apple orchards surveyed in Shimla, Kinnaur and Kullu district of HP revealed the presence of viral infection with an incidence ranging from 5.41-92.18%. Direct Double Antibody Sandwich (DAS ) ELISA assays resulted in detection of ACLSV, ApMV, ASGV and ASPV in apple trees of different commercial cultivars being grown in different orchards. The ACLSV, ApMV, ASGV and ASPV were found to be present in serologically detectable limit in leaves, bark and petals parts of an infected plant. However, leaf sample drawn during April and May, bark during August and petals taken during April found to contain highest relative concentration of these viruses based on the OD values obtained in serological reactions. About 50% of the diseased plants revealed the presence of two or more than two viruses, thus indicating prevalence of mixed infection.

Key words: Apple viruses, survey, cultivars, plant parts, DAS-ELISA

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## INTRODUCTION

Apple is an important fruit crop being grown throughout the temperate hilly regions of India and plays a pivotal role in the economy of growers due to their commercial importance. Tremendous progress has been made in the field of temperate horticulture mainly in apple production in Himachal Pradesh, which is also known as "Apple State of India". The total area and production of apple in India is 313.04 thousand ha and 2497.68 thousand Mt, respectively. Jammu and Kashmir is the leading state in both area and production with 160.87 thousand ha and production of 1647.69 thousand Mt. The area under apple in Himachal Pradesh is 107.69 thousand ha and production is 738.72 thousand Mt. Production is hampered by a Number of diseases including viral etiology, which are of unique nature in their importance (Thakur *et al*., 2009). The Apple Chlorotic Leaf Spot Virus (ACLSV) is a member of genus Trichovirus in the Flexiviridae family (Martelli *et al*., 1994). These are flexuous filamentous particles 680-780 nm long and 12 nm in width containing linear positive-sense and ssRNA. It occurs in woody plants of the family Rosaceae. The Apple Mosaic Virus (ApMV) is a member of subgroup III of the Ilarvirus genus in the Bromoviridae family. The virus possesses a tripartite, positive-sense, single-stranded RNA genome encapsulated by a CP of approximately 25-30 kDa (Brunt *et al*., 1990). The Apple Stem Grooving Virus (ASGV) is a member of genus Capillovirus. It is flexuous filamentous particles of 600-700x12 nm in size. It was reported in *Malus sylvestris* cv. virginia crab, from the USA (Lister *et al*., 1965). The genome is unipartite RNA. The Apple Stem Pitting Virus (ASPV) particles are flexuous filamentous having size of 1250 nm long with ssRNA genome of 9306 nucleotides (Jelkmann, 1994). Like fungal and bacterial pathogens, viral diseases cannot be managed by chemical treatment and moreover their infection passes to successive generations through vegetatively propagating planting material (Cieszlinska and Malinowski, 2002; Pupola *et al*., 2011) resulting in decline of plant health and poor productivity over a No. of years. Unlike fungal and bacterial diseases, viruses and related organisms causing the infection are systemic in nature and symptoms depend on the variety, type and concentration of the agent. Viral diseases cause significant economic damage in orchard plants because there is no effective safety net of viral diseases. Only healthy, virus and related organisms (phytoplasma, viroid, rickettsia) free plants can produce high yields of quality fruit (Stankiene *et al*., 2012). Therefore, selection and propagation of disease free planting material is the only way to tackle such problems and this is dependent on the technology for quick and reliable detection of viruses and related pathogens.

## MATERIALS AND METHODS

**Survey of apple orchards:** Surveys were conducted in different apple growing areas of Himachal Pradesh to record virus disease incidence along with marking of virus infected trees of important commercial cultivars such as royal delicious, golden delicious, tydeman's early worcester, red chief and vance delicious through visual indexing and symptoms observed on the apple plants. Orchards were surveyed in each district and randomly hundred trees per orchard were taken for recording the disease incidence. During surveys diseased trees of different cultivars were also marked for collection of bud-wood and maintenance of cultures at experimental block of Department of Plant Pathology, UHF, Solan.

During surveys good quality and apparently healthy trees of important commercial cultivars namely royal delicious, golden delicious, tydeman's early worcester, red chief etc., were marked especially in the orchards of selected registered nursery growers at Kotkhai, Jubbal and Rohroo (Shimla district), Seobagh and Naggar (Kullu district) and Rekong Peo (Kinnaur district) for further indexing by using ELISA. The commercial cultivars of apple being grown in apple orchards of HP were first screened visually to mark the apparently healthy trees by discarding the trees exhibiting the symptoms of virus or virus-like diseases for further serological indexing. However, the detection of ApMV, ACLSV, ASGV and ASPV were made in the orchards of Kotkhai, Jubbal and Rohroo (Shimla district), Seobagh and Naggar (Kullu district) and Rekong peo (Kinnaur district) areas of commercial cultivars like royal delicious, red gold, red chief, golden spur, vance delicious, red fuji, granny smith, shallet spur, gale gala, scarlet spur, super chief, top red, silver spur, tydeman's early worcestor and oregon spur.

**Collection and maintenance of virus isolates:** Cultures of the virus isolates representing different localities were maintained under isolated field conditions in the experimental block of Department of Plant Pathology, UHF, Solan. Through grafting of budwood taken from infected apple trees on to the healthy seedlings, they were done in the month of March and raised at the farm at a distance of 60×30 cm.

**Serological detection of viruses in different isolates through direct-DAS form of ELISA:** Leaves exhibiting typical symptoms on plant cultures of each isolate were taken from the field and

brought to the laboratory in separate polythene bags for the serological detection of ACLSV, ApMV, ASGV and ASPV. Alkaline phosphatase based double antibody sandwich enzyme-linked immunosorbent assay was used to detect the virus as per the protocol of the supplier (BIOREBA Switzerland). The plates were kept in humid box in the dark condition at room temperature after giving a brief incubation of 15 min at 37°C. The plates were incubated at room temperature until a yellow colour was clearly visible in the positive controls (usually between 30 and 90 min). If desired reaction was stopped by adding 50 µL of 3 M NaOH to each well (the components were mixed by agitating the plate carefully). The results were assessed either by measurement of the absorbance value of the hydrolyzed substrate (p-nitrophenyl) at 405 nm wavelength in a microtitre plate reader. The results of the ELISA for detection were interpreted by Lemmetty (1988).

**Serological detection of ACLSV, ApMV, ASGV AND ASPV in different plant parts through DAS-ELISA:** Double antibody sandwich ELISA procedure was used to detect the viruses in leaf, bark, fruit pulp, fruit peel and petal of ACLSV, ApMV, ASGV and ASPV from infected plants. The samples from leaf, bark, fruit pulp, petal and fruit peel were drawn throughout, the year at different months i.e., March-October for leaf, January-December for bark, August for both fruit peel and fruit pulp and April for petals.

## RESULTS

**Survey of apple orchards:** Surveys were conducted to determine the occurrence and distribution of different virus es affecting apple plantation in Shimla, Kinnaur and Kullu district of Himachal Pradesh. Typical viral symptoms were seen on different cultivars of apple viz., royal delicious, golden delicious, kesari, golden spur, red gold, rich-a-red and vance delicious. Symptoms like chlorotic leaf spot, necrotic lesions, mosaic and puckering of leaves were exhibited by the diseased trees of different cultivars of apple. Chlorotic leaf spot and mosaic mixed with necrotic lesions were recorded to be the predominant symptoms in most of the cultivars at all the location surveyed. During surveys in different orchards (Table 1), it was found that most of the different cultivars viz., royal delicious, golden delicious, kesari, red chief, golden spur, red gold, rich-a-red and vance delicious were carrying symptoms of virus diseases as evident from the information presented in Table 2.

Table 1: Disease incidence of apple virus in different districts of Himachal Pradesh

| District | No. of orchard (s) | Village (location) | Disease incidence (%) |
|---|---|---|---|
| Shimla | 1 | Darbar (Kotkhai) | 42.54 |
| | 2 | Dhangvi (Kotkhai) | 69.03 |
| | 2 | Chainthla (Kotkhai) | 50.41 |
| | 2 | Tehtoli (Kotkhai) | 92.18 |
| | 1 | Shahoon (Kotkhai) | 31.76 |
| | 2 | Bathara (Sarahan) | 43.17 |
| | 1 | PCDO Annu (Jubbal) | 18.25 |
| | 2 | Pangla (Jubbal) | 90.30 |
| | 2 | Karalash (Rohroo) | 21.03 |
| | 1 | KVK (Rohroo) | 15.42 |
| Kinnaur | 4 | Kalpa (Rekong Peo) | 23.38 |
| | 1 | PCDO Bocktu (Rekong Peo) | 90.71 |
| | 3 | Pangi (Rekong Peo) | 16.43 |
| Kullu | 2 | Seo bagh (Kullu) | 11.89 |
| | 1 | Barteli (Manali) | 80.45 |
| | 1 | Dawari (Manali) | 60.09 |
| | 2 | Dhabi (Manali) | 12.57 |

**Collection and maintenance of virus isolates:** Budwood from the symptomatic trees marked in Shimla, Kinnaur and Kullu districts were collected during dormant stage (January-Febuary). Grafting of budwood from 45 virus isolates so, collected on healthy apple seedling was carried out to record the transmission as well as for maintaining the isolates. Observations on graft transmissibility presented in Table 2 reveals that all the isolates were graft transmissible with the production of typical viral symptoms such as chlorotic leaf spots, necrotic lesions, mosaic and puckering of the leaves. These isolates have been maintained in isolation at experimental farm of Department of Plant Pathology for further studies on serological detection of viruses infecting apple.

**Serological detection of viruses in different isolates through DAS-ELISA:** For the detection of apple viruses, DAS ELISA method was performed in test samples. The information provided in Table 3 clearly indicated that out of these 45 virus isolates 21 virus isolates (C1, C2, C6, C7, C8, C9, C12, C17, C18, C19, C20, C21, C22, C23, C33, C35, C36, C37, C41, C42 and C45) were found positive for ACLSV. Among these 45 virus isolates, 12 virus isolates (C10, C11, C13, C18, C19, C22, C23, C27, C28, C32, C34 and C39) were found positive for the presence of ApMV and 8 virus isolates (C2, C3, C5, C7, C8, C10, C14 and C21) were found positive for the presence of ASGV and ASPV. Observations recorded on the detection of ACLSV, ApMV, ASGV and ASPV in different isolates through the use of DAS ELISA are presented in Table 3. Serological reaction indicated positive detection of one or another virus in single or mixed

Table 2: Virus isolates collected from different locations of Himachal Pradesh

| Isolate | Location | District | Cultivar | Symptoms |
|---|---|---|---|---|
| C1 | Annu | Shimla | Royal delicious | Chlorotic leaf spots |
| C2 | Annu | Shimla | Royal delicious | Mosaic and necrotic lesion |
| C3 | Pangi | Kinnaur | Golden delicious | Mosaic and necrotic lesion |
| C4 | Pangi | Kinnaur | Golden delicious | Mosaic and necrotic lesion |
| C5 | Pangi | Kinnaur | Golden delicious | Mosaic and necrotic lesion |
| C6 | Kalpa | Kinnaur | Royal delicious | Chlorotic leaf spots |
| C7 | Kalpa | Kinnaur | Royal delicious | Chlorotic leaf spots |
| C8 | Kalpa | Kinnaur | Royal delicious | Puckering and chlorotic spots |
| C9 | Jubbal | Shimla | Royal delicious | Chlorotic leaf spots |
| C10 | Jubbal | Shimla | Golden delicious | Mosaic and necrotic lesion |
| C11 | Jubbal | Shimla | Golden delicious | Mosaic and necrotic lesion |
| C12 | Jubbal | Shimla | Golden delicious | Mosaic and necrotic lesion |
| C13 | Kotkhai | Shimla | Kesari | Mosaic |
| C14 | Kotkhai | Shimla | Golden delicious | Mosaic and necrotic lesion |
| C15 | Nihari | Shimla | Red delicious | Mosaic and necrotic lesion |
| C16 | Kotkhai | Shimla | Seedling | Mosaic and necrotic lesion |
| C17 | Nihari | Shimla | Golden delicious | Mosaic and necrotic lesion |
| C18 | Kotkhai | Shimla | Kesari | Mosaic |
| C19 | Kotkhai | Shimla | Red chief | Chlorotic leaf spots |
| C20 | Kotkhai | Shimla | Red chief | Chlorotic leaf spots |
| C21 | Kotkhai | Shimla | Red chief | Chlorotic leaf spots |
| C22 | Kotkhai | Shimla | Royal delicious | Mosaic and necrotic lesion |
| C23 | Kotkhai | Shimla | Golden spur | Chlorotic leaf spots |
| C24 | Kotkhai | Shimla | Golden delicious | Chlorotic leaf spots |
| C25 | Kotkhai | Shimla | Golden spur | Mosaic and necrotic lesion |
| C26 | Nihari | Shimla | Red gold | Mosaic and necrotic lesion |
| C27 | Sarahan | Shimla | Golden delicious | Mosaic and necrotic lesion |
| C28 | Kotgarh | Shimla | Red delicious | Mosaic and necrotic lesion |
| C29 | Kotgarh | Shimla | Red gold | Mosaic and necrotic lesion |
| C30 | Jubbal | Shimla | Royal delicious | Mosaic and necrotic lesion |
| C31 | Jubbal | Shimla | Royal delicious | Mosaic and necrotic lesion |
| C32 | Jubbal | Shimla | Golden delicious | Mosaic and necrotic lesion |
| C33 | Jubbal | Shimla | Red delicious | Mosaic and necrotic lesion |
| C34 | Jubbal | Shimla | Rich-a-red | Mosaic and necrotic lesion |
| C35 | Barteli | Kullu | Golden delicious | Chlorotic leaf spots |
| C36 | Manali | Kullu | Golden delicious | Chlorotic leaf spots |
| C37 | Dawari | Kullu | Royal delicious | Mosaic and necrotic lesion |
| C38 | Manali | Kullu | Royal delicious | Mosaic and necrotic lesion |
| C39 | Manali | Kullu | Royal delicious | Mosaic and necrotic lesion |
| C40 | Manali | Kullu | Royal delicious | Mosaic and necrotic lesion |
| C41 | Manali | Kullu | Royal delicious | Chlorotic leaf spots |
| C42 | Manali | Kullu | Royal delicious | Chlorotic leaf spots |
| C43 | Manali | Kullu | Golden delicious | Mosaic and necrotic lesion |
| C44 | Dhabi | Kullu | Golden delicious | Mosaic and necrotic lesion |

infection manner in case of 30 isolates. Out of these 30; 16 isolates (C1, C6, C9, C11, C12, C13, C17, C20, C32, C35, C36, C39, C41, C42, C44 and C45) were carrying infection of only one virus, whereas 14 isolates (C2, C3, C5, C7, C8, C10, C14, C18, C19, C21, C22, C23, C33 and C37) were carrying infection of two or more than two viruses, which resulted in depiction of mixed infection. Out of 45; 15 isolates (C4, C15, C16, C24, C25, C26, C27, C28, C29, C30, C31, C34, C38, C40 and C43) did not show any positive reaction with the antibodies of ACLSV, ApMV, ASGV and ASPV. Mixed infections involving more than two viruses were of common occurrence in a large No. of apple plants. Serological indexing has been done for the selection of virus free clones of important commercial cultivars of apple.

**Serological detection of ACLSV, ApMV, ASGV and ASPV in different plant parts through DAS-ELISA:** Periodic detection of all the four viruses in different plant parts was done during the entire year. The ACLSV was detectable in low concentration in March, August, September and October months in leaves, while in bark it is also detectable in June month. Petals, fruit peel and fruit pulp didn't detected ACLSV

Table 3: DAS-ELISA based serological reaction of different virus isolates against ACLSV, ApMV, ASGV and ASPV

| Isolate | Location | District | Cultivar | ACLSV | ApMV | ASGV | ASPV | Mixed infection |
|---|---|---|---|---|---|---|---|---|
| C1 | Annu | Shimla | Royal delicious | + | - | - | - | Absent |
| C2 | Annu | Shimla | Royal delicious | + | - | + | + | Present |
| C3 | Pangi | Kinnaur | Golden delicious | - | - | + | + | Present |
| C4 | Pangi | Kinnaur | Golden delicious | - | - | - | - | - |
| C5 | Pangi | Kinnaur | Golden delicious | - | - | + | + | Present |
| C6 | Kalpa | Kinnaur | Royal delicious | + | - | - | - | Absent |
| C7 | Kalpa | Kinnaur | Royal delicious | + | - | + | + | Present |
| C8 | Kalpa | Kinnaur | Royal delicious | + | - | + | + | Present |
| C9 | Jubbal | Shimla | Royal delicious | + | - | - | - | Absent |
| C10 | Jubbal | Shimla | Golden delicious | - | + | + | + | Present |
| C11 | Jubbal | Shimla | Golden delicious | - | + | - | - | Absent |
| C12 | Jubbal | Shimla | Golden delicious | + | - | - | - | Absent |
| C13 | Kotkhai | Shimla | Kesari | - | + | - | - | Absent |
| C14 | Kotkhai | Shimla | Golden delicious | - | - | + | + | Present |
| C15 | Nihari | Shimla | Red delicious | - | - | - | - | - |
| C16 | Kotkhai | Shimla | Seedling | - | - | - | - | - |
| C17 | Nihari | Shimla | Golden delicious | + | - | - | - | Absent |
| C18 | Kotkhai | Shimla | Kesari | + | + | - | - | Present |
| C19 | Kotkhai | Shimla | Red chief | + | + | - | - | Present |
| C20 | Kotkhai | Shimla | Red chief | + | - | - | - | Absent |
| C21 | Kotkhai | Shimla | Red chief | + | - | + | + | Present |
| C22 | Kotkhai | Shimla | Royal delicious | + | + | - | - | Present |
| C23 | Kotkhai | Shimla | Golden spur | + | + | - | - | Present |
| C24 | Kotkhai | Shimla | Golden delicious | - | - | - | - | - |
| C25 | Kotkhai | Shimla | Golden spur | - | - | - | - | - |
| C26 | Nihari | Shimla | Red gold | - | - | - | - | - |
| C27 | Sarahan | Shimla | Golden delicious | - | - | - | - | - |
| C28 | Kotgarh | Shimla | Red delicious | - | - | - | - | - |
| C29 | Kotgarh | Shimla | Red gold | - | - | - | - | - |
| C30 | Jubbal | Shimla | Royal delicious | - | - | - | - | - |
| C31 | Jubbal | Shimla | Royal delicious | - | - | - | - | - |
| C32 | Jubbal | Shimla | Golden delicious | - | + | - | - | Absent |
| C33 | Jubbal | Shimla | Red delicious | + | + | - | - | Present |
| C34 | Jubbal | Shimla | Rich-a-red | - | - | - | - | - |
| C35 | Barteli | Kullu | Golden delicious | + | - | - | - | Absent |
| C36 | Manali | Kullu | Golden delicious | + | - | - | - | Absent |
| C37 | Dawari | Kullu | Royal delicious | + | + | - | - | Present |
| C38 | Manali | Kullu | Royal delicious | - | - | - | - | - |
| C39 | Manali | Kullu | Royal delicious | - | + | - | - | Absent |
| C40 | Manali | Kullu | Royal delicious | - | - | - | - | - |
| C41 | Manali | Kullu | Royal delicious | + | - | - | - | Absent |
| C42 | Manali | Kullu | Royal delicious | + | - | - | - | Absent |
| C43 | Manali | Kullu | Golden delicious | - | - | - | - | - |
| C44 | Dhabi | Kullu | Golden delicious | - | + | - | - | Absent |
| C45 | Kotkhai | Shimla | Vance delicious | + | - | - | - | Absent |
| Positive control | | | | + | + | + | + | |
| Negative control | | | | - | - | - | - | |

concentration during the season (Table 4). However, in April, May, June and July, there is medium to high concentration of ACLSV in leaves. The ApMV was detected in low concentration in leaves collected in April and May and also in petals in the month of April, while there was no detectable amount of ApMV concentration was observed in bark, fruit peel and fruit pulp. The ASGV was detectable in medium concentration in April and May, while it was low in June and also detectable in bark during April and May in low and medium concentration, respectively. Petal is also a source of virus detection, which was collected in the month of April, while in fruit peel and fruit pulp there is no ASGV concentration was found. The ASPV was also detected in leaves in April and May in low concentration and in bark the detectable amount of virus was present in May. Petal was also a source of low concentration of ASPV, which was collected during April month. Fruit peel and fruit pulp was free of all the viruses. The DAS-ELISA using different plant parts during the entire season confirmed the presence of all the four viruses in different tissues (Table 4). Similar studies were conducted by Clark and Adams (1977), James

Table 4: Detection of apple viruses in different plant parts by using DAS-ELISA

| | | Mean OD values ($A_{405\ nm}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Jan. | Feb. | Mar. | Apr. | May | June | July | Aug. | Sep. | Oct. | Nov. | Dec. |
| Plant part | Virus | (months) | | | | | | | | | | | |
| Leaf | ACLSV | - | - | + | +++ | +++ | ++ | ++ | + | + | + | - | - |
| | ApMV | - | - | - | + | + | - | - | - | - | - | - | - |
| | ASGV | - | - | - | ++ | ++ | + | - | - | - | - | - | - |
| | ASPV | - | - | - | + | + | - | - | - | - | + | - | - |
| Bark | ACLSV | - | - | - | - | - | + | - | - | - | - | - | - |
| | ApMV | - | - | - | - | - | - | - | - | - | - | - | - |
| | ASGV | - | - | - | + | ++ | - | - | - | - | - | - | - |
| | ASPV | - | - | - | - | + | - | - | - | - | - | - | - |
| Petal | ACLSV | - | - | - | - | - | - | - | - | - | - | - | - |
| | ApMV | - | - | - | + | - | - | - | | - | - | - | - |
| | ASGV | - | - | - | + | - | - | - | - | - | - | - | - |
| | ASPV | - | - | - | + | - | - | - | - | - | - | - | - |
| Fruit peel | ACLSV | - | - | - | - | - | - | - | - | - | - | - | - |
| | ApMV | - | - | - | - | - | - | - | - | - | - | - | - |
| | ASGV | - | - | - | - | - | - | - | - | - | - | - | - |
| | ASPV | - | - | - | - | - | - | - | - | - | - | - | - |
| Fruit pulp | ACLSV | - | - | - | - | - | - | - | - | - | - | - | - |
| | ApMV | - | - | - | - | - | - | - | - | - | - | - | - |
| | ASGV | - | - | - | - | - | - | - | - | - | - | - | |
| | ASPV | - | - | - | - | - | - | - | - | - | - | - | - |
| Positive control | | + | + | + | + | + | + | + | + | + | + | + | + |
| Negative control | | - | - | - | - | - | - | - | - | - | - | - | - |

-: No concentration, +: Low concentration, ++: Medium concentration and +++: High concentration

(1999), Corvo and Barros (2001), Kundu *et al*. (2003) and Svoboda and Polak (2010) also reported the presence of apple viruses in different plant parts except ApMV for which petal samples drawn during April or May were more suitable for reliable detection.

## DISCUSSION

Apple is one of the most important temperate fruit crops of India and is mainly grown in temperate regions of North-western Himalayan states like Himachal Pradesh, Jammu and Kashmir, Uttarakhand, hill districts of Uttar Pradesh, Arunachal Pradesh and Sikkim. Its cultivation has revolutioned the socio-economic conditions of hilly farmers and has become the number one commercial fruit crop of Himachal Pradesh. It is affected by a number of fungal and bacterial diseases, beside these diseases, viral diseases are also of economic importance and reduce the yield of the crop to some extent. The present studu was conducted on the ELISA based serological detection of viruses infecting apple in the state in addition to record viral disease incidence in different orchards and indexing of apple trees to work virus tested healthy mother trees.

During surveys of 17 orchards situated in Shimla, Kinnaur and Kullu district, it was found that virus infected trees were present in all the orchards with varying incidence ranging from 5.41-92.18%. Prominent symptoms associated with ailing trees were chlorotic spots, mosaic along with necrotic lesions, puckering and some distortions of leaves. Such types of symptoms are reported to be prevalent on apple trees due to infection of viral pathogens (Nemeth, 1986). Fidan (1994) surveyed the apple orchards in Turkey and reported that 15.45% of apple trees were infected with ApMV, 28.6% were infected with ACLSV and 23.6% were infected with ASGV. Padder *et al*. (2011) surveyed apple orchards in Kashmir valley and found the prevalence of ApMV in different districts of Jammu and Kashmir State. Kumar *et al*. (2012) reported that ASPV (17.2%), ACLSV (16.8%), ApMV (15.2%) and ASGV (12%) were present in different locations of Shimla district of Himachal Pradesh through DAS-ELISA, NASH and RT-PCR. Routine surveys conducted in different apple growing areas of Himachal Pradesh revealed that the apple plantation is marred by the virus infections in different orchards. Mixed infection of these viruses was frequently detected in apple trees in Himachal Pradesh (Thakur *et al*., 2009). The trees, which were positive for viruses and viroids, showed a variety of fruit deformation and rusting symptoms besides leaf deformation, mosaic and chlorosis. Variable incidence were observed in different orchards, which might be due to the inadvertent use of budwood for raising planting material (nursery plants) without taking into consideration health status of the mother trees. Keeping in view the infection of viruses in apple

orchards it becomes essentially important to use virus free propagation material for raising nurseries. There is a need to strengthen the certification programs just like those that exists in many developed countries of the world (Nemeth, 1986), so as to get rid of viral infections in future plantation of apple in different orchards.

For serological studies, budwood from 45 naturally infected trees of apple existing in different orchards were collected to maintain isolates (cultures) in an isolated field of the department through grafting on healthy apple seedlings all the 45 isolates produced typical symptoms of viruses, thus indicated the transmissible nature of the incitant (s). Serological probing by using antisera against ACLSV, ApMV, ASGV and ASPV in both DAC and DAS-ELISA resulted in successful detection of these viruses. The ELISA techniques because of its reliability, rapidity and sensitivity are commonly used all over the world for the detection and identification of ACLSV, ApMV, ASGV and ASPV. These DAC and DAS forms of ELISA have been used by different workers all over the world for detection of viruses not only in apple but in other fruit crops also (Fuchs *et al*., 1979; Detienne *et al*., 1981; Adams *et al*., 1984; Tozzi *et al*., 1995; Akbas and Ilhan, 2005; Ylmaz *et al*., 2005). The DAS-ELISA is specific and issued for closely related strains (Hampton *et al*., 1990).

Similarly, DAS-ELISA used under present studies also resulted in detection of ACLSV, ApMV, ASGV and ASPV in 21, 11, 8 and 8 isolates, respectively. As stated earlier, DAS-ELISA is specific and is being widely used not only for detection of viruses but also employed in various indexing and certification programs (Fridlund, 1989; Tozzi *et al*., 1995; Turk, 1996). Moreover, all the commercially available ELISA kits also contain reagents with major emphasis on DAS-ELISA based protocols. In the present investigation DAS ELISA was used to detect the presence of ACLSV, ApMV, ASGV and ASPV in different plant parts (leaves, bark, petals, fruits peel and fruit pulp) of an infected plant along with recording of relative concentration of the test virus in these tissues during different period of the year. These viruses were found to be present in the detectable limit in leaf during active growth period and in bark tissues throughout the year. All these viruses were easily detectable in flower petals during the bloom period. However, in fruit pulp and peels ACLSV and ApMV were detected but not ASGV and ASPV. However, keeping in view the concentration of virus in these plant parts, leaf sample drawn during June month, bark tissue taken during September and petals in April seems to be highly suitable for the serological detection of infection of ACLSV. For ApMV leaf sample drawn during April and May month and petals in April seems to be highly suitable for the serological detection of infection. For ASGV leaf sample drawn during June month, bark tissue taken during September and petals in April seems to be highly suitable for the serological detection of infection. For ASPV leaf sample drawn during July month, bark tissue taken during October and petals in April, seems to be highly suitable for the serological detection of infection. Flegg and Clark (1979), Fuchs (1982, 1983), Rankovic and Vuksanovic (1983), Adams *et al*. (1984), Llacer *et al*. (1985), Machita *et al*. (1986), Cieslinska *et al*. (1995), Topchiiska (1995), Svoboda and Polak (2010) and Kundu *et al*. (2003) also conducted the similar studies and reported the suitability of the use of different plant parts of an apple tree for the detection of these viruses.

Present studies, on the serological detection of ACLSV, ApMV, ASGV and ASPV infecting in apple also revealed the presence of mixed infection of two or more than two viruses. Out of 45 isolates serologically probed, 14 isolates showed the presence of mixed infection of viruses (Savino *et al*., 1991; Tozzi *et al*., 1995; Myrta *et al*., 1995; Caglayan *et al*., 2006). Therefore, it is likely to come across with mixed infection of viruses in apple trees, which may result in the production of variable symptoms as observed during surveys in the present studies. So, it is important to focus on the detection of multiple infections of viruses in any indexing and classification program. Under natural conditions mixed infection of viruses in apple is of common occurrence as reported by other workers from different parts of the world. Caglayan *et al*. (2006) from Turkey reported that among the mixed infection of apple viruses, the most common was ASPV+ACLSV (84.21%), ASPV+ASGV (36.84%), ACLSV+ASGV (26.32%) and ASPV+ApMV (5.26%).

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