DISCUSSION
Discussions

5.1 SURVEYS AND VIRUS INCIDENCE ON POME AND STONE FRUITS IN HP AND J&K

Viruses cause direct damage to the infected plants by flower distortion, reduced fruit production, distortion of fruit and finally decreasing market value of the yield. Viral diseases are thus of huge concern to both farmers and scientists due to severe loss in quality and quantity of yield produced. Identification and characterization of virus is the first step in virus management. The problem is escalated because of lack of definite control measures and potent chemicals effective against them. ACLSV alone causes 30-40% losses in apple fruit yield in addition to reducing the productive age and rendering the crop more susceptible to other pathogens and insect pests (Ahlawat and Chenulu 1986; Azad and Sehgal 1958; Cembali et al., 2003; Wu et al., 1998). Identification and characterization of virus is the first step in virus management. During the present investigation incidence of ACLSV was estimated based on visual symptoms, serological methods, RT-PCR, slot blot hybridization and at molecular level through cloning and sequencing of complete coat protein gene.

5.1.1 Based on Visual Symptoms

Characteristic viral symptoms of leaf mosaic, severe chlorosis, deformation, curling etc. were common in apple orchards. While plum, cherry, peach/nectarine, almond, cherry and apricots exhibited various symptoms like yellow flecking on leaves, mosaic, mottling, enations, necrotic ring spots and shot holes. The symptoms were not persistent throughout the year, and disappeared late in the season making the plant seem healthy. ACLSV was difficult to identify based on symptoms, as it is a latent virus (Nemec, 1967; Németh, 1986a). However, characteristic leaf chlorosis, deformation and curling were observed in some sensitive cultivars (Golden Delicious and Gala Mast). The symptoms were present discontinuously (one branch healthy other symptomatic) in many apple trees. However, most of the apple trees were apparently healthy and no diagnostic symptoms were observed. An estimated disease incidence of ACLSV infection on apple, other pome and stone fruits based on visual symptoms would be misleading due to latent infections. However, estimated percent
disease incidence based on visual symptoms (Table 4.2) was highest for apple (50%) and pear (40%).

5.1.2 Based on Enzyme Linked Immunosorbent Assay (ELISA)

With the introduction of ELISA tests in plant sciences by Clark and Adams (1977) the ability of plant pathologists to detect and study plant viruses has increased tremendously. ELISA has been found to be a best way for large scale routine testing of virus due to its rapid, cost effective, simple and fast assay properties. Procedures for the identification of ACLSV pose specific problems in apple owing to higher concentration of phenolics (Nassuth et al., 2000; Singh et al., 2002) in apple ultimately interfering with release of virus and its detection. The erratic distribution of virus in plant system coupled with the presence in low concentration adds to the problem (Barbara and Clark, 1982). Workers have used ELISA for preliminary detection of ACLSV and other related viruses, for estimating the disease incidences and the mixed viral infections in various other pome and stone fruits (Van der Meer, 1976; Cambra et al., 1982; Lla´cer et al., 1986, 1997; Savino et al., 1995 Caglayan et al., 2006; Klerks et al., 2001; Myrta et al., 2004; Kundu and Yoshikawa, 2008).

According to the proposed plan extensive surveys (2007-2009) of pome and stone fruit growing areas in HP (21 locations) and J&K (4 locations) were conducted. DAS-ELISA was performed to check the presence of virus in 290 apple plants (90-symptomatic and 200-apparently healthy). More than half of the healthy samples were latently infected with one or more of the viruses. ACLSV came across as a major virus on apple with disease incidence based on ELISA of upto ~75 % in India. The change in concentration of ACLSV titre in bark, buds, flowers, young and mature leaf stage was very evident. ACLSV detection was more reliable in spring season (March-April) using flower petals, buds and young leaves in apple, other pome and stone fruits. Even ACLSV positive samples gave ambiguous or negative ELISA readings in dormant season (July- September). Similar observations about young leaves and flower/buds being best source for ACLSV detection are present (Fuchs, 1983; Bucharest Minoiu et al., 1990; Varveri and Bem, 1997; )The decrease in concentration of virus may be due to erratic distribution of virus in plant system coupled with seasonal changes. The change of viral concentration with season was mentioned earlier by Flegg and Clark (1979), Fuchs (1981, 1983), Adams et al.
Discussions

The decrease in ACLSV concentration may also be attributed to activation of some defense mechanisms in plants against viral infection, although the details were not confirmed in this direction. Due to mixed cultivation of fruit trees ACLSV was also detected in other pome (pear, quince) and stone fruits (plum, peach, apricot, almond, cherry). Some wild plants (wild Himalayan cherry, wild apricot) also tested positive for ACLSV.

Other important viruses infecting apples viz. ApMV, ASGV, ASPV, PNRSV, PPV were also tested as ACLSV in nature occurs mostly in mix infection. ApMV was also best detected with flowers while ASPV and ASGV could be detected throughout the year using mature leaves and to some extent using bark also.

Among other viruses, ASPV followed by ASGV were major viruses on apples. Symptoms of mosaic on apple leaves were most common in HP. However, ApMV infection was more prevalent in cultivars from J&K in comparison to cultivars from HP. The reason could be use of older cultivars and relatively low temperature conditions (as ApMV is thermolabile) in J&K.

5.1.3 Standardization of Diagnostics

5.1.3.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Limitations of ELISA to detect virus may be overcome by the use of RT-PCR technique (Jelkmann, 1994; MacKenzie et al., 1997), which is more sensitive than ELISA and can detect as low as 50-200ng concentration of virus (Kinard et al., 1996). Many of the earlier workers (Menzel et al., 2002; Choi et al., 2003; MacKenzie et al., 1997; Kulshrestha, 2005; Faggioli and Ragozzino, 2002, Caglayan et al., 2006) have already used RT-PCR for confirming the presence of ACLSV and other apple viruses/viroid(s) on various pome and stone fruits.

In the present study RT-PCR was performed using virus specific primers (ASPV, ACLSV, ASGV, ApMV) and indigenously designed primers for ACLSV to confirm presence of various viruses. All the ELISA positive cultivars and most of the apparently healthy samples were also found positive by RT-PCR. This was for the first time that incidence of these viruses (ACLSV, ASGV and ApMV) was confirmed on various pome and stone fruits from India at molecular level. An apple viroid (Apple scar skin viroid, ASSVd) was also confirmed at molecular level from
India for the first time. PCR was more sensitive than direct probing or serological techniques for detecting and characterizing ACLSV. Similar observation was made by Hadidi et al. (1995) for various plant pathogens.

5.1.3.1 *Slot Blot Hybridization for Diagnosis*

Slot blot hybridization using specific DNA probes is an alternative technique for identification and detection of viruses. It is a very sensitive technique and has found to detect virus concentration in pg compared to μg in ELISA (Boonekamp et al., 1990). Already sequenced ACLSV-CP from apple was labeled with α<sup>32</sup>-P dATP and used as probe in hybridization. Apart from the samples ELISA positive some new samples in apple (Scarlet Gala from CITH), Almond (Solan) and Cherry (Shimla) not positive by ELISA also tested positive by hybridization. Similarly more positive samples (apart from ELISA positive samples) were identified by using radiolabelled probes of ASGV, ApMV and PNRSV (Table 4.10).

The percentage of infection observed through slot blot hybridization was greater than ELISA which confirmed that slot blot hybridization is more sensitive method than ELISA. Similar observations were mentioned by Knapp et al. (1995a). This method has the added advantage of being able to localize virus within the plant (Mansky et al., 1990; Chia et al., 1995; Knapp et al. (1995b).

5.2 VIRUS TRANSMISSION AND HOST RANGE

5.2.1 Virus Transmission

In this study virus transmission studies were performed by mechanical inoculation of some plant samples showing maximum and borderline ELISA values were selected.

5.2.1.1 *Mechanical Transmission of ACLSV*

During the present study ACLSV was transferred mechanically by sap inoculation on to the indicator herbaceous plants like *Chenopodium quinoa*, *C. amaranticolor*, *Vigna sinensis* (var. Chitlidana) and *Phaseolus vulgaris*. Hosts like *Cucumis sativus*, *Gomphrena globosa*, *Dianthus barbatus*, *Lycopersicon esculentum*, *Nicotiana glutinosa*, *Cymopsis tetragonoloba* and *N. tabacum* were also tested. After mechanical transmission necrotic and/or chlorotic local lesions appeared on inoculated leaves followed by systemic chlorosis and leaf deformations 8-12 days post inoculation on some herbaceous host range plants indicating that the virus was
transmitted mechanically. Similar observations and method of transmission has been reported by many groups (Cation and Carison, 1960; Lister et al., 1964; Hillegonda, 1967; Chairez and Lister, 1973a, b; Walt and Engelbrecht, 1974; Mink, 1989a; Brunt et al., 1996).

5.2.2 Host Range and Symptomatology

Twenty-five plant species belonging to six families were used for host range studies. Of these 16 plant species only five were found to be susceptible for ACLSV infection. After back inoculation from these plants local lesions were produced on C. quinoa and/ or P. vulgaris. Few ACLSV isolates also showed chlorosis on some of the Nicotiana spp. (Fig 4.8). All the symptomatic N. benthamiana plants were ELISA positive for ACLSV but did not confirm by RT-PCR at molecular level.

Host range studies (for six isolates India11, 13, 15, 16 [apple]; India27 [cherry] and India20 [peach]) showed that ACLSV triggered systemic infection on several hosts including Chenopodium quinoa, C. amaranticolor, Vigna sinensis (var. Chitlidana) and Phaseolus vulgaris. Hosts like C. sativus, G. globosa, D. barbatus, L. esculentum, N. glutinosa, C. tetragonoloba and N. tabacam were symptomless for ACLSV indicating that these are diagnostically insusceptible host species. C. quinoa and C. amaranticolor were found to be the best maintenance and propagation hosts. However, N. megalosiphon and N. benthamiana showed mild mosaic and curling symptoms for isolates India11, apple and India27, cherry) respectively. All the symptomatic N. benthamiana plants were ELISA positive for ACLSV. An evident difference in infectivity was observed in the isolates used for host range studies viz. India13 [apple] being the least infective while India11 [apple] and India27 [cherry] being most infectious.

Majority of workers commonly employed C. amaranticolor for the host range studies of ACLSV (Anonymous, 1992; Németh, 1986a; Mink, 1989a; Hollings, 1956). Moreover, the symptoms exhibited by this indicator to the infection of present isolates are also similar to those reported by these workers. Similar observations were mentioned on C. sativus by Walt and Engelbrecht (1974), even though no literature was found regarding symptom development and serological detection of ACLSV on N. benthamiana.
**5.2.3 Maintenance of Virus Culture**

The culture of ACLSV used in this study was obtained from apple and wild Himalayan cherry. The pure culture was maintained both *in vivo* and *in vitro*.

**5.2.3.1 In vivo**

Pure virus culture for apple cultivar Gala Mast from Gandarbal (J&K), Gala from Uttarakhand and wild Himalayan cherry from Palampur (HP) was maintained *in vivo* on *C. quinoa*, *C. amaranticolor* (inoculation at 4-8 leaf stage) and *P. vulgaris* (inoculation at cotyledonary leaf stage). Purity of the virus culture was checked periodically by back inoculation on *C. quinoa / P. vulgaris* and reconfirmation at molecular level.

**5.2.3.2 In vitro**

To maintain pure culture of the virus *in vitro* tissue culture was used. The apical/lateral meristems (0.2-1.0mm) from explants of apple cultivar Gala from Uttarakhand found to be infected only by ACLSV were cultured on Murashighe and Skoog (MS) medium with 1mg/ml BAP, 0.1mg/ml NAA, 3% sucrose and 0.7% agar. The explants were routinely subcultured at 6 weeks interval to maintain the stock and for further use in molecular studies (Fig 4.10). The presence of other apple viruses such as ApMV, ASPV, ASGV and PNRSV was regularly checked by ELISA and RT-PCR.

**5.3 PURIFICATION AND SEROLOGICAL CHARACTERIZATION OF ACLSV**

Virus purification was performed using established protocols (Lister, 1970a; Saksena and Mink, 1969) with minor modification. Crude virus extract (CVE) from (1) the systemically infected *C. quinoa* plants inoculated with extracts of leaves showing chlorotic leaf spots symptoms from Gala Mast cultivar (J&K), 10-15 days post inoculation (2) shoots/leaves from *in vitro* maintained pure culture (Gala from Uttarakhand) and (3) young buds and flower petals (wild Himalayan cherry from HP) was obtained.

**5.3.1 UV Absorption Spectrum**

The CVE of ACLSV preparation showed absorption maxima ($A_{max}$) at 260 nm
wavelength and absorption ratio $A_{260}/A_{280}$ were recorded to be 1.5, 1.53 and 1.43 for India15, India27 and India16 isolates respectively.

5.3.2 **Electron Microscopy**

The crude virus extract prepared from wild Himalayan cherry flowers and Gala Mast leaves was examined under electron microscope at 80kV and 21,000X magnification. Dimensions of the virions were determined by plotting a size distribution frequency curve of 100 virions. End to end aggregation of flexuous filamentous particles of ACLSV (~700nm) was observed in CVE by EM. Similarly aggregation, entangling, clumping and fragmentation in ACLSV has been observed in earlier EM studies (Dunez *et al.*, 1973; Francki *et al.*, 1985; Lister, 1970; Dunez *et al.*, 1973; Lister and Bar-Joseph, 1981; Thomas, 1983).

5.3.3 **DAS-ELISA**

Commercial antibodies of ACLSV detected partially purified virus up to 1:500 dilutions. The absorbance at 405nm for the optimum dilution (1:1000) of purified ACLSV preparation was 1.56, while the absorbance values of ACLSV positive and negative controls were 1.70 and 0.154, respectively.

5.3.4 **SDS-PAGE and Western Blotting of the Partially Purified Virus Preparation**

Heat denatured CVE preparation of ACLSV was analyzed on 12% SDS-PAGE, which revealed one major band of approx. size 25 kDa. Different sizes of CP ranging from 21.5-28K in various isolates of ACLSV has been reported (German *et al.*, 1990; Martelli *et al.*, 1994; German *et al.*, 1997; Marini *et al.*, 2008). Also possible phosphorylation of CP in infected tissue or a larger ORF containing the 23kDa fragment inframe CP could be the reason for larger than expected size of CP. Similar, report for phosphorylated ACLSV-MP in infected tissue and larger sized CP is available (Sato *et al.*, 1995; German *et al.*, 1997 respectively). The western blots were prepared by transferring the protein bands to PVDF membrane. When the membrane was probed with antibodies specific to ACLSV, it reacted with the coat protein bands of the virus, confirming presence of the virus.
5.4 MOLECULAR CHARACTERIZATION OF ACLSV

For the characterization of ACLSV at molecular level RNA isolated from various samples by using slightly modified RNA extraction buffer (Foissac et al., 2001) along with the commercial RNA extraction kit. Classical CTAB method of RNA extraction by Gasic et al. (2004) was used for large quantity of RNA. This RNA was used in RT-PCR to amplify coat protein (CP), movement protein (MP) and replicase using indigenously designed gene specific primer pairs (Table 4.12). The PCR conditions were standardized and desired amplicons were cloned, sequenced and analyzed with already established sequences in the database.

Sequencing of amplicons obtained from Indian isolates revealed that CP (~800bp) gave CP gene [584bp i.e. 193 amino acid (aa)] and 3’end un-translated region (UTR). Twenty-nine CP isolates were characterized from various hosts and locations in India. About 1405bp of MP amplicons had 1386bp (460aa) MP gene as expected. Two partial MP 5’end sequences (~141aa) from HP (Palampur and Kinnaur) were also obtained. The replicase gene was amplified in two parts - first ~1572bp (from 5’- 28nt to 1580nt - 3’) and second ~420bp (from 5’- 4502nt to 4922nt - 3’). Apart from this, deduced amino acid sequence of 3’end of MP (~110aa) overlapping with the CP was obtained by frame shift reading of 29 CP isolates from India. Similarly, from a frame shift reading of 5’end of MP amplicons a 36aa replicase-MP overlapping region was obtained. All the sequences obtained have been submitted to EMBL database (Table 4.15, 4.16).

Most of the workers have characterized partial CP genes (Al-Rwahnih et al., 2004; Cieślińska et al., 1995, 2007). However, analyzing the complete nucleotide sequences of ACLSV isolates from apple (Jelkmann, 1996), cherry (German et al., 1997), peach (Marini et al., 2008) and plum (German et al., 1990; Sato et al., 1993a) show similar amino acid sizes for CP, MP and partial replicase genes as determined in the present study. The work done for this thesis characterized ACLSV from apple, wild Himalayan cherry, almond, quince, apricot and peach (Rana et al., 2007b; 2007a; 2008a; 2008b; 2008c; 2009 respectively) from India for the first time. ACLSV infection on plum and pear at molecular level was also confirmed (Ferretti et al., 2010; Rana et al., 2010).
5.5 ASSESSMENT OF DIVERSITY IN THE GENOME OF ACLSV INFECTING POME AND STONE FRUIT

The complete nucleotide sequences of ACLSV isolates from apple (Sato et al., 1993a), cherry (German et al., 1997), peach (Marini et al., 2008) and plum (German et al., 1990; Jelkmann, 1996) have been analyzed with respect to diversity in genome using sequence features and in some cases by phylogenetic tree construction. In the present endeavour the sequences of Indian isolates of ACLSV CP, MP and replicase were analyzed separately using various bioinformatics tools with corresponding sequences of other established ACLSV sequences from the database to characterize the Indian isolate of ACLSV.

5.5.1 Phylogenetic Analysis of ACLSV Coat Protein (CP) gene Sequences

Most of the earlier workers had used partial CP sequences for diversity analysis (Al-Rwahnih et al., 2004; Serce and Rosner, 2006). The CP isolates 29 from the present study and those from earlier deposited sequences (partial and complete) in the database were subjected to multiple sequence alignment and phylogenetic analysis. This study of sequence variability analyses of complete ACLSV-CP from India is the first of its kind.

5.5.1.1 Apple

The percent sequence identity of forty-one apple isolates, (16 Indian characterized in this study [91-100% identity at aa level] and 25 others) at amino acid (aa) level ranged from 89-100%. A few CP isolates from India shared 100% sequence identity at nucleotide and amino acid levels. However, no region wise clustering was observed. Multiple alignment of the all CP isolates showed considerable conservation in first 36 and last 55 amino acids at N and C-terminals respectively. However, maximum non conserved changes creating variability in amino acid sequences were in the middle portion of the coat protein gene (Fig 4.15). Phylogenetic trees constructed (Fig 4.14 A, B) clearly indicated formation of two groups viz. Group 1 and Group 2, based on distance in phylogeny and variation / co variation of amino acids (Table 4.18). The isolates from Group 1 had more variability as compared to Group 2.
5.5.1.2 Other Pome Fruits (Pear and Quince)

Among all the CP isolates from pome fruits, the Kuerel (pear) and MO-5 (apple) isolates showed maximum variability at amino acid level (Fig 4.16) and also clustered separately from most of the Group 2 isolates (Fig 4.17). Radiated phylogenetic tree (Fig 4.17) showed clustering of the India19 (pear), Kuerel (pear, China) and quince (Greece) CP isolates in Group 2 while the India23 (quince) isolate falls in Group 1.

5.5.1.3 Plum

All the CP isolates from plum showed maximum variability at amino acid positions 20-98 (Fig 4.18 A). Phylogenetic tree clearly showed grouping of related isolates (Fig 4.19 A). Both the Indian isolates (India17, 18) infecting plum were 99 and 100% identical at nucleotide and amino acid levels respectively, and clustered in Group 1. These isolates were obtained from trees growing in different orchards. All the other CP (plum) isolates from the world fall in Group 2 (Fig 4.20 C, D). Among plum isolates, the Indian sequences were unique in having valine and phenylalanine at positions 59 and 75, respectively.

5.5.1.4 Almond

The coat protein sequence of Indian isolate of ACLSV infecting almond (isolate India24) was unique as it had asparatic acid (D) residue at 151 position instead of asparagine (N) when compared to other two partial CP sequences from almond. All the CP isolates from almond clustered in Group 2 (Fig 4.20 C, D).

5.5.1.5 Cherry

The percent sequence identity of CP sequences isolated from wild Himalayan cherry (isolates India27, India28 and India29) with other cherry isolates at amino acid level ranged from 86-98%. Isolates India28 and India29 amplified by IC-RT-PCR using flower extract from the same tree shared 98% and 92-93% sequence similarity with each other and India27 isolate respectively. Balton-1, the only complete CP isolate from cherry had sequence similarity of 88, 90 and 91% with India27, India28 and India29 isolates respectively. Middle portion of the coat protein gene showed maximum variability in amino acids (Fig 4.18 C). The India27 isolate along with the
C-2, ASwC43 and P1R9D9 isolates clustered together (Fig 4.19 B) and fall in Group 1 (Fig 4.20 C, D).

5.5.1.6 Apricot
The India25 (cultivated apricot) and India26 (wild apricot) isolates sharing (94%) falls in Group 1 and Group 2 respectively (Fig 4.20 D). Multiple sequence alignment of all CP sequences isolated from apricot at the amino acid level showed that most of the variability was present in the N-terminal part of the CP cistron (overlapping with the movement protein i.e. from 60-100 amino acids) whereas the C-terminus was significantly less divergent (Fig 4.18 D).

5.5.1.7 Peach/Nectarine
Multiple sequence alignment indicated that maximum variability occurred between amino acid positions 33rd and 98th (Fig 4.18 E). The India20 isolate was the only ACLSV peach isolate falling in Group 1 with maximum variability at amino acid level (Fig 4.18 E) and thus sharing sequence identity of less than 87.1% with most of the peach isolates from the world.

5.5.1.8 Pome and Stone Fruits
The CP isolates from India shared high sequence identity of 91-100% at aa level with world isolates. The highest degree of variability was observed in the middle portion with 9 amino acid substitutions in contrast to the N-terminal and C-terminal ends which were maximally conserved with only 4 amino acid substitutions at each end. However, maximum non conserved changes creating variability in amino acid sequences were in the middle portion of the coat protein gene. Al-Rwahni and workers (2004) analyzed partial sequence of CP gene of 35 ACLSV isolates at nucleotide level and reported that isolates vary among themselves slightly in N-terminal and while the C-terminal was mostly conserved. Similar observation of conserved C-terminal of CP though at aa level was evident for present analyses. Sequence analysis showed that India24 (almond) and India9 (apple) were the only CP isolates having aspartic acid (D) residue at 151 position instead of asparagine (N) (Fig 4.20 B). The isolates shared 93% sequence identity at amino acid level and fell in Group 2 (Fig 4.20 C, D). The CP isolates India1, India3, India 4 (apple); India20 (peach); India23 (quince) and India17, 18 (plum) were the only sequences
Discussions

having valine and phenylalanine amino acids at positions 59 and 75 respectively. An important factor responsible for high sequence similarities among pome and stone fruit isolates in India could be the mixed cultivation of these fruits in Indian orchards. Most of the CP isolates from stone fruit clustered in Group 2 suggesting co-evolution however, isolates P1R9D9 (DQ329160, cherry) and C-2 (AY677106, cherry) were exceptions. The Indian CP (stone fruit) isolates however, did not show such co-evolution. Indian isolates (India17, 18) infecting plum (100% identical at amino acid level) clustered in Group 1. These isolates were obtained from trees growing in different orchards from same area. Similarly, India21 (peach) and India19 (pear) isolates (sharing 100% identity) were obtained from different corners of the same orchard. Identical isolates from different locations, hosts and in isolates from same location or orchard could be attributed to unclean horticultural practices, common seedling source – nursery/mother plant (Al-Rwahni et al., 2004), mixed cultivation and may be involvement of an unidentified vector or transmission route. The India24 (almond) and India3 (apple) isolates (93% identity at amino acid level) were from the same orchard and in close proximity to each other. However, there were differences in phylogeny (India3- Group 1, India 24 - Group 2). The clustering into Group 1 and Group 2 is supported by the covariation / changes in amino acid positions in CP listed in Table 4.18. Similar observations for phylogenetic differences based on co variation at five amino acids at positions (highly conserved within each cluster) were proposed by Yaegashi et al. (2007a).

The CP isolates from plum (India17, 18; Palampur), quince (India23; Salooni), peach (India22; Solan) and various CP isolates from apple (different locations in HP) clustered together (Fig 4.20 D) indicating no host or region wise clustering or similarity (Fig 4.20 D). A correlation between nucleotide sequence divergence and geographic origin of the ACLSV isolates was proposed by Serce and Rosner (2006) after characterizing ACLSV isolates at molecular level by RFLP. However, no such host or region wise correlation was observed in this study. The India20 isolate falling in Group 1 shared sequence identity of less than 87.1% with most of the peach isolates from the world and could be considered as the most divergent isolate obtained in the study. Amino acid sequence identity of CPs differing by more than 15% is considered as the molecular criteria for demarcation of species in Trichovirus genus (Van Regenmortel et al., 2000). Later, Adams et al.,
Discussions

(2004) worked out that minimum 87.1% variation within a species in *Trichovirus* genus was acceptable. The conserved residues RQ/FDF (102-103/154-156) identified as a salt bridge structure (highlighted in green) were also present in all the CP sequences (Fig 20 A, B). The conserved residues RQ/FDF for salt bridge structure have been reported (Dolja *et al.*, 1991) in CPs of filamentous viruses.

### 5.5.1.9 Recombination Analysis: Understanding Evolutionary Relationship Based on Coat Protein Gene

Recombination analysis of all available complete CP sequences from India and elsewhere gave five potential recombination events (PREs). However, only event 1 seemed significant (Table 4.19) as it was detected by five of the recombination detection programs with identical breakpoints between nucleotides from 186-498 in India7 (major parent) and India12 (minor parent) isolates. The presence of this recombination event has been depicted with the help of a graphical representation (Fig 4.21) which clearly shows break points and location of recombination sites. The break points were also confirmed by drawing phylogenetic tree of 1-185, 186-498 and 499-582 nt of all the Indian CP isolates. All the sequences involved including the daughter isolate (India20, peach) fall in Group 1. Recombination events with insignificant average P-values were detected by single RDP program for most of the Indian CP (apple) isolates among themselves and with Japanese (AB326230, B6) apple isolate. Recombination analysis performed for the CP isolates from cherry, almond and apricot individually indicated no possible recombination event.

This is also the first evidence of homologous recombination in ACLSV. The present analysis thus confirms the existence of differences in phylogeny among Indian isolates (irrespective of host species and/or geographic origin) though they share high sequence identity (91-100% at aa level). The variability among Indian isolates was supported by host range studies and molecular data analysis (indicating variation in amino acids). The lack of enough significant recombination among ACLSV-CP isolates (Indian and world) points toward coat protein gene being a not a much targeted sequence for virus recombination and subsequent evolution of the virus. It is thus the most conserved region of the ACLSV genome based on sequence similarities.
5.5.2 Sequence, Phylogenetic and Recombination Analysis of ACLSV Movement Protein (MP) gene

The MP amplicon was analyzed in four parts viz. complete MP gene (460aa), 5’end amplicons (200aa), deduced amino acid sequence of 3’end of MP overlapping the CP gene (316aa) and combined MP-CP fragment (1856bp) with 8 complete MP isolates reported from the world (Table 4.20). The complete MP from India (FN673831, India15Apmp isolate) showed low (61-86%) similarity at amino acid level with other MP isolates of the world. The MP is reported to have the highest divergence level in ACLSV genome (German-Retana et al., 1997). The Indian isolate was found to be maximally (86%) similar to P-205 (apple; D14996) isolate and least to TaTao-5 (61%; peach, EU223295) isolate. The phylogenetic analysis of complete MP isolates revealed that Indian isolate (India15Apmp, apple) and MO-5 (apple) isolate were more closely related though sharing only 81% sequence similarity at amino acid level. However, MP of B6 isolate (apple) having 84% identity to Indian isolate shared phylogeny with stone fruit isolates. Infact, it was the only MP isolate from apple in stone fruit cluster. Three clusters for MP isolates (Fig 4.22 B) were identified viz. apple, stone fruit and isolates having very different phylogenies (viz. Balton-1 and TaTao-5). Multiple sequence alignment of complete MP isolates from India and the world revealed some high conservation in N-terminal portion (230aa) except few changes, while C-terminal half showed a much higher variability, with only a few blocks of fully conserved residues. As reported (Isogai and Yoshikawa, 2005), the MP isolates from India at 5’end had two adjacent independently active conserved RNA-binding domains (A; 82-126 and B; 127-287aa) with ‘characteristic D/G’ motifs of ‘30K Super Family’ of movement proteins (Fig 4.22A). Most of the amino acid changes in the replicase-MP overlapping (~67nt / 22aa) region were conserved except a few unique substitutions. An amplicons of MP from 5’end viz. isolate IndiaApRC-Plp (AM888397) was phlogenetically closer to MP isolates from stone fruit and distant from the other two MP 5’end Indian isolates.

Sequence analysis of the deduced amino acid sequence of 3’end of MP (last 316aa) overlapping with the CP were obtained by frame shift reading of 29 CP isolates (India) revealed that all these Indian isolates of MP (3’end segment) among themselves shared identity ranging from 57-100% at amino acid basis indicating maximum variability in the C-terminal half (Fig 4.23 C). Similar observation for 3’-
end variability was made by German-Retana et al. (1997). Phylogenetic relationship of the 3’-end MP isolates shows separation into Group 1 and Group 2 clusters (Fig 4.23 D). All the Group 2 MP isolates have more variability between 351-377aa as compared to Group 1 isolates (Fig 4.23 C).

Recombination analysis for complete MP sequences indicated maximum recombination signals at 3’-end of MP which is least conserved (Fig 4.22 A) and overlaps with CP gene (~316nt). Evidence for a high variability in region overlapping between the 50K and 28K ORF of ACLSV has previously been obtained by the sequencing of a short 353nt fragment amplified by PCR in the (Candresse et al., 1995). These 3’-end recombination events had significant average P-values but were detected by only one of the programmes and hence considered inconsequential. However, only one event with breakpoints in the MP-CP overlapping segment from almost the same region as detected in recombination analyses of CP was found significant (Table 4.23). Similarly, only one significant recombination in 5’end MP sequence at the replicase-MP overlapping part was obtained.

By aligning complete MP (FN673831) and CP (FN550875) sequences of India15 (apple) isolate amplified in the study, two probable hypothetical nucleotide sequences (~1856bp, Fig 4.25) viz. India15Ap-1 and India15Ap-2 (sharing 98% sequence identity) could be obtained. The phylogenetic analysis at nucleotide level of this complete MP-CP region showed distinct clustering of apple and stone fruit isolates (Fig 4.26). Recombination results show more recombination events in MP-CP overlapping region (316nt) than in replicase-MP (~67nt) region. As obtained by earlier analysis, the recombination events at MP-CP overlapping region were relatively significant, clearly indicating that the former region is important with respect to recombination and subsequent evolution. Two significant events (with break points 1753-1820 and 1732-1828) were also detected in the 3’-end non/untranslated region (NTR /UTR) region (Table 4.23). Literature does not report any study of MP sequence of ACLSV w.r.t. recombination analyses. Though comparisons of complete genome sequences of ACLSV on sequence identity and phylogenetic basis is present (Sato et al., 1993a; German et al., 1997; Marini et al., 2008)
5.5.3 Sequence, Phylogenetic and Recombination Analysis of ACLSV - Replicase Gene

Replicase was likewise analyzed, in three parts (5’end, middle portion and 3’end), for sequence variability, phylogeny differences and recombination evidences. Three Indian isolates of replicase from the 5’end of ACLSV genome were used for diversity analysis of replicase gene. The putative methyltransferase (MET) domain was identified in the N-terminal region (43-336 amino acids) and was mostly conserved. The presence of MET domain is already reported (Rozanov et al., 1992, German-Retana et al., 1997). The MET domain of Indian isolates showed 15 amino acid changes most of which were conserved. However, six unique changes were detected (Fig 4.27 A). The sequence identity among the Indian isolates ranged from 92-98% at aa level while it was 88-96% with rest of the world isolates. The TaTao-5 peach isolates being the most variable with 76-77% identity. The India15ApRep1 (FN826784) isolate was maximally (96%) similar at aa level to P-205 isolate. The other two Indian isolates shared maximum (94%) similarity to B6 isolate (Table 4.25 A). The last 86aa had maximum variability. All the Indian isolates clustered together with other apple isolates while stone fruit isolates clearly showed difference in evolution.

Sequence analysis of middle part of ACLSV-Replicase gene (123aa) indicated low variability (Fig 4.27 C) and 91-95% identity at aa level (Table 4.25 B) with all the isolates used in the study irrespective of isolation host. The Indian isolate FN555394 (India15ApRepMid) showed closer phylogeny to stone fruit isolates even after sharing same sequence identity (95% at aa level) with P-205 apple isolate (Fig 4.27 E). Similarly the 3’end portion of ACLSV-Replicase (36aa) was much conserved with only four unique changes in the Indian isolates (Fig 4.27 D). The sequence identity among the Indian isolates ranged from 71-88% at aa level while it was 71-97% with rest of the world isolates. The Indian isolates India15ApRep3’ (FN673831) and IndiaApRep3’RC-Plp (AM888397) shared 88% sequence identity at amino acid level and were least similar (68-83%) to rest of the world isolates (Table 4.25 C). The clustering in phylogenetic tree shows difference in evolution of these two of the Indian isolates. Five significant recombination events with significant average P values from 5’end ACLSV-Replicase sequences (130nt region downstream the MET domain) were detected in 5’end segment of replicase. The 3’ end replicase sequences
Discussions

gave only one recombination that was significant but not in the replicase-MP overlapping region. Middle portion of replicase was conserved and gave no recombination.

The results obtained in the present study by recombination studies supported by sequence variations at amino acid level and divergence in phylogenies in CP, MP and replicase is supported by similar observations in literature (Sato et al., 1993a; German et al., 1997) German et al. (1997) reported that most of the variation between isolates was observed in three main regions: the hyper-variable segment downstream of the methyl-transferase domain in the 216KORF, the C-terminal part of the 50K movement protein ORF, and the N-terminal part of the 28K ORF upstream of the CP coding region.

5.6 DEVELOPMENT OF DIAGNOSTIC TOOL THROUGH HETEROLOGOUS EXPRESSION OF COAT PROTEIN

5.6.1 ELISA Based Diagnostics

5.6.1.1 Amplification, Cloning and Transformation of Cloned Fragment in E.coli

Primers were so designed (Table 4.13) to amplify the complete CP gene of ACLSV that could be cloned inframe with in the expression vector (pET-32). For inframe cloning, the restriction enzyme sites of Bam HI and Hind III were added in upstream and downstream primers, respectively. The CP gene thus amplified (584 bp) was successfully cloned in the pET-32a expression vector. Similarly, (Sato et al., 1993a) had used different restriction enzyme sites for inframe cloning of the CP gene into the E. coli. using pTrc99A expression vector. Later, in 1995 Sato et al. reportedly cloned MP gene in pKK223-3 expression vector for heterologous protein expression in E. coli. BL 21, a widely used strain for expression (Bragard et al., 2000; Liu et al., 2001; Jacob and Usha, 2002; Saini and Varti, 2003) was used for the purpose.

5.6.1.2 Standardization of Optimal Expression Conditions for ACLSV CP

The ACLSV coat protein was over-expressed in E. coli BL 21 strain using pET32a vector and the conditions for optimal expression were standardized in this study at 28°C, with 1 mM IPTG and 3 hr incubation. Similarly, the duration for expression of gene of interest at a particular IPTG concentration may vary according to the gene of interest or by other conditions, as 37°C for 3-4 h has been used for the expression by
Bragard et al. (2000), Liu et al. (2001), Jacob and Usha (2002) and Saini and Varti (2003), and 16°C for overnight by Kadkhodayan et al. (2000).

### 5.6.1.3 Purification of Expressed Protein

The CP expressed in the system was in the form of 6XHis-fusion protein, and was purified using His bind protein purification kit. For purification of expressed fusion protein, cells expressing the protein were pelleted and suspended in PBS. Then the cells were lysed by sonication and the fusion protein was purified from the supernatant using the kit. The column containing the resin provided with the kit has the capacity to bind His and the proteins containing His as a tag.

### 5.6.1.4 Raising of Antiserum

The immunization procedures and bleeding schedule may also have an influence but relatively little reliable information is available. In the present study, for the development of diagnostic kit, polyclonal antiserum was raised against purified E. coli expressed coat protein of ACLSV. For raising antiserum against ACLSV CP immunizations were performed by priming 2 Angora female rabbits, approximately 5 months old, four times at weekly intervals. Aliquots of purified ACLSV-CP/His fusion protein (0.6 mg) were emulsified with complete Freund’s adjuvant (FA) (1:1 v/v) and injected subcutaneously into the hind legs. After 5 weeks, a booster injection emulsified with incomplete FA (0.6mg protein) was given and rabbits were bled beginning 1 week later at weekly intervals for 7 weeks. Antiserum / whole serum (obtained after clotting of the blood) against the ACLSV CP was collected and stored in aliquots at -20°C along with 0.02% sodium azide.

In the present study, IgG was separated from whole serum by ammonium sulfate precipitation and by affinity chromatography using Nab antibody purification kit. It contains protein A bound to sepharose column, that specifically binds to Fc region of antibodies from the whole serum which was then eluted in low pH buffer. IgG specific for ACLSV were separated from the mixture of antibodies. Purified ACLSV IgG was conjugated with alkaline phosphatase. The best ELISA readings were obtained at dilution 1:300 which were less ambiguous and more reliable than those given by commercial kit at 1:1000 dilution (Table 4.26). Sensitivity and specificity of purified IgG against the ACLSV-CP were also evaluated by western blotting of fusion proteins using indigenously raised antiserum. Signals obtained by western
Discussions

blotting also indicated to the indigenous antiserum being specific to ACLSV (Fig 4.30 D).

5.6.1.8 Field Screening to Check the Activity of the Kit

To check efficacy of the indigenous (test) kit (Fig 4.31), different apple cultivars, pome and stone fruits along with herbaceous hosts were checked for presence or absence of ACLSV using DAS-ELISA and DTBIA. At the same time samples were also checked by a standard/commercial (reference) kit Bioreba (USA). Purified fusion protein and Indian ACLSV positive samples (apples and peach), herbaceous hosts (*Chenopodium amaranticolor* and *Phaseolous vulgaris*) inoculated with positive apple and peach isolate and few random samples were screened. Negative and positive thresholds for DAS-ELISA were set at two times the mean of healthy control sample absorbance at 405nm. For reference checking commercial ACLSV positive control and negative control were used. The test was carried out in triplicates. It was observed that in some cases the test works better than the standard or reference kit thus test kit gave stronger positive signal in DAS-ELISA for some cultivars infected with ACLSV (Table 4.26). The positive control used from the Bioreba (USA) kit was detected better by the Indian antiserum. The ELISA results indicated that antiserum prepared in the present study was more sensitive for the detection of Indian isolates of ACLSV in comparison with standard kit. It was found that in some cases the test kit works better than the standard kit *i.e.* test kit gave strong positive signal in DAS-ELISA for some cultivars infected with ACLSV while the standard kit gave somewhat lesser reaction. Thus kit prepared in the present study was more sensitive for the detection of Indian strain of ACLSV in comparison to the standard kit.

For DTBIA same plant extracts were plotted on nitrocellulose membranes (Biorad, USA) and screened with indigenousACL SV antibodies and detected using HRP conjugated anti-rabbit antibodies (Roche, USA). Results were similar to ELISA readings though at 1:500 dilution (Fig 4.32). Similarly, Wang *et al.* (1998) used dot-immuno binding assay (DIBA) for detecting ACLSV and ASGV in the plant extracts. Other workers have mostly used tissue hybridization for virus localization studies (Mansky *et al.*, 1990; Chia *et al.*, 1995; Knapp *et al.*, 1995a, b).
5.6.2 Development of PCR Based Diagnostics

Indigenous degenerate primer pair for CP was designed from all the available full genomes of ACLSV in GenBank and the RT-PCR conditions were standardized for molecular detection of ACLSV. Sequenced CP was enzyme/radiolabelled to be used as a probe for large scale detection of ACLSV positive samples by tissue slot blot hybridization. Conditions for IC-RT-PCR using the indigenously raised ACLSV antibodies and designed primers were also standardized for a more reliable diagnosis of ACLSV infection.

Earlier workers have also used sequenced probes and IC-RT-PCR techniques for ACLSV detection (Candresse et al., 1995; Nemchinov et al., 1995; Deng et al., 2004). However, keeping in view the variability in ACLSV strains (German et al., 1997), the diagnostics developed would be more specific and reliable in detecting Indian isolates of ACLSV. The various nucleic acid and ELISA based indigenous diagnostics developed could be useful in certification system for pome and stone fruit nursery material and in quarantine.