CHAPTER 4

RESULTS
4.1 SURVEY OF VIRUS (ES) INFECTING CHERRY

During surveys in H.P. and J&K (Fig 4.1), virus like symptoms such as chlorosis, mosaic, deformations, necrotic spots, shot holes, oak leaf pattern, enations, curling or folding, epinasty, vein bending, distortion, yellowish bent and asymmetric leaf, bronzing were noticed on leaves and small, pointed and deformed fruits were observed on cherry trees. Symptoms like gummosis, grooves and pits were also observed on bark.

Fig 4.1: Areas surveyed in J&K and H.P. for viruses infecting cherry indicated by red dots
4.2 DETECTION OF VIRUS (ES) INFECTING CHERRY

4.2.1 Detection based on visual symptoms

On the basis of visual symptoms (Fig. 4.2), our observations and comparison with published data from literature, viruses were tentatively identified on the sweet and sour cherry plants. The suspected symptoms caused by them are summarized in Table 4.1.
Fig 4.2: Diverse symptoms observed on cherry in various orchards. Arrow indicates pointed fruit.
Table 4.1: Tentative identification of suspected virus based on visual symptoms

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Symptoms</th>
<th>Suspected virus</th>
<th>Host</th>
<th>Variety</th>
<th>Location (Area, District, State)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2a</td>
<td>Vein bending</td>
<td>CVA</td>
<td>Sweet cherry</td>
<td>Gold</td>
<td>Rohru, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2b</td>
<td>Vein bending</td>
<td>CVA</td>
<td>Sour cherry</td>
<td>-</td>
<td>Palampur, Kangra, H.P.</td>
</tr>
<tr>
<td>4.2c</td>
<td>Vein bending</td>
<td>CVA</td>
<td>Sweet cherry</td>
<td>Manigam</td>
<td>CITH*, Srinagar, J&amp;K</td>
</tr>
<tr>
<td>4.2d</td>
<td>Enation</td>
<td>PNRSV/ CRLV</td>
<td>Sweet cherry</td>
<td>Black</td>
<td>Bhutti, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2e</td>
<td>Enation</td>
<td>PNRSV/ CRLV</td>
<td>Sweet cherry</td>
<td>Black</td>
<td>Bhutti, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2f</td>
<td>Rugose</td>
<td>CGRMV</td>
<td>Sweet cherry</td>
<td>Black</td>
<td>Bhutti, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2g</td>
<td>Mosaic</td>
<td>ApMV</td>
<td>Sweet cherry</td>
<td>Misri</td>
<td>CITH, Srinagar, J&amp;K</td>
</tr>
<tr>
<td>4.2h</td>
<td>Necrosis</td>
<td>PNRSV</td>
<td>Sweet cherry</td>
<td>Lapin</td>
<td>CITH, Srinagar, J&amp;K</td>
</tr>
<tr>
<td>4.2i</td>
<td>Rusty necrosis</td>
<td>CNRMV</td>
<td>Sweet cherry</td>
<td>Gold</td>
<td>Rohru, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2j</td>
<td>Necrosis</td>
<td>PNRSV</td>
<td>Sweet cherry</td>
<td>Misri</td>
<td>Sopore, Baramulla, J&amp;K</td>
</tr>
<tr>
<td>4.2k</td>
<td>Folded leaf</td>
<td>CLRV</td>
<td>Sweet cherry</td>
<td>Stella</td>
<td>Bhutti, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2l</td>
<td>Epinasty</td>
<td>CGRMV</td>
<td>Sweet cherry</td>
<td>Stella</td>
<td>Bhutti, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2m</td>
<td>Leaf distortion</td>
<td>TBSV</td>
<td>Sweet cherry</td>
<td>Misri</td>
<td>Palampur, Kangra, H.P.</td>
</tr>
<tr>
<td>4.2n</td>
<td>Yellowish bent and asymmetric leaf</td>
<td>RpRSV</td>
<td>Sweet cherry</td>
<td>Misri</td>
<td>Palampur, Kangra, H.P.</td>
</tr>
<tr>
<td>4.2o</td>
<td>Chlorosis</td>
<td>PDV</td>
<td>Sweet cherry</td>
<td>-</td>
<td>Rohru, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2p</td>
<td>Bronzing</td>
<td>LChV-2</td>
<td>Sweet cherry</td>
<td>Double</td>
<td>CITH, Srinagar, J&amp;K</td>
</tr>
<tr>
<td>4.2q</td>
<td>Vein clearing</td>
<td></td>
<td>Sour cherry</td>
<td>-</td>
<td>Palampur, Kangra, H.P.</td>
</tr>
<tr>
<td>4.2r</td>
<td>Rusting</td>
<td>CNRMV</td>
<td>Sour cherry</td>
<td>-</td>
<td>Palampur, Kangra, H.P.</td>
</tr>
<tr>
<td>4.2s</td>
<td>Angular and pointed fruit</td>
<td>LChV-1 &amp; 2</td>
<td>Sweet cherry</td>
<td>Black</td>
<td>Rohru, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2t</td>
<td>Angular and pointed fruit</td>
<td>LChV-1 &amp; 2</td>
<td>Sweet cherry</td>
<td>Stella</td>
<td>Rohru, Shimla, H.P.</td>
</tr>
<tr>
<td>4.2u</td>
<td>Healthy fruit</td>
<td></td>
<td>Sweet cherry</td>
<td>Stella</td>
<td>Rohru, Shimla, H.P.</td>
</tr>
</tbody>
</table>
4.2.2 Detection based on Enzyme linked immunosorbent assay (ELISA)

Leaf samples (symptomatic and non-symptomatic) were collected randomly from each orchard and subjected to ELISA for the diagnosis of Apple chlorotic leaf spot virus (ACLSV), Apple mosaic virus (ApMV), Arabis mosaic virus (ArMV), Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV), Cherry raps leaf virus (CRLV), Cherry leaf roll virus (CLRv), Prune dwarf virus (PDV), Prunus necrotic ringspot virus (PNRSV), Plum pox virus (PPV), Strawberry latent ringspot virus (SLRSV), Raspberry ringspot virus (RpRSV), Apple stem pitting virus (ASPV) and Tomato ring spot virus (ToRSV). For all the viruses DAS-ELISA was performed (Clark and Adams, 1977). ELISA results are presented in Table 4.2 and 4.3.

Table 4.2: Viruses detected by DAS-ELISA in cherry samples collected from various orchards of H.P.

<table>
<thead>
<tr>
<th>Serial No</th>
<th>Virus</th>
<th>Samples tested</th>
<th>Positive samples</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CLRV</td>
<td>38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>CRLV</td>
<td>38</td>
<td>6</td>
<td>15.78</td>
</tr>
<tr>
<td>3</td>
<td>PDV</td>
<td>38</td>
<td>6</td>
<td>15.78</td>
</tr>
<tr>
<td>4</td>
<td>PNRSV</td>
<td>38</td>
<td>9</td>
<td>23.68</td>
</tr>
<tr>
<td>5</td>
<td>PPV</td>
<td>38</td>
<td>4</td>
<td>10.5</td>
</tr>
<tr>
<td>6</td>
<td>RpRSV</td>
<td>38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>SLRSV</td>
<td>38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>ToRSV</td>
<td>38</td>
<td>0</td>
<td>0</td>
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</table>

Table 4.3: Viruses detected by DAS-ELISA in cherry samples collected from various orchards of J&K

<table>
<thead>
<tr>
<th>Serial No</th>
<th>Virus</th>
<th>Samples tested</th>
<th>Positive samples</th>
<th>Incidence (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>ArMV</td>
<td>48</td>
<td>6</td>
<td>12.5</td>
</tr>
<tr>
<td>2</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>3</td>
<td>CRLV</td>
<td>48</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>PDV</td>
<td>48</td>
<td>3</td>
<td>6.25</td>
</tr>
<tr>
<td>5</td>
<td>PNRSV</td>
<td>48</td>
<td>3</td>
<td>6.25</td>
</tr>
<tr>
<td>6</td>
<td>PPV</td>
<td>48</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>RpRSV</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>SLRSV</td>
<td>48</td>
<td>9</td>
<td>18.75</td>
</tr>
<tr>
<td>9</td>
<td>ToRSV</td>
<td>48</td>
<td>0</td>
<td>0</td>
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<tr>
<td>10</td>
<td>ApMV</td>
<td>48</td>
<td>10</td>
<td>20.80</td>
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4.2.3 Detection based on Dot-blot hybridization

The leaf samples of cherry plants collected from J&K and H.P. were checked by dot-blot hybridization for detection of *Cherry virus A* (CVA), *Cherry green ring mottle virus* (CGRMV), *Prune dwarf virus* (PDV), *Plum pox virus* (PPV), *Little cherry virus 1* and 2 (LChV-1 and 2) and *Cherry necrotic rusty mottle virus* (CNRMV). Results obtained are described in various figures and tables (Fig 4.3 - 4.4 and Table 4.4 - 4.5).

**Fig 4.3:** Isotopic dot-blot of samples from J&K hybridized with CVA (a), CGRMV (b), PPV (c) and PDV (d) cloned DNA as probe. Black spots were treated as positives samples and eluted PCR products act as a positive control indicated by arrow. The intensity of the black spot shows virus titer of the positive samples.

**Fig 4.4:** Non isotopic dot-blot of samples from H.P. hybridized with LChV-1 (a), LChV-2 (b) and PDV (c) cloned DNA as probe (biotin labeled). Blue or purple spots were treated as positives samples and eluted PCR products act as a positive control indicated by C. The intensity of the spot shows virus titer of the positive samples.
Table 4.4: Viruses detected by Dot-blot hybridization in cherry samples collected from various orchards of J&K

<table>
<thead>
<tr>
<th>Serial No</th>
<th>Viruses</th>
<th>Samples tested</th>
<th>Infected samples</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PDV</td>
<td>47</td>
<td>18</td>
<td>38.29</td>
</tr>
<tr>
<td>2</td>
<td>PPV</td>
<td>42</td>
<td>16</td>
<td>38.09</td>
</tr>
<tr>
<td>3</td>
<td>CNRMV</td>
<td>45</td>
<td>17</td>
<td>37.77</td>
</tr>
<tr>
<td>4</td>
<td>CGRMV</td>
<td>46</td>
<td>19</td>
<td>41.30</td>
</tr>
<tr>
<td>5</td>
<td>CVA</td>
<td>47</td>
<td>19</td>
<td>40.42</td>
</tr>
<tr>
<td>6</td>
<td>LChV I</td>
<td>47</td>
<td>18</td>
<td>38.29</td>
</tr>
<tr>
<td>8</td>
<td>LChV II</td>
<td>44</td>
<td>8</td>
<td>18.18</td>
</tr>
<tr>
<td>9</td>
<td>PNRSV</td>
<td>40</td>
<td>8</td>
<td>20.00</td>
</tr>
</tbody>
</table>

Table 4.5: Viruses detected by Dot-blot hybridization in cherry samples collected from various orchards of H.P.

<table>
<thead>
<tr>
<th>Serial No</th>
<th>Viruses</th>
<th>Samples tested</th>
<th>Infected samples</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PDV</td>
<td>31</td>
<td>16</td>
<td>51.61</td>
</tr>
<tr>
<td>2</td>
<td>PPV</td>
<td>31</td>
<td>12</td>
<td>38.70</td>
</tr>
<tr>
<td>3</td>
<td>CGRMV</td>
<td>31</td>
<td>19</td>
<td>61.29</td>
</tr>
<tr>
<td>4</td>
<td>LChV-2</td>
<td>31</td>
<td>11</td>
<td>35.48</td>
</tr>
</tbody>
</table>

4.2.4 Detection based on Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to elucidate the identity of the viruses such as LChV-1, LChV-2, CVA, PNRSV, CGRMV, CNRMV, ArMV, PPV (strain D and M), PDV, SLRSV, RpRSV and CRLV. Which were found positive in various samples by ELISA and dot-blot hybridization. Various primer pairs (from published literature or designed by multiple sequence alignment) were used to confirm the identity of above viruses from sweet cherry plants. Only four viruses LChV-1, CVA, PNRSV and CNRMV were identified from sweet cherry plants by RT-PCR analysis. Presence of other viruses LChV-2, CGRMV, ArMV, PPV (strain D and M), PDV, SLRSV, RpRSV and CRLV which were found positive by ELISA and dot blot hybridization could not be confirmed by RT-PCR.
4.3 MOLECULAR CHARACTERIZATION AND VARIABILITY ANALYSIS OF LITTLE CHERRY VIRUS 1 (LChV-1)

Fragments of approximately 400 bp (Fig 4.5) and 300 bp (Fig 4.6-4.7) were amplified by RT-PCR from the leaf samples collected from H.P. using primer pair LChV1-16390U/LChV1-16809L and LCUW7090/LCUWc7389, respectively. The amplicons from six different isolates obtained from Shimla district of H.P. by primer pair LChV1-16390U/LChV1-16809L were cloned and sequenced. The amplicons of 387 bp were shown to include 339 bp sequence of the ORF8 (hypothetical protein) and 48 bp of 3’-UTR region of virus genome (accession no: FM211646, FN557587, FN557588, FN557589, FN557590 and FN557591). The nucleotide sequences were analyzed and compared with all the available sequences of ORF8 (hypothetical protein) and 3’-UTR region of LChV-1 reported from the world. Multiple sequence alignment showed that the amplified regions in the Indian isolates (S, C2-C6) are short of 35 nucleotides in comparison with type isolate UW2 (Germany), ITMAR (Italy), W2 (Poland), WAYa1, WAGr1, WACH1a, WACH1b, CAJS1, WACH2 (USA), BCOS1 (Canada) and 98Fi10R2 (Turkey) at 5’ end of 3’UTR region. Therefore the Indian isolates have a shorter 3’-UTR (variable region: Fig 4.8). The sequence exhibited nucleotide and amino acid similarity level 90-100% and 85-90% with the available sequences. A phylogenetic analysis based on the available partial deduced nucleotide sequences of ORF8 gene and 3’UTR in the GenBank Database with Indian isolates of LChV-1 of the same region divides all isolates into two clusters (Fig 4.9). Indian isolates are more closely related to the German, Italian, Turkish and Polish isolates, respectively and are part of the same cluster. In another cluster American, Canadian and isolates are grouped together.
Fig 4.5: Detection of LChV-1 by RT-PCR. Lane 1-8: Amplification of ~ 400 bp of ORF8 and 3’ UTR region of LChV-1 using primer pair LChV1-16390U/LChV1-16809L from sweet cherry leaf samples collected from Shimla district. Lane 9: Water as a negative control. Lane M: 1 kb ladder DNA.

Fig 4.6: Detection of LChV-1 by RT-PCR. Lanes 1-12: Amplification of 300 bp of RdRp region of LChV-1 by primer pair LCUW7090/LCUWc7389 from sweet cherry leaf samples collected from Rohru region of Shimla district. Lane M: 1 kb ladder DNA.

Fig 4.7: Detection of LChV-1 by RT-PCR. Lanes 13-24: Amplification of 300 bp of RdRp region LChV-1 by primer pair LCUW7090/LCUWc7389 from sweet cherry leaf samples collected from Rohru region of Shimla district. Lane M: 1 kb ladder DNA.
Fig 4.8: Multiple sequence alignment of 3' UTR (4-87 nt) showing the comparison of amplified products obtained from six isolates (S, C2-C6) of LChV-1 RT-PCR fragments with UW2 (Germany), ITMAR (Italy), W2 (Poland) WAYa1, WAGr1, WACH1a, WACH1b, CAJS1, WACH2 (USA), BCOS1 (Canada) 98Fi10R2 (Turkey) sequence. Stretch missing in Indian (H.P.) isolates is indicated by dash
Fig 4.9: A phylogenetic relationship generated from the deduced ORF8 and 3’ UTR sequence regions (419 nt) of LChV-1. The analysis involved 18 nucleotide sequences. Each representative isolate was designed using GenBank accession number followed by isolate name and host location. Sequence alignment and phylogenetic tree were constructed by MEGA5 with 1000 replicates of bootstrapping shown next to the branches.

4.4 MOLECULAR CHARACTERIZATION AND VARIABILITY ANALYSIS OF CHERRY VIRUS A (CVA)

4.4.1 Molecular Characterization of CVA

Fragments of approximately 400 (Fig 4.10), 1100 (Fig 4.11) and 1500 bp (Fig 4.12) were amplified by RT-PCR from the leaf samples collected from H.P and J&K by primer pair CVAU6508/CVAL6905, CVAU5839/CVAL6905 and CVAU5839/CVAL, respectively. Twenty-five out of 89 samples from J&K and 3 out of 24 samples from H.P. were found positive for CVA by RT-PCR. The amplified PCR products were cloned and sequenced and the sequences GenBank accession nos. FN557253, FN557254, FN557255, FN669545, FN669546, FN66947 and FN669548 were obtained. On sequencing these fragments were of exactly 419, 1089 and 1539 bp.
**Fig 4.10**: Detection of CVA by RT-PCR. Lane 1-2: Amplification of 419 bp of polyprotein gene of CVA by primer pair CVAU6508/ CVAL6905. Lane 3: Healthy sample as a negative control with nad5 (181 bp) as an internal control. Lane M: 1 kb DNA ladder

**Fig 4.12**: Detection of CVA by RT-PCR. Lane 1-2: Amplification of 1089 bp of polyprotein gene of CVA by primer pair CVAU5839/CVAL6905. Lane 3: Healthy sample as a negative control with nad5 (181 bp) as an internal control. Lane M: 1 kb DNA ladder

**Fig 4.12**: Detection of CVA by RT-PCR. Lane 1, 4, 5 and 6: Amplification of 1539 bp of polyprotein gene of CVA by primer pair CVAU5839/CVAL. Lane 2-3: CVA negative samples. Lane M: 1 kb DNA
4.4.2 Molecular characterization and recombination analysis of movement protein (MP) of CVA

A full length (1392 bp) of movement protein of CVA (Fig 4.13) was amplified by RT-PCR from the leaf samples collected from H.P. and J&K by primer pair CVAMPF/CVAMPR. The amplified PCR products were cloned and sequenced, and the sequences were submitted to the GenBank Database (FR718887, FR718888, FR718889 and FR718890).

Fig 4.13: RT-PCR analysis of CVA MP. Lane 1-3: Amplification of 1392 bp of complete MP of CVA by primer pair CVAMPF/CVAMPR. Lane 4: Water as a negative control. Lane M: 1 kb DNA ladder.

Fig. 4.14: Locations of unique recombination event identified by RDP4 in relation to the full-length sequence alignment of CVA MP. The recombination event was identified by 6 different programs viz. RDP (average P-value = 1.089 10^{-02}), BootScan (average P-value = 2.833 10^{-03}), MaxChi (average P-value = 4.079 10^{-03}), Chimaera (average P-value = 3.247 10^{-03}), SiScan (average P-value = 1.234 10^{-03}), LARD (average P-value >1) and 3 Seq (average P-value = 1.531 10^{-02}). This recombination event shows
recombination between present French plum isolate ‘PF’ (HQ267856) as the major parent and the J&K isolate (FN691959) as the minor parent, which led to a recombinant isolate of HPSK25 (FR718888). In this recombination event, a region (314-696 nt) of the French isolate (HQ267856) was replaced with the MP sequence of J&K isolate (FN691959).

![Graphs showing recombination breakpoint distribution plot](image)

Fig 4.15: Recombination breakpoint distribution plot (A=distance plot, B=Bootscan evidence and C= Pairwise identity) for the complete CVA MP. The breakpoint for the recombination event lies between positions 314-697 as shown in pink region. The broken line indicates Bonferroni corrected chi square Pvalue = 0.05. Bootscan evidence for the recombination origin on the basis of pairwise distance model with 1000 Bootstrap replicates.
4.4.3 Complete genome organization of CVA

For complete genome organization of CVA, total RNA was extracted from the infected leaf tissue of sweet cherry plants collected from Srinagar and Sopore regions of J&K and Rohru region of H.P. (Fig 4.17). As expected, a fragment of approximately 7.4 kb was amplified by RT-PCR from all three CVA infected samples (Fig 4.18 - 4.19). PCR products were either purified or eluted from agarose gel using the gel extraction kit (RBC, Taiwan) and ligated into pGEM-T Easy Vector (Promega, USA) or T&A cloning vector (RBC, Taiwan). Chemically competent Escherichia coli cells (strain DH5α) were transformed with the ligated vectors. Recombinants in pGEM-T-easy cloning system were checked by restriction digestion with EcoRI (confirmed by digestion with NotI if needed). After EcoRI digestion, bands were obtained at 3685, 3015, 2204, 1046 and 444 bp where as only two bands were obtained when digested by NotI (Fig 4.20).

Recombincants in T&A cloning vector were checked by restriction digestion with HindIII and positive colony was further confirmed by double digestion with SmaI and SalI. After HindIII digestion band was obtained at ~ 2700, 2511, 1465, 1457, 1273, 540 and 133 bp where as only two bands were obtained when digested by SmaI and SalI (Fig 4.21). The colonies, found positive in restriction digestion, were selected for the pure plasmid extraction. The
pure plasmids were extracted from small overnight cultures and sequenced. Complete genome of one J&K isolate was sequenced by primer walking (FN691959) where as other two isolates (JKSPMi and K12) were partially sequenced and confirmed by end sequencing and submitted to GenBank Database under accession number FN669548, HE574819 and HE5748120.

Fig 4.17: Agarose gel electrophoresis of total RNA from sweet cherry leaf tissue infected with CVA

Fig 4.18: RT-PCR analysis of complete genome of CVA. Lane 1: Amplification of full length (7.4 kb) of CVA genome (variety Manigam) by primer pair CVAU/CVAL. Lane M: 1 kb DNA ladder

Fig 4.19: RT-PCR analysis of complete genome of CVA. Lane 1 & 2: Amplification of full length (7.4 kb) of CVA genome (isolate K12) by primer pair CVAU/CVAL. Lane M: 1 kb DNA ladder
Fig 4.20: Restriction analysis of recombinant plasmid (pGEM-T Easy) containing complete genome (7.4 kb) of CVA. Lane 1: 7.4 kb (insert) and 3 kb (vector) fragment digested by *Not*I. Lane 2: Fragments of 3685, 3015 (vector), 2204, 1046 and 444 bp digested by *Eco*RI. Lane M: 1Kb DNA ladder.

Fig 4.21: Restriction analysis of recombinant plasmid (T&A) containing complete genome of CVA. Lane 1: Fragments of 2700, 2511, 1465, 1457, 1273, 540 and 133 bp digested by *Hind*III. Lane 2: 7.4 kb and 2.7 kb fragment of recombinant plasmid digestion by *Sma*I and *Sal*I. Lane M: 1Kb DNA ladder.

Fig 4.22: Diagrammatic representation of complete genome of Indian isolate of CVA.
Chapter 4

Results

Complete nucleotide sequence of CVA cDNA

tcacttccatcaattttcaaacacttccaacacacacacaagtcagtaatggca 60
M A
ttttggtgctaaatggtgagagagatattcactcactccctgtgaatagtacactgtat 120
F V A K F A E E N Y F N S L P S N V T D
gcgtttcttaaggctaggattcactgacaagacaacacacacagtctggtagaccccttctcaaaacac 180
A F L R D G F N A E H N R E T L S K H
ttcgcttttgaattaaaccacagctcaggattactgaagtattggtgcattcacta 240
F A F A L P S Q R N Y L N D C G I Q L
gcacattgcatcacaagacacactcaccacctgtgtcaaaactattgaaatcatctc 300
A P I A S K T H P V S K T I E N H L
cttactgtgtgattttcataaatatgtgtaaataatgatatgtttttacttcatcgcac 360
L Y C V V S N M I S N F K F L V F L S I
aaggaagaacaggtcataatactttggaataaacaacacatcagacagagagagact 420
K E S K A E Y I W N K N T S D T V R E I
tctaattgccgtcctgtatataaagagatcctttttctgtgcacccagtcaccaacagtcata 480
S N R V L D I K D A F R Y S P V N T V N
ggtgtgttgtaataactttagctctctttctgtgcacactttggagagatttattcttaga 540
G G L N N F C A N L A R R F N S R
gccatcaacacctgacgctctctctatctccatcagatgaagtgcattttttgagaccttcc 600
A I K P D C F F I H D E V H F W S P S N
tctgtgtgtgatctttctgttcacccgtgagaacaaaaattgtgtggaactggaggattatacc 660
L C E F L T E P K N V L A T V V I P
cacagattgataaggagactgaatatacgcttttaattcagctgtttatgattttgttaaag 720
P E L I E G L N Y S F N S V A Y D F V K
gtggagtagcagcaacttttatattctctctgtgacaatccaaagcagcttatcaacaaacct 780
V D G N L Y Y F P D K S K G K P Y Q P
acagacccgtgtttgtctccagatgcataaaatattcataatgataagggtggagaacactttt 840
T D P W L L R C N K I S M I K G G E T F
tctttacatcatatagttctcttctgaggctttgtgggagcaactctctttctttctttcaagg 900
S Y S I G L L E S V G A N H L F S F Q R
aacaagtagctgtgaactgtttgtagatctctctcttcatgatatgtttaggattgaat 960
N K V V S V P R F N D F D C L D L R N
tttttcccatcaatggaatagatagattttgagattttgatttagtttaggattgaat 1020
L L P I N V E N K N I M K G Y N I R T W V
tttttaaataaatgtttttcattttgatttttctttttaaaagagagattctgtaaatcttcttt 1080
F K K I L S Y I V C L K K G D S E S S L
gctaatggtgagacaatattagcattttcctcccaagaggcttgagctttactattattgt 1140
A K L R Q L S S P S S D E L L I G
gactcttttgatattgagactagtcacaatttcataaaccgcagcttttcgtgttttt 1200
D F F D L M T R V K I F N K R S P W S F
tataagtcgcagaaaaatatgttgattcatgggtatctctcaatctctcctttctttgaaga 1260
L S D A K N Y V D S W V I Q S P F L R R
atattttcccaagttgtgtagcagaaacagcaactacagtgaatttataaaagagattttgattgaacagct 1320
I F P V G S K A I T E L I R D W I A N
gaatctttttaaatctcagacacacctgtttctctctgtcttctccagactccccctgaacc 1380
E S L K I Q T C T S S L V F S D S F E P
attagagctgcaaacctccctctctccagtcttgatagtctgagcactctttgaaagttttctgtatagc 1440
I R A D N L S S V C D G I L E S V S D S
tttttccaaactgtttgtaagctctgaaatattgtttttgatttttctttcaaaaaagtttagttggaaga 1500
Chapter 4

Results

FSKTVKACRNWLICPRFSGG
tgtagtcattctgatgatgagaaaaaggtatatatcatagttgtgttcatagtaatgacatcctg 1560
YSMSVMRSKGILOVGSMinimumTS
tactcttccaggttattttttgtggacctttctcttcattctttagacgacattttactccc 1620
YSSEFVDDLFPSSVRPAFYS
gacctctgtttttctcaattggagtttggagacccaatggaattaatctctcatggtctcc 1680
DSAFSOLSEFPEPKWNLYHGS
tgggattacgatcaagcaattaagactttgtgtaatattcgttctggacttaatgacaat 1740
WDYDQRFLORCLESGLSDNDN
agttgagatgtaaggctgaaatgttgtccagacctttttcttcagacgcttaagagaata 1800
ESVVKVECVKTSSEPDKDEN
agtcaattctcaagctcaattttttagactccaaaagcagcatgatgagaaaccctcaaaaaa 1860
MLKSNSFLDSKMSMGPSQK
attgagatgacagatgaattataaaaacaaaggggaccaatcacaaggaatgtctttctctc 1920
IEDADELKQDKQITRKNACF
ssaattctcagtagttaggactatcggagtttcagctgattcgatgataacaaaattctc 1980
FNANVSETIQVSADSMINKIL
tggactctgcattctgctcttcacccaccaagctgtggagaccaattgatttttagataatcggatt 2040
CSDLPLAPVKVQEPVLVDNPI
agttcacaattactggaatattgtgctgtttttggctatagtgctcattataactatat 2100
SSKLMIECVFVFLGRYRVHIYY
ggtagtagataatattagctcaatgacacaaaacagcatgcacattctatattggaggt 2160
GDSIIMLDNPNMHAIHGG
aaacctgagcatctttttttgctacccaaacagagatgattccatctcatgacagccaa 2220
KPGHLFQIQKEKVEIPHDQS
ctgaaattctctggaataggccccagcattcattgtgctctattcctcacaataacttat 2280
LKIPEIGPQAFIGSFSKTY
ggcatgggacatccagctcacaattctctgtagtgaaaatctttttcaaaacttat 2340
GMGSSAPIHLSEIDITRALT
tcggtagtattgacatcttttttttcacccaaacagagatgattccatctcatgacagccaa 2400
LIAAFESSMNGLIRVDRKAII
Gaggtatatctctgataatcaatattgtttttgcctttcacaattgagagataatagggg 2460
EGNLSNSFLAFLKSKSNEG
tataagaaccatactccttctccagtttccctttcttttatttgtagttgctgtcttt 2520
YKTISIPYPVFPIFAGSG
ggccaatctttttggattaatagagaaattaattgtatggtgattttgctcagcaatattcatt 2580
GKSFGLEYKLIDGCQNSFM
ttcactgcacctagaaaaagatcatagaggcaagtctcagcacaattaatggctcaagggaa 2640
FTAAPRKLIGQVHDKIDSRQ
-tagggataaataaccaataagcaggaagaagaatttttagacctctttgaaatatcattt 2700
YEDKLLKIISRKKNKSFENTL
ctttctctttgcacacagccataattttgtcatgttagatgtaatgctcctttgacacaccttga 2760
LSLVBNKPLIVMDCESLNPPLG
ttcatgtctgacctgtttggaggtgctctcattctatataggaagacgtgaagat 2820
FIDLVLVNLDSIIRKSSKDI
cttatattcctttttctcaaggctagtgtctttaggagaaatattcgaatgctgcttt 2880
LNYYFSSAVSEGIIIANVAS
ccaattgtttgcatagctgcaacagagttgacatactctatcaatcaagcactttcctattcttgagatg 2940
PIACIATAAGTGIDLIQSSFYESST
ttgtagtaatattgaccgatataaattgtttggaaaaacccctttttgtgctaagagccattta 3000
CGKLMQHKNDLKTLCALSHL
Chapter 4

Results

agattgccctaccttttttgctcaaaaagattttggtatatctcataatggtcataagctt 3060
RLPYLFGSKRFGFYNGFIKL
gttattacagtcaatatgggtcataatggtcataatggtcataagctt 3120
GYSQMEKAFTDNMETTL
aaagctgtgacacttcaatgtgataaaatttggttggttgctacttagcagcagcaaa 3180
KAVGTSMDKFGVLTSTRADK
tcagatttttgacacttagtttccccataatgtcataatgctacacagcagcagcaaa 3240
SDFELPNVCTINESQGST
tcaaatagttgtattttgatagctacaagagacacttttctcccataaatttgctaca 3300
FNSVIILIVTRDFSNPIESI
tagttacactacaaaggtctcaaaatatctcttctgtcatcagttcaaccc 3360
IVAITHQKNLLILYFPAAIQ
gtgagatttttctttgacagagtttcaatcttcaatggtcataatggtcataagctt 3420
GEMDFLSRRFPPIHSSVgLKN
nttagtctttgtgataatttacataatgcaaaataatccattttcattgacactggaa 3480
FSVLDNKLDKLPFQLIEQ
gatccatcgagactattttggagttaaatctcttgaggggtcattttcctgaagagttgaa 3540
DPFHVDFVKEGDPFLKSE
ttgagcctgtgtagtgcacacctcccataatttgagaaggtcataatggtcataatggtcata 3600
LSLGVNDVKLPPQIEKAEAEV
qaaatctccaaacttactttccataattctgagactgtggtgagtcgtgaaatataggt 3660
ENLKTHPLISYCLGWDLIES
qagattgagctagggaataatcagaggttttggttcgtgctgagcagcaaa 3720
EMNARENFEFKFGGVWSKQ
tccaaagtagtgacggagacaaatttgagaagctgcaatgcctccagaa 3780
FKDEPNQRDPIEDNCAMLPE
gcagttttttcacaagcttcaaatctgcagctgtggtgagtcgtgaaatataggt 3840
AVFPRHFANDDLTFWSAVKK
agactagtcttttcataacatatagttctgtcatgatttttgaggtggtaaaccatttt 3900
RLVFKNPLNNVHDVKEAKPF
ggttaagagatgtgctgacatcttttctttaagaagaattgctccacttattcctagtttagctcag 3960
GKEMLIDFLKKVPLIPSFDQ
agagttgggaatctttttttcttgaatttgagaagagtaagataagcaaatgcggcc 4020
RMYESISEFEEEKKISKNAA
atgattggggcccatctgatcgatcaacaaactgcactggcccaataaacagagatctttttct 4080
MIGAHHDRTTDDWPINEIFL
tccataaaaccttcttcgacaaaggaagaaatgatttttggtgatctaaagccggc 4140
FIKSKLCTKKKEKMFCDAKAG
caaaaactttttgctcttttcttccatatttttgaattttgcaccactaaacaggtat 4200
QTLACFSHILCCKFAPLLNY
attgagaagaaatgacccagttctgctcagagtaaatctctacattatacatcagaaagaataat 4260
IEKKVTCLPGLPNFYIHQKN
ntttagattacaggttaaaatgtggtgaaacatagatttttagttgagctctgcagctaca 4320
FDELEKVKSYDFSVCSTES
gattatagctttagtgcacataacattcaccctctttgcgatgaatagt 4380
DYEAYDASPQDSYTLAFEYEL
atgaagttcrtttttgcttcacaagcatatgattttgagattatctttttactttctcaaaaaa 4440
MKLYGVSNSMIEDLYLKLKMH
tttataattccagctagggaaacctagctatcatagctacactgggaaatttttgcatctttc 4500
LNCKLGNLAIMRFTGEFCFT
ttgattaccttttgcacaaacatgtttttcaatctcacttcatgagttctggtgcagaaact 4560
Chapter 4

Results

Tctataggccaaattatccactctgtcagctccaattatgatcgaactaggtgtctagtga 5880
S I G Q I I Q C P H M I Q T C I G L L E
Y R P N Y P M S T H D P N M H R A R I
Tcaagtttaagattcgatgcaatcactatttggtcttctactcattg 5940
S S L N Q S M Q L L T I P T C S S S I
K F E F D A I V N P T V A L I D V S V D Q
Ttgaggctagtcataaaccactagcaacaaagcactgcagaataacctgctgctgaagttgtaa 6000
L E S C T N L A T K A L Q K R Q L P Q M
G V M Y Q L N Q S T E A K T T A A D V
Ttggggcgcagttctcaagcacttttggtcttcgactccaaatctcgaattctccttc 6060
L G R S F K H F L G L D Y Q I L N P F
G A Q F Q A L F G S S G L P N P E S F L
Tcgaggatgaagataatgtctacacctctacaactgctgctctcctcgatgttagttgtg 6120
S R M K I L S T Q L W L S S M L V L I
E D E D I V N P P T V A L I D V S V D Q
agagctccgcgaagggtggtttttcgacacatcgagctccaaagagccagccaggaagttg 6180
R A S G R V V S S K V R H A Q E P E G
S F R K G F F K G P P R S T A R R Y
atcatgccagaagaatattgtcaggttttgaatcatcactccaaagatttgctgaaataagtt 6240
I M P E V L V R V L N Q S Q D L L K K I
H A R S I Q G F E S I P R F A E K D S
cctgagcgaaaggagaatttggcttagtcgaattatcgaggtgctgaataatctcagtt 6300
L N S K G K I L G Q I H V G L K I F S
E Q Q R R N L L R S N S C R A E N F Q F
tcaatccagagaaagtttctcagttgatcaagatttcacacaaatggatgtaggtc 6360
S I Q N K G F Q K N S T N L I S A
N P E Q R F S V D Q E F I N K F D K C N
attctcagagagggactcaaatcctcacacatgcttttgacatttggcagcagaaattac 6420
I L R E G T Q I S H M A L S I V E Q N Y
S Q R R D S N L T H G F E H C G A E L Q
aacagaaataagaagggcctttggaacaactacactgtaagaataatgatagataatactgcaca 6480
N E I R R G L G N Y I W K N M I D P R D
R N K K G P W K L H L E E Y D R S K G S
cctgttaacatcaactcgcagaaacgagcagtggagcatcacagggaggtgccagcacaacc 6540
L L H L T A K P A V E A S E G V A A T P
V T S N C E T S S G G I R G S G S N P S
gccatagtagttctgcagaaaccagagagctgttaaaaaacacatcaggaattattaccttg 6600
A I V L S E N Q R A V K N T R N Y Y L
H S I V R E P E S C K K H H Q E L L P E
aggataatgtttggagactcttcagatattggacacagcagcacagcactacaccaggg 6660
R I M F G N L A V M G T S E Q T D Y P G
D N V W E S C N G N K R T D R L P R G
gagcatctagcattccgagaccagcagctagtagagagaatcaggaggtcctgactgcacatctc 6720
E H L A I P R P V I E N Q E A L T A H L
A S S D E S T D R E S G G S D C T S P
cjacagacagctgtacattttcgcacacacattggtaaaagatcaggtggtatagttggt 6780
P A G M S L L T F A T N V K A W G V V G
S R H V I V N F C H K C E S M G C S W C
gcagaaaggttaagttttgctgattaaccccttagagcaattttagtgtagcagctcctgacaa 6840
A E G K F A G L T F R Q L C E P F A E Q
R R
Fig 4.23: Nucleotide sequence of CVA cDNA in small letters. (A)n indicates the 3’ poly(A) tail. The amino acid sequences of two putative ORFs are shown below the nucleotide sequence. Amino acid of ORF 1 is indicated by black capital letter and ORF 2 by red capital letter.
4.4.4 Phylogenetic analysis

Phylogenetic analysis based on the replicase and trichovirus coat protein genes with selected members of the family Betaflexiviridae showed that the isolate is closely related to the German isolate (Fig 4.24 - 4.25).

Fig 4.24: Phylogenetic analysis based on the complete deduced amino acid sequence of the replicase protein of the Indian isolate of CVA and selected members of the family Betaflexiviridae. The analysis included the following viruses: Apple chlorotic leaf spot virus (ACLSV, genus Trichovirus), Apple stem grooving virus (ASGV, genus Capillovirus), Apple stem pitting virus (ASPV, genus Foveavirus), Cherry green ring mottle virus (CGRMV, unassigned member of the family Betaflexiviridae), Cherry necrotic rusty mottle virus (CNRMV, unassigned member of the family Betaflexiviridae), Cherry virus A (CVA, genus Capillovirus).

Fig 4.25: Phylogenetic analysis based on partial or complete deduced amino acid sequences of the trichovirus coat protein of the Indian isolate of CVA and selected members of the family Betaflexiviridae. The analysis included the following viruses: Apple chlorotic leaf spot virus (ACLSV, genus Trichovirus), Apricot pseudo-chlorotic leaf spot virus (APCLSV, genus Trichovirus), Apple stem grooving virus (ASGV, genus Capillovirus), Citrus tatter leaf virus (CTLV, genus Capillovirus), Cherry virus A (CVA, genus Capillovirus).
4.5 DEVELOPMENT OF DIFFERENT PCR BASED DETECTION METHODS FOR CVA AND OTHER STONE FRUIT VIRUSES

4.5.1 Immunocapture-RT-PCR (IC-RT-PCR) for CVA

Fragments of approximately 900 bp (Fig 4.26 - 4.27) and 400 bp (Fig 4.27) were amplified by IC-RT-PCR from the CVA positive leaf sample using primer pair CVAU6508/CVAL and CVAU6508/CVAL6905. No amplification was observed in the healthy sample by IC-RT-PCR.

Fig 4.26: Detection of CVA by IC-RT-PCR. Lane 1: Healthy plant as a negative control. Lane 2-3: Amplification of 875 bp of polyprotein of CVA using primer pair CVAU6508/CVAL. Lane M: 100 bp DNA ladder

Fig 4.27: Detection of CVA by IC-RT-PCR. Lane 1: Healthy sample as a negative control. Lane 2: Amplification of 419 bp of polyprotein of CVA using primer pair CVAU6508/CVAL6905. Lane 3: Amplification of 875 bp of polyprotein of CVA using primer pair CVAU6508/CVAL. Lane M: 100 bp DNA ladder
4.5.2 Nested and Hemi-nested RT-PCR for CVA

A nested and hemi-nested PCR (Diagrammatic representation shown in Fig 4.28) assay was used to enhance the specificity of amplified product of CVA. In first round of PCR, CVA5839U and CVA6905R primer pair (external) gave an amplification of 1089 bp. In second round of amplification, CVA6508U and CVAMPR primer pair (internal) gave 282 bp fragments in nested PCR whereas primer pair CVA6508U (internal)/CVA6905R (external) gave 419 bp in heminested PCR. Healthy sample did not show any amplification in RT-PCR (Fig 4.29).

Fig 4.28: Diagrammatic representation of nested and heminested RT-PCR. A: First strand cDNA synthesis. B: Initial PCR amplification using two original flanking primers CVA5839U/CVA6905R. C: Heminested PCR from the primary PCR amplification using one original flanking prime (CVA6905R) and one internal nested primer (CVA6508U). D: Nested PCR from the primary PCR amplification using two internal nested primers CVA6508U/CVAMPR.
Fig 4.29: Detection of CVA by nested and hemi-nested RT-PCR. Lane 1: Healthy plant as a negative control. Lane 2: Amplification of 419 bp of polyprotein of CVA using one original flanking prime (CVA6905R) and one internal nested primer (CVA6508U). Lane 3: Amplification of 282 bp of polyprotein of CVA using two internal nested primers CVA6508U/CVAMPR. Lane 4: Amplification of 1089 bp of polyprotein of CVA using two original flanking primers CVA5839U/CVA6905R. Lane M: 100 bp DNA ladder

4.5.3 Multiplex RT-PCR (mRT-PCR) for the simultaneous detection of CVA, CNRMV, LChV-1 and PNRSV

4.5.3.1 Specificity of primers

To test the specificity of designed primers as well as primers taken from published article, total RNA extracted from healthy and virus-infected plants were used for RT-PCR amplification. A cDNA fragment of the expected size (181 bp) was consistently amplified by nad5 mRNA-specific primers (nad5s/nad5as) as plant internal control, irrespective of whether total RNA came from healthy or infected plants.

Specific PCR amplification products of the expected sizes were obtained from all positive virus samples. Four amplified products of expected sizes (1051 bp for CVA; 675 bp for PNRSV; 553 bp for CNRMV and 300 bp for LChV-1) were observed only in infected samples (Fig 4.30). No amplification products were obtained from extracts of healthy plants (Fig 4.30).

To assess specificity of the primers when more than one primer pair was present in RT-PCR reaction, the multiplex primer set containing CVA3568F/MPLEXR, PNRSVCP-F/PNRSV-CPR, CNRMV5291F/MPLEXR, LCUW7090/LCUWC7389 and nad5s/nad5as were used for
RT-PCR assay. As previously described, total RNAs extracted from healthy and mix-infected cherry were tested for mRT-PCR amplification. The expected RT-PCR product of internal control was amplified from all cherry samples. There were no differences at the level of amplification in simplex RT-PCR than in multiplex RT-PCR. RT-PCR results clearly indicated that both single- and five-pair of primers could produce specific cDNA fragments of plant *nad5* mRNA, CVA, PNRSV, CNRMV and LChV-1. Therefore, the specificity of these designed primers in mRT-PCR was as good as that in simplex RT-PCR.

### 4.5.3.2 Optimization of mRT-PCR

For the optimization of mRT-PCR reaction parameters such as the concentration of each primer pairs, PCR annealing temperature, annealing time, magnesium chloride concentration, amount of dNTP mix, and amount of cDNA were evaluated. The concentration CVA, PNRSV, CNRMV, LChV-1 and internal control (*nad5*) specific primers were tested from 0.25 µl (0.05 µM) to 1 µl (0.2 µM) concentration. The final concentrations of each viral specific forward and reverse primer were 0.2 µM for CNRMV, 0.15 µM for PNRSV, 0.1 µM for CVA, LChV-1 and internal control respectively. CVA and *nad5* show clear and sharp band even at 0.05 µM primer concentration whereas band intensity of CNRMV significantly reduced below 0.2 µM primer concentration. In PCR reaction, the annealing temperature was tested from 50°C to 55°C. Finally, an annealing temperature of 53°C was used for further multiplex RT-PCR because multiple bands were observed at the temperature below 53°C whereas above 53°C some desirable bands were not seen properly. The annealing time was tested from 30 to 40 s and best result was obtained at 40 s. Band intensity was significantly improved when the concentration of MgCl₂ increased from 1.5 to 2.0 mM and final concentration of dNTP mix decreased from 0.6 mM to 0.3 mM. The amount of cDNA also affected the band intensity. It was seen that the band intensity was significantly decreased above and below 4 µl concentration of cDNA from 25 µl of RT reaction.

### 4.5.3.3 Detection sensitivity of mRT-PCR

To determine the detection limits of mRT-PCR, a 10-fold serial dilution (10⁰ to 10⁻⁵) of cDNA obtained from artificially mixed total RNA (~1µg) from infected sweet cherry leaf
were made. CVA showed positive result at highest $10^4$ dilution whereas LChV-1, PNRSV, CNRMV and internal control were detected at $10^2$ dilution, respectively (Fig 4.31).

4.5.3.4 Determination of the specificity of genome amplification

In order to assess the specificity of the PCR assays, all positive amplicons from simplex and mRT-PCR were cut from the agarose gel, purified and sequenced. The sequences obtained were submitted to the GenBank Database under accession number FR773524 for PNRSV, FR748229 for LChV-1 and FR748228 for CNRMV. BLAST search of these isolates in GenBank showed 80-90%, 94-99%, 89-92% and 79-99% similarity with the available isolates of CVA, PNRSV, CNRMV and LChV-1, respectively at nucleotide level confirming identity of the amplicons.

4.5.3.5 Detection of more than one cherry virus from field samples by mRT-PCR

The sweet cherry leaf samples collected from Rohru region of Shimla district were used to detect infecting viruses by mRT-PCR. A total of 9 sweet cherry plants were tested. Out of 9, 7 plants showed mixed infection and 2 showed single infections. All 9 plants were infected with LChV-1. One plant was found to be infected with three viruses viz., CVA, CNRMV and LChV-1. Six plants were infected with at least two viruses; three were with PNRSV and LChV-1 and other three with CNRMV and LChV-1 (Fig 4.32).

![Fig 4.30: Multiplex and simplex RT-PCR for the detection of four cherry viruses viz. CVA, PNRSV, CNRMVand LChV-1. Lane 1: Multiplex RT-PCR of all four viruses from artificial mixed infection. Lane 2: Simplex RT-PCR for CVA (1051 bp). Lane 3: Simplex RT-PCR for PNRSV (675 bp). Lane 4: Simplex RT-PCR for CNRMV (553 bp). Lane 5: Simplex RT-PCR for LChV-1 (300 bp). Lane 6: Simplex RT-PCR for internal control from plant mitochondrial gene nad5 (181 bp). Lane 7: Positive control. Lane M: 100 bp DNA ladder]
Fig 4.32: Sensitivity limits of multiplex RT-PCR for all four cherry viruses (CVA, PNRSV, CNRNV and LChV-1) with internal control using 10 fold serial dilutions of cDNA. Lane 1-6: $10^0$ to $10^{-5}$ serial dilutions. Lane M: 100 bp DNA ladder

Fig 4.32: Simultaneous detection of CVA, PNRSV, CNRMV and LChV-1 from sweet cherry plants by mRT-PCR. Lane 1: Positive control of all four viruses from artificial mixed infection. Lane 2: Cherry plant infected with CNRMV and LChV-1. Lane 3: CVA, CNRMV and LChV-1. Lane 4: PNRSV and LChV-1. Lane 5: LChV-1. Lane 6: LChV-1. Lane 7: PNRSV and LChV-1. Lane 8: PNRSV and LChV-1. Lane 9: CNRMV and LChV-1. Lane 10: CNRMV and LChV-1. Arrows indicates specific amplification products of 1051 bp for CVA, 675 bp for PNRSV, 553 bp for CNRMV 300 bp for LChV-1 and 185 bp for nad5. Lane M: 100 bp DNA ladder

4.5.4 Development of Polyvalent Degenerate Oligonucleotide based RT-PCR (PDO-RT-PCR) for the detection of different members of the family *Betaflexiviridae*

To screen the presence of multiple infections of viruses such as CVA, CNRMV, CGRMV, ASPV and ACLSV in the stone and pome fruit plants a degenerate primer pair (PDO-F/PDO-R) was developed from the conserved region of RdRP gene after multiple alignment. A fragment of approximately 300 bp was amplified by primer pair (PDO-F/PDO-R) from the infected leaf sample of sweet cherry and apple plants (Fig 4.33 - 4.35). No amplification was observed in the healthy sample by RT-PCR.

A fragment of approximately 300 bp was amplified corresponding to ASPV, CVA and CNRMV (Fig 4.33 and 4.34) by this primer pair from the respective positive samples. The
amplified PCR products were purified and directly sequenced, and submitted to the GenBank Database under accessions HE574815, HE574816 and HE574817. Blast search of isolates of CVA, CNRMV and ASPV in NCBI showed 82-99% similarity for the available isolates of CVA, CNRMV and ASPV, respectively.

Fig 4.33: Detection of ASPV by POD RT-PCR. Lane 1: Amplification of 290 bp of replicase gene of ASPV using primer pair PODF/PODR. Lane M: 100 bp DNA ladder.

Fig 4.34: Detection of CVA and CNRMV by POD RT-PCR. Lane 1: Amplification of 290 bp of replicase gene of CVA. Lane 1: Amplification of 290 bp of replicase gene of CNRMV using primer pair PODF/PODR. Lane M: DNA ladder (100 bp/1 Kb DNA ladder)

Fig 4.35: Detection of viruses belonging to the family Betaflexiviridae by POD RT-PCR. Lane 1-12: Amplification of 290 bp of replicase gene of member of the family Betaflexiviridae using primer pair PODF/PODR from the sweet cherry samples collected from Rohru region of Shimla district. Lane M: 1kb DNA ladder.
4.6 EXPRESSION OF COAT PROTEIN (CP) IN HETEROLOGOUS SYSTEM FOR DEVELOPMENT OF ANTIBODY BASED DIAGNOSTICS

4.6.1 Expression of CVA CP in *E. coli* for development of diagnostic kit

4.6.1.1 Construct preparation

DNA fragment containing the tricho coat protein region of CVA (621 bp) was amplified using designed primers (Table 3.8; Fig 4.36) from CVA CP cloned in T&A vector as template. Eluted PCR product was cloned into T&A cloning vector. Chemically competent *E. coli* cells (strain DH5α) were transformed with the ligated vectors. Ten clones were selected as white, ampicillin-resistant colonies, and plasmids were extracted from small overnight cultures by the boiling method. The clones containing inserts were checked by restriction digestion with *Hind*III. T&A cloning vector containing CP region of CVA (Fig 4.37) and pET-32a expression vector were double digested with *Sac*I and *Bam*HI and, ligated overnight at 16°C and cloned. Pure plasmids were extracted from positive colony grown overnight. Now these pure plasmids (pET-32a vector) were used to transform the competent *E. coli* (BL21) cells. After transformation, recombinant clones were screened by colony PCR to check desired insert. Plasmid was isolated from several recombinant clones and all were sequenced in an automated sequencer in order to identify the orientation and frame of insert. After getting the sequence, one clone was selected containing CVA CP in the correct orientation and in correct frame that was used for expression studies.
4.6.1.2 Standardization of optimal expression conditions for CVA CP

Different temperature (30°C and 37°C) and IPTG concentrations (0.4 mM and 1 mM) for different time intervals (0 hour to 4 hours) were tried at different combinations to standardize the conditions for optimum expression of recombinant CVA CP. The best expression level of CVA CP was observed at 37°C with 1 mM IPTG concentration after four hours of IPTG addition into the insoluble fraction (pellet) (Fig 4.38).

![Image](image_url)

**Fig 4.38**: Expression of fusion proteins containing CVA-CP in *E. coli*. Lane 1: Approximately 40 kDa fusion proteins expressed after 4 h of induction into insoluble fraction at 37°C. Lane 2: Insoluble fraction of cells having pET-32a after 4 hours of induction at 37°C. Lane 3: 40 kDa fusion protein expressed after 3 h of induction into insoluble fraction at 37°C. Lane 4: Insoluble fraction of cells having pET-32a after 3 hours of induction at 37°C. Lane 5: Insoluble fraction having recombinant vector at 0 hour of induction at 37°C. Lane 6: Insoluble fraction having pET-32a vector after 0 hour of induction at 37°C. Lane M: Prestained protein marker (SM0672: Fermantas)

4.6.1.3 Purification of expressed protein

Expressed fusion protein was purified using His tag purification kit (Novagen, USA) based on affinity purification of His tagged proteins. Purity of the purified protein was checked by electrophoresis on 12% SDS-PAGE gel that revealed one distinct band of expected size after coomassie staining (Fig 4.39) and confirmed by western blotting (Fig 4.40). The concentration of the purified protein was determined by using Nanodrop (Thermofischer, USA) and found to be 5 mg/ml. This was further diluted to make final concentration of 1 mg/ml.
Purified protein obtained after purification from *E. coli* was used as antigen for immunization of rabbit. A polyclonal antisera against CVA coat protein was raised through applying six weekly consecutive injections of 500 µl purified coat protein (1 mg/ml, each) into a healthy white New Zealander male rabbits (approximately four months old). In the first injection, the purified coat protein was emulsified with complete Freund’s adjuvant in an equal volume ratio and injected subcutaneously and intramuscularly at ten different sites. In the subsequent injections, incomplete Freund’s adjuvant was used and the coat protein was injected both subcutaneously and intramuscularly. Finally, the rabbit was bled after 6th weeks. The animal was bled from the marginal ear vein. The blood was collected into 50 ml falcon tube and allowed to clot at room temperature for an hour. Subsequently the tube containing the clotted blood was kept at 4°C overnight. The serum was collected by using pasture pipette and centrifuged at 5000 rpm for 10 min at 2-6°C. The supernatant was collected and stored at -80°C, either frozen or mixed with 50 % glycerol or at 4°C after adding sodium azide to a concentration of 0.02% (w/v).

### 4.6.1.4 Raising of antiserum

Antiserum was raised in healthy New Zealander white male Albino rabbits by injecting *E. coli* expressed coat protein along with Freund’s adjuvant. In the first injection, the purified coat protein 500 µl (500 µg) was emulsified with 500 µl of complete Freund’s adjuvant and
injected subcutaneously and intramuscularly at ten different sites. Another five injections were given with 500 µl (500 µg) of purified coat protein with 500 µl of incomplete Freund’s adjuvant both subcutaneously and intramuscularly at weekly intervals. Finally, the rabbit was bled after 6th weeks. The animal was bled from the marginal ear vein. The supernatant was collected and stored at -80°C, either frozen or mixed with 50% glycerol or at 4°C after adding sodium azide to a concentration of 0.02% (w/v).

4.6.1.5 Purification of antibody (separation of IgG from serum)

The IgG was extracted from the antiserum using IgG purification Kit (Genei, India). Concentration of IgG in the purified preparation was 7 mg/ml. The antibodies were diluted to final concentration of 1 mg/ml IgG. The extracted IgG was checked by electrophoresis on 12% SDS-PAGE gel that revealed one distinct band of expected size after coomassie staining (Fig 4.41).

![Fig 4.41: SDS PAGE analysis of purified rabbit IgG produced against CVA CP.](image)

4.6.1.6 Preparation of antibody-enzyme conjugate

Separated CVA antibodies 0.2 ml (1 mg/ml concentration) was conjugated with alkaline phosphatase 0.1 ml (10 mg/ml, Sigma). Activity of conjugate was checked by DAS-ELISA using antibodies produced in rabbit as coating antibodies at optimum dilution. It reacted well with the purified protein as a positive control up to the dilution of 1:200.
4.6.1.7 Evaluation of antibodies

Antibodies produced in rabbit were used as coating antibodies in various dilutions (1:100, 1:200, 1:300, 1:500, 1:1000 and 1:2000) in DAS-ELISA along with the antibody enzyme conjugate produced after alkaline phosphatase to identify maximum dilution of the antibodies at which virus can be detected successfully. Antibodies produced in the rabbit reacted specifically in DAS-ELISA with the known positive samples or purified protein, positive controls, negative controls and healthy plants. The maximum titer to which the antibodies can be diluted and gave confirmed positive result is 1:200 dilutions.

4.6.1.8 Field screening to check the activity of the kit

To check efficacy of the kit, different stone and pome fruits were checked for presence or absence of CVA using DAS-ELISA as earlier described. A total of 74 samples including 15 positives (by RT-PCR) were checked, out of which 40 were found positive by DAS-ELISA (Tables 4.6 - 4.7). These 40 includes 12 positive samples which were already positive in RT-PCR. Three RT-PCR positive samples were found negative in DAS ELISA test. Presently no ELISA based diagnostics available is for the detection of CVA. The developed kit by us will be useful for the detection of CVA from different stone fruits plants in plant quarantine and certification programs.

Table 4.6: OD values of DAS-ELISA for different stone fruits infected with CVA

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Plants tested</th>
<th>Mean OD values 405nm</th>
<th>Serial No.</th>
<th>Plants tested</th>
<th>Mean OD values 405nm</th>
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Table 4.7: Result of ELISA test
4.6.2 Expression of PNRSV CP in *E. coli* for development of diagnostic kit

4.6.2.1 Construct preparation

DNA fragment (675 bp) containing coat protein region of PNRSV was amplified using designed primers (Table 3.8; Fig 4.42) from PNRSV CP cloned in T&A vector as template. Eluted PCR product was cloned into pGEM-T Easy cloning vector. Chemically competent *E. coli* cells (strain DH5α) were transformed with the ligated vectors. Eight clones were selected as white, ampicillin-resistant colonies, and inserts were checked by colony PCR (Fig 4.43). pGEM-T Easy vector containing CP region of PNRSV and pET-32a vector were double digested (Fig 4.44), ligated overnight at 16°C and transformed into *E. coli*. The positive colonies were checked by colony PCR (Fig 4.45) and pET-32a with cloned CP was used to transform competent *E. coli* (BL21) cells. Plasmid was isolated from several recombinant clones and few of them were sequenced in an automated sequencer in order to identify the orientation and frame of insert. After getting the sequence, one clone was selected containing PNRSV CP in the correct orientation and in correct frame that was used for expression studies *in vitro*.

![Fig 4.42: Amplification of PNRSV CP gene using specific primer pair PNRSVexpU/ PNRSVexpD. Lane 1- 2: 675 bp of PNRSV CP gene. Lane M: 1 kb DNA ladder](image)
Fig 4.43: Colony PCR analysis of PNRSV CP gene for bacterial expression. Lane 1: water as a negative control. Lane 2-9: Amplification of 675bp of PNRSV CP gene by primer pair PNRSVexpU/ PNRSVexpD. Lane M: 100bp DNA ladder

Fig 4.44: Restriction analysis of recombinant pGEM-T Easy vector containing modified CP gene of PNRSV and double digestion of pET-32a expression vector. Lane 1: pET-32a vector digested by BamHI and NofI. Lane 2: Undigested pET 32a vector. Lane 3: Fragment of 3 kb and 675 bp digested by BamHI and NofI. Lane M; 1kb DNA ladder

Fig 4.45: Colony PCR analysis of PNRSV CP gene cloned into pET-32a vector. Lane 1: Water as a negative control. Lane 2-6: Amplification of 675bp of PNRSV CP gene by primer pair PNRSVexpU/PNRSVexpD. Lane M: 100bp DNA ladder
4.6.2.2 Standardization of optimal expression conditions for PNRSV CP

Different temperature (30°C and 37°C) and IPTG concentrations (0.4 mM and 1mM) for different time intervals (0 hour to 4 hours) were tried at different combinations to standardize the conditions for optimum expression of recombinant PNRSV CP. The best expression level of PNRSV CP was observed at 37°C with 1 mM IPTG concentration after four hours of IPTG addition into the insoluble fraction (Fig-4.46).

![Image of gel electrophoresis](image-url)

**Fig 4.46**: Expression of fusion proteins containing PNRSV-CP in E. coli. Lane 1: 43 kDa fusion protein expressed after 3 h of induction at 37°C into insoluble fraction (pellet). Lane 2: supernatant + 3 hours + 37°C. Lane 3: Pellet + 4 hours + 37°C. Lane 4: supernatant + 4 hours + 37°C. Lane 5: Pellet + 4 hours + 30°C. Lane 6: supernatant + 4 hours + 30°C. Lane 7: Pellet + 3 hours + 30°C. Lane 8: Control with no IPTG at 0 hour. Lane M: Prestained protein marker (Fermentas)

4.6.2.3 Purification of expressed protein

Expressed fusion protein was purified using His tag purification kit (Novagen, USA) based on affinity purification of His or His tagged proteins. Purity of the purified protein was checked by electrophoresis on 12% SDS-PAGE gel that revealed one distinct band of expected size after coomassie staining (Fig 4.47) and confirmed by western blotting (Fig 4.48). The concentration of the purified protein was determined by using Nanodrop (Thermofischer, USA) and found to be 3 mg/ml. This was further diluted to make final concentration up to 1 mg/ml.
Chapter 4

Results

4.6.2.4 Raising of antiserum

Antiserum was raised in healthy New Zealander white male Albino rabbits by injecting *E. coli* expressed coat protein along with Freund’s adjuvant. In the first injection, the purified coat protein of 500 µl (500 µg) was emulsified with complete Freund’s adjuvant in an equal ratio and injected subcutaneously and intramuscularly at ten different sites. In the subsequent five injections, purified coat protein of 500 µl (500 µg) emulsified with incomplete Freund’s adjuvant in an equal ratio and injected both subcutaneously and intramuscularly at weekly interval. Finally, the rabbit was bled after 6th weeks. The animal was bled from the marginal ear vein. The supernatant was collected and stored at -80°C, either frozen or mixed with 50% glycerol or at 4°C after adding sodium azide to a concentration of 0.02% (w/v).

4.6.2.5 Purification of antibody (separation of IgG from serum)

The IgG was extracted from the antiserum using IgG purification Kit (Genei). Concentration of IgG in the purified preparation was 7 mg/ml. The antibodies were diluted to final concentration of 1 mg/ml IgG. The extracted IgG was checked by electrophoresis on 12% SDS-PAGE gel that revealed one distinct band of expected size after coomassie staining (Fig 4.49).

![Fig 4.47: SDS PAGE analysis of purified His Tag fusion protein. Lane 1- Purified 43 kDa His Tag fusion protein. Lane M - Prestained protein marker (Fermantas)](image)

![Fig 4.48: Western blot analysis of purified fusion protein. Lane 1: ~43 kDa fusion protein. Lane 2: ~17kDa His tag. Lane M: protein marker (Fermantas)](image)
4.6.2.6 Preparation of antibody-enzyme conjugate

Separated PNRSV antibodies 0.2 ml (1mg/ml concentration) was conjugated with alkaline phosphatase 0.1 ml (10 mg/ml, Sigma). Activity of conjugate was checked by DAS-ELISA using antibodies produced in rabbit as coating antibodies at optimum dilution. It reacted well with the purified protein as a positive control up to the dilution of 1:200.

4.6.2.7 Evaluation of antibodies

Antibodies produced in the rabbit were used as coating antibodies in various dilutions (1:100, 1:200, 1:300, 1:500, 1:1000 and 1:2000) in DAS-ELISA along with the antibody enzyme conjugate produced after alkaline phosphatase identify maximum dilution of the antibodies at which virus can be detected successfully. Antibodies produced in the rabbit reacted specifically in DAS-ELISA with the known positive samples (earlier RT-PCR tested confirmed positives or purified protein), positive controls, negative controls and healthy plants. The maximum titer at which the antibodies can be diluted and gave confirmed positive result is 1:200.

4.6.2.8 Field screening to check the activity of the kit

To check efficacy of the kit, different stone and pome fruits were tested for presence or absence of PNRSV using DAS-ELISA as earlier described. Total 54 samples were checked out of which 4 were found positive by DAS ELISA (Table 4.8 - 4.9). These samples were also checked by commercial kit (Boireba, Switzerland). All four samples found positive for
PNRSV by test kit were also found positive by commercial kit. All these samples were also confirmed by RT-PCR.

Table 4.8: Comparative OD values of DAS-ELISA for different stone fruits infected with PNRSV, Positive control and negative control using the reference ELISA kit and test kit.

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Plants tested</th>
<th>Mean OD values 405 nm</th>
<th>Serial No.</th>
<th>Plants tested</th>
<th>Mean OD values 405 nm</th>
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<td>Ref. kit</td>
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<td>Wild cherry</td>
<td>0.094</td>
<td>0.154</td>
<td>51</td>
<td>Plum</td>
</tr>
<tr>
<td>26</td>
<td>Wild cherry</td>
<td>0.113</td>
<td>0.153</td>
<td>52</td>
<td>Plum</td>
</tr>
</tbody>
</table>

Positive control: 1.343 | 1.413
Healthy: 0.080 | 0.106
Table 4.9: Result of ELISA test

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Sample</th>
<th>Samples tested</th>
<th>Positive in Test Kit</th>
<th>Positive in Reference kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sweet cherry</td>
<td>27</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Sour cherry</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Plum</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Quince</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Wild cherry</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Apricot</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>54</strong></td>
<td></td>
<td><strong>4</strong></td>
<td><strong>4</strong></td>
</tr>
</tbody>
</table>

4.6.2.9 Keeping quality of the kit

When keeping quality of the formulated kit was checked it was observed that the kit works well and gave optimum results when kept at 4°C. Keeping the kit reagents at room temperature diminishes their quality. Therefore, it was recommended to keep the reagents in a refrigerator with temperature of 4°C.