3. MATERIALS AND METHODS

The present investigation was conducted in the Department of Chemistry and Biochemistry, College of Basic Sciences, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur and Virology Laboratory, Council for Scientific and Industrial Research-Institute of Himalayan Bioresources technology (CSIR-IHBT), Palampur during 2012-2015. The studies were conducted to explore the phytochemical constituents viz. antioxidant activity, total phenols, flavonoids and quinones, polyphenol oxidase (PPO), total chlorophylls, total carotenoids, ascorbic acid and molecular characterization of causal agent of chlorotic leaf spot disease in C. gigantea grown in green house condition. The chapter elaborates the methodology followed for the achievement of objectives pertaining to the research problem and has been dealt under following sub-heads:

3.1 Sample collection

3.2 Phytochemical analysis

3.2.1 Antioxidant activity

3.2.2 Total phenols

3.2.3 Flavonoids and Quinones

3.2.4 Estimation of quinones

3.2.5 Polyphenol oxidase (PPO)

3.2.6 Total chlorophylls and total carotenoids

3.2.7 Ascorbic acid

3.3 Molecular characterization

3.3.1 Host range studies

3.3.1.1 Raising and maintenance of herbaceous indicator plants

3.3.1.2 Preparation of inoculum
3.3.1.3 Mechanical sap inoculation

3.3.2 Virus purification

3.3.2.1 Purification of Virus Like Particles (VLP)

3.3.2.2 Short protocol for Virus Like Particle preparation

3.3.3 Electron microscopy

3.3.4 Analysis of purified VLP on SDS-PAGE

3.3.5 MALDI TOF-TOF MS-MS analysis

3.3.6 Isolation of nucleic acid from VLP

3.3.6.1 Agarose gel electrophoresis

3.3.7 RNase A treatment to VLP genomic VNA

3.3.8 DNase and S1 nuclease treatment for checking of strandedness of Viral Nucleic Acid (VNA)

3.3.9 Polymerase Chain Reaction (PCR) using Degenerate Oligonucleotide Primer (DOP-PCR)

3.3.10 Cloning and sequencing

3.3.10.1 Ligation reaction

3.3.10.2 Preparation of competent cells and their transformation

3.3.10.3 Transformation

3.3.10.4 Plasmid isolation from transformed cells

3.3.10.5 Digestion of the plasmid DNA for checking positive clones

3.3.10.6 Purification of plasmid DNA for sequencing

3.3.10.7 Sequencing

3.3.10.8 Purification of the reaction mixture

3.3.10.9 Sequence analysis using bioinformatics tools
3.1 Sample collection

Collection of infected *C. gigantea* samples

Plants of *C. gigantea* infected with chlorotic leaf spots and mosaic like symptoms were collected and maintained at green house under suitable conditions at ± 20°C. The *C. gigantea* leaves that showed the symptoms or which had infection were used in this study as the source of inoculum (Plate 3.1.1 and 3.1.2).

Plate No. 3.1.1 Infected *C. gigantea*

Plate No. 3.1.2 Green House View of *C. gigantea*
3.2 Phytochemical analysis

3.2.1 Antioxidant Activity

**DPPH radical scavenging activity**

DPPH radical scavenging activity or 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity was evaluated by the methods of Kordali et al. (2005) and Sharma and Bhatt (2009). It was calculated in the aqueous extracts of samples and for standard monomer of (+) catechin was used. Protocol for standard monomer is given in Table 3.1

**Reagents:**

1) Stock solution (0.1 mg/ml): 2 mg of (+) catechin (Sigma) was dissolved in 20 ml of methanol.

2) Stock DPPH solution: In 50 ml of methanol 3.9 mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved. It was wrapped with aluminium foil and left for half an hour. Weighing was carried out in dark.

3) Methanol (AR).

**Table 3.1 Protocol for preparation of standard curve for (+) catechin**

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>Concentration of stock solution (μg/ml)</th>
<th>Stock Solution (μl)</th>
<th>Methanol (ml)</th>
<th>DPPH solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>3.000</td>
<td>1.000</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>20.0</td>
<td>2.980</td>
<td>1.000</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>40.0</td>
<td>2.960</td>
<td>1.000</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>60.0</td>
<td>2.940</td>
<td>1.000</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>80.0</td>
<td>2.920</td>
<td>1.000</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>100.0</td>
<td>2.900</td>
<td>1.000</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>120.0</td>
<td>2.880</td>
<td>1.000</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>140.0</td>
<td>2.860</td>
<td>1.000</td>
</tr>
</tbody>
</table>
The absorbance was recorded at 517 nm with spectrophotometer model Merck Spectroquant Pharo 100 after 30 minutes incubation in the dark at 30°C. The percentage of DPPH free radical scavenging activity (% inhibition) was calculated with the help of following equation:

$$\text{% inhibition} = \frac{\text{Abs (Control)} - \text{Abs (Test)}}{\text{Abs (Control)}} \times 100$$

IC$_{50}$ value (the amount of antioxidant necessary to decrease the initial DPPH free radical concentration by 50 per cent) was calculated from the regression line obtained from the plot of per cent inhibition against concentration of each solution using the following equation:

$$\text{IC}_{50} \text{ value} = \frac{(50 - \text{y intercept})}{\text{Slope}}$$

The percent inhibition which indicates the antioxidant activity of catechin is shown in Figure 3.1

![Figure 3.1 Per cent inhibition of standard monomer (+) catechin](image)

**Figure 3.1** Per cent inhibition of standard monomer (+) catechin

Equation of line: $y = 5.119x + 14.03$

Antioxidants activity was calculated by the following equation

$$\text{Concentration (µg)} = \frac{\text{Absorbance at 517- (y-intercept)}}{\text{Slope}}$$
3.2.2 Total Phenols

Total phenol was estimated in dried extract of *calotropis* leaves by the method of Makkar (2003).

**Standard curve of tannic acid**

Standard curve was plotted using tannic acid solution (E. Merck, India) as a standard for total phenol.

**Reagents:**

1. Folin-Ciocalteu (FC) reagent (1N): FC reagent (2N) was diluted in the ratio of 1:1 with double distilled water. The reagent was always stored at 4°C.

2. Sodium carbonate (20 per cent): 40 g of Na$_2$CO$_3$ was dissolved in about 150 ml distilled water and made it up to 200 ml in a volumetric flask.

3. Standard tannic acid solution (0.5 mg/ml): 25 mg of tannic acid (TA) was dissolved in 50 ml of distilled water.

The protocol for the preparation of standard curve is given below in Table 3.2

**Table 3.2 Protocol for preparation of standard curve of Tannic acid**

<table>
<thead>
<tr>
<th>Test tube</th>
<th>TA Solution (ml)</th>
<th>Distilled water (ml)</th>
<th>FC Reagent (ml)</th>
<th>Na$_2$CO$_3$ (ml)</th>
<th>Concentration (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.00</td>
<td>1.00</td>
<td>0.50</td>
<td>2.50</td>
<td>0.000</td>
</tr>
<tr>
<td>T$_1$</td>
<td>0.01</td>
<td>0.99</td>
<td>0.50</td>
<td>2.50</td>
<td>5.00</td>
</tr>
<tr>
<td>T$_2$</td>
<td>0.02</td>
<td>0.98</td>
<td>0.50</td>
<td>2.50</td>
<td>10.00</td>
</tr>
<tr>
<td>T$_3$</td>
<td>0.03</td>
<td>0.97</td>
<td>0.50</td>
<td>2.50</td>
<td>15.00</td>
</tr>
<tr>
<td>T$_4$</td>
<td>0.04</td>
<td>0.96</td>
<td>0.50</td>
<td>2.50</td>
<td>20.00</td>
</tr>
<tr>
<td>T$_5$</td>
<td>0.05</td>
<td>0.95</td>
<td>0.50</td>
<td>2.50</td>
<td>25.00</td>
</tr>
<tr>
<td>T$_6$</td>
<td>0.06</td>
<td>0.94</td>
<td>0.50</td>
<td>2.50</td>
<td>30.00</td>
</tr>
<tr>
<td>T$_7$</td>
<td>0.07</td>
<td>0.93</td>
<td>0.50</td>
<td>2.50</td>
<td>35.00</td>
</tr>
<tr>
<td>T$_8$</td>
<td>0.08</td>
<td>0.92</td>
<td>0.50</td>
<td>2.50</td>
<td>40.00</td>
</tr>
<tr>
<td>T$_9$</td>
<td>0.09</td>
<td>0.91</td>
<td>0.50</td>
<td>2.50</td>
<td>45.00</td>
</tr>
<tr>
<td>T$_{10}$</td>
<td>0.10</td>
<td>0.90</td>
<td>0.50</td>
<td>2.50</td>
<td>50.00</td>
</tr>
</tbody>
</table>
Absorbance was recorded at 725 nm after 40 minutes incubation at 30°C

**Figure 3.2** Standard curve of tannic acid

Equation of line: \( y = 0.01053 \times + 0.004467 \)

Total phenols were calculated by the following equation

\[
\text{Concentration (µg)} = \frac{\text{Absorbance at 725 nm - (y-intercept)}}{\text{Slope}}
\]

**Total Phenols**

**Reagents**

1. \( \text{Na}_2\text{CO}_3 \) (20 per cent)

2. Folin Ciocalteu reagent (FCR) or Phenol reagent (1N)

**Extraction**

Finely ground 0.2 g sample was taken in a 100 ml beaker. To this sample, added 10 ml of 70 per cent acetone and kept it on a shaking water bath for 2 hours at 30°C. The beaker was tightly covered with aluminium foil to avoid evaporation. After the expiry of time, the contents were centrifuged at 10,000 rpm for 20 minutes; supernatant was used for the estimation of total phenols.

**Estimation**

Aliquot (0.1) ml was taken and final volume was made upto 1ml with distilled water. To this 2.5 ml of 20 per cent \( \text{Na}_2\text{CO}_3 \) followed by 0.5 ml of Folin Ciocalteu
reagent (1N) was added. The content was incubated for 40 minutes at room temperature. Developed blue colour was measured at 725 nm on spectrophotometer model Merck Spectroquant Pharo 100.

3.2.2.1 Simple phenols

Reagents

1. Polyvinylpyrrolidone (1 per cent)
2. Na₂CO₃ (20 per cent)
3. Folin Ciocalteu reagent (FCR) or Phenol reagent (1N)

Estimation

One ml of polyvinylpyrrolidone (1 per cent) was added to 1 ml of extract and the solution was vortexed for 15 minutes under ice cold conditions. 0.2 ml of aliquot was taken in test tube and the final volume was made 1 ml with distilled water. To it 2.5 ml of 20 per cent Na₂CO₃ was added followed by 0.5 ml of Folin Ciocalteu reagent. The content was incubated for 30 to 40 minutes at room temperature. The precipitates were filtered and the absorbance of filtrate was measured at 725 nm on spectrophotometer model Merck Spectroquant Pharo 100.

3.2.2.2 Tannins

Total tannins were calculated after subtracting simple phenols from total phenols

Total tannins = Total phenols – Simple Phenols

3.3.3 Flavonoids

Reagents:

1. NaNO₂ (5 per cent): Dissolved 5 g Sodium Nitrite in 100 ml distilled water
2. Aluminium chloride (10 per cent): Dissolved 10 g AlCl₃ in 100 ml distilled water
3. NaOH (1 M): 0.4 gm NaOH in 10 ml distilled water
4. Standard (+) Catechin: 0.01mg/ 100 μl

Preparation of standard curve of (+) catechin for flavonoid

To a known volume (50, 100, 150, 200, 250, 300, 350μl) of (+) catechin taken in sets of test tubes comprising of seven test tubes, was added double distilled water to make
the final volume of 1250 µl. 75µl of 5 per cent NaNO\textsubscript{2} was added to the mixture. The contents were mixed thoroughly with the help of vortex and allowed to stand for 5 minutes. After 5 minutes 150 µl of 10 per cent AlCl\textsubscript{3} solution was added. After 6 minutes 500 µl of NaOH and 275 µl of double distilled water was added to the mixture. The solution was mixed well and the intensity of pink colour was measured at 510 nm with the help of Merck Spectroquant Pharo 100 spectrophotometer.

### Table 3.3 Protocol for preparation of standard curve of (+) Catechin for flavonoids

<table>
<thead>
<tr>
<th>Test Tube no.</th>
<th>Catechin solution (µl)</th>
<th>Distilled water (µl)</th>
<th>NaNO\textsubscript{2} (µl)</th>
<th>AlCl\textsubscript{3} (µl)</th>
<th>NaOH (µl)</th>
<th>Distilled water (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T\textsubscript{1}</td>
<td>000</td>
<td>1250</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>T\textsubscript{2}</td>
<td>50</td>
<td>1200</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>T\textsubscript{3}</td>
<td>100</td>
<td>1150</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>T\textsubscript{4}</td>
<td>150</td>
<td>1100</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>T\textsubscript{5}</td>
<td>200</td>
<td>1050</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>T\textsubscript{6}</td>
<td>250</td>
<td>1000</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>T\textsubscript{7}</td>
<td>300</td>
<td>950</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
<tr>
<td>T\textsubscript{8}</td>
<td>350</td>
<td>900</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>275</td>
</tr>
</tbody>
</table>

**Standard curve for catechin**

![Standard curve for catechin](image)

$r^2 = 0.9973$

Y-intercept = 0.008929 ± 0.004535

Slope = 0.008777 ± 0.0002028

**Figure 3.3** Standard curve of (+) Catechin
Equation of line: $y = 0.008777 \times + 0.008929$

Flavonoids were calculated by the following equation

$$\text{Concentration (µg)} = \frac{\text{Absorbance at 510- (y-intercept)}}{\text{Slope}}$$

**Extraction of total flavonoids from the sample**

Flavonoids were extracted following method of (Swain and Hills 1959; Mahadevan and Sridhar 1986). The dried leaf sample (1.0 g) was taken in a test tube and 10 ml of methanol was added to it. The test tubes were placed on a water bath shaker maintained at 37°C for shaking. After 12 hours, the test tubes were removed and centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected and evaporated to dryness. The resulting extract was re-dissolved in 1ml of methanol for further use.

**Estimation of total flavonoids**

An aliquot of 250 µl of re-dissolved methanol extract was used for the estimation of flavonoids. After subjecting it to similar treatment as for standard curve preparation given in Table 3.3. The intensity of pink colour developed was measured at 510 nm and the result was expressed as mg of (+) Catechin equivalent (CEs) per g of extract using following formula:

$$\text{Flavonoids (mg/g)} = \frac{\text{Absorbance at 510- y intercept}}{\text{Slope}}$$

Y intercept and slope were calculated from the standard curve drawn with (+) Catechin.

**3.3.4 Estimation of quinones**

Quinones were estimated by using fresh leaf samples of *C. gigantea* by using catechol as a standard by the method of Mahadevan (1966) followed by Mahadevan and Sridhar (1986).

**Reagents**

1. Sodium phosphate buffer (0.1M, pH 6.6)
2. Standard catechol or caffeic acid ($5 \times 10^{-3}$M in water)
3. Trichloroacetic acid in 60 per cent ethanol (0.5N)

4. Trichloroacetic acid in 60 per cent ethanol (0.5N) containing 0.05M ascorbic acid

Two gram tissue was crushed in pre chilled mortar and pestle with chilled phosphate buffer (5 ml) for each gram of tissue and centrifuged at 2,000g for 30 minutes at 4°C. Discarded pellet and supernatant was used for further estimation. This was used as an enzyme extract. Pipetted out 3 ml of buffer, 3 ml of standard catechol or caffeic acid and 1.5 ml of enzyme extract into a test tube. This was shaken gently and incubated in the water bath. Withdrawn 2 ml of samples in duplicate at different time intervals. Added 4 ml of TCA reagent (without ascorbic acid) to one sample and to the other corresponding sample added 4 ml of TCA containing ascorbic acid. Precipitated sample was filtered and recorded the absorbance at 400 nm. Calculated the amount of quinones produced by the enzyme extract from ortho-dihydric phenols.

Quinones were calculated by following equation:

\[
\text{Total activity} = \frac{\text{Standard concentration (mM/min/g tissue)} \times \text{O.D. of sample/g}}{\text{Standard O.D.}}
\]

3.3.5 Estimation of Polyphenol Oxidase (PPO) activity (Farkas and Kirlay 1962)

Polyphenol oxidase activity of fresh C. gigantea leaves was estimated in freshly prepared acetone powder by the method of Farkas and Kirlay (1962).

Reagents

1. Acetone

2. Na₂SO₄ (0.2 M)

3. Phosphate buffer (0.2 M, pH 6.0)

4. Pyrogallol (1.0 per cent)
**Procedure**

**Preparation of acetone powder**

Accurately weighed 4.0 g fresh *C. gigantea* leaves were transferred to a cleaned pre-chilled pestle and mortar. Then chilled acetone (kept at -20°C) was added just enough to cover the sample, which was ground slowly in clockwise direction for 2-3 minutes, followed by fast anticlockwise grinding for 3-5 minutes. The contents were filtered through Whatman No.1 filter paper and washed repeatedly with chilled acetone till a colourless filtrate was obtained. The residue was air dried on the filter paper and immediately stored in air tight sealed vials at -4°C for further use.

**Preparation of Enzyme extract**

To a clean centrifuge tube kept in ice cold conditions, 0.1 g of acetone powder was added, followed by 4.0 ml of double distilled water. The content was vortexed and centrifuged in cold at 4,000 rpm for 10 minutes (4°C). The supernatant containing water-soluble enzyme was filtered through cotton and collected into a test tube, already placed in icebox. The residual bound enzyme in the pellet were re-extracted with 5.0 ml of 0.2 M NaSO₄ solution and centrifuged at 4,000 rpm for 10 minutes at 4°C. The resulting supernatant was collected similarly and pooled with the previous lot. The contents were mixed properly to get the enzyme extract for further use. This was termed as enzyme extract.

**Enzyme assay with Pyrogallol as a substrate**

Two ml of enzyme extract was taken in a test tube (already placed in icebox) to which 2.0 ml of 0.2 M phosphate buffer (pH 6.0) and 0.5 ml of 1.0 per cent pyrogallol solution (E. Merck, India) as substrate was added. Enzymatic activity (OD/minutes) was observed at 410 nm at 30 seconds interval for 3 minutes on spectrophotometer model Merck Spectroquant Pharo 100.

**3.3.6 Estimation of chlorophylls**

Chlorophylls in fresh *calotropis* leaves were estimated spectrophotometrically by the method of Jayraman (1981) and Davies (1976).

**Reagents**

Acetone (80 per cent)
Procedure

For total chlorophyll extraction the process was carried out into clean pestle and mortar by grinding sample of *calotropis* leaves (0.2 g) with 80 per cent acetone. The extracts were centrifuged at 4,000 rpm for 1 minute and content was re-extracted 4 times in the same manner with 80 per cent acetone until residues became colorless. Finally, this volume was made up to 20 ml with 80 per cent acetone and absorbance was measured at 663 nm, 645 nm and 480 nm on spectrophotometer model Merck Spectroquant Pharo 100.

The extent of chlorophylls (mg/g) was calculated by the following equation:

\[
\text{Chlorophyll 'a'} = \frac{12.7 \times A_{663} - 2.69 \times A_{645} \times \text{Volume made up}}{1000 \times \text{wt. of sample}}
\]

\[
\text{Chlorophyll 'b'} = \frac{22.9 \times A_{645} - 4.68 \times A_{663} \times \text{Volume made up}}{1000 \times \text{wt. of sample}}
\]

\[
\text{Total chlorophylls} = \frac{20.2 \times A_{645} + 8.02 \times A_{663} \times \text{Volume made up}}{1000 \times \text{wt. of sample}}
\]

Total Carotenoids (*Jayraman 1981 and Davies 1976*)

Procedure

Extraction of total carotenoids was carried out into cleaned pestle and mortar by grinding fresh *calotropis* leaves (0.2 g) with 80 per cent acetone. The extracts were centrifuged at 4,000 rpm for 15 minutes and then carotenoids were repeatedly extracted out with 20 ml (5-5 ml repeatedly, 4 times) 80 per cent acetone until residues became colourless. Finally, the volume was made up to 20 ml with 80 per cent acetone and absorbance was measured at 663 nm, 645 nm and 480 nm on spectrophotometer model Merck Spectroquant Pharo 100.
The amount of total carotenoids (µg/g) was calculated with following equation:

\[
\text{Total carotenoids} = A_{480} + 0.114 \times A_{663} - 0.638 \times A_{645} \times \frac{\text{Volume made up}}{\text{Wt. of Sample}}
\]

3.3.7 Ascorbic acid (AOAC 2010)

**Reagents**

1. Oxalic acid (1 per cent)

2. L-ascorbic acid

3. 2,6 dichlorophenol indophenols dye: The dye solution was prepared by taking 52 mg of dye in 200 ml volumetric flask adding 100 ml of hot distilled water. The volume was then made to 200 ml with distilled water. After cooling 42 mg of NaHCO₃ was added and dissolved properly.

**Preparation of standard for estimation of ascorbic acid**

Accurately 100 mg of L-ascorbic acid was dissolved in 500 ml of 1.0 per cent oxalic acid solution and was used as standard (always prepared fresh). The amount of ascorbic acid in sample is expressed as mg/100g.

**Procedure**

Fresh, just after plucking *calotropis* leaves samples (25 g) was accurately weighed, and ground with 25 ml of 2.0 per cent oxalic acid as extraction medium in order to get slurry. The total weight of slurry was recorded. 10 g slurry was taken in a 100 ml beaker and volume was made up to 25 ml with 1 per cent oxalic acid. This content of the beaker was filtered properly through Whatman filter paper No.1 and 5.0 ml of this filtrate was titrated against 2, 6 dichlorophenol indophenol dye. From these titrations three concordant readings were observed.

\[
\text{mg/100g Ascorbic acid} = \frac{\text{Standard conc. of ascorbic acid} \times A \times \text{Volume made up} \times B}{\text{Titer value of Standard} \times \text{Wt. of slurry} \times S \times \text{Vol. used for titration}} \times 100
\]

Where,

\[
A = \text{Titer value}
\]

\[
B = \text{Total weight of slurry}
\]

\[
S = \text{Weight of sample}
\]
3.3 Molecular characterization of virus

3.3.1 Host range studies

3.3.1.1 Raising and maintenance of herbaceous indicator plants

Healthy seeds of susceptible host species viz. *Nicotiana tabacum* cv. Xanthi, *N. edwardsonii*, *N. glutinosa*, *N. benthamiana*, *N. clevelandii*, *N. tabacum* cv. Samsun, *N. rustica*, *Chenopodium amaranticolor*, *Datura stramonium*, *Datura metel*, eggplant (*Solanum melongena*), cowpea (*V. unguiculata*) and white beans (*Phaseolus vulgaris*) etc. were grown in plastic pots (5 inches) filled with autoclaved soil under insect proof glass house condition (22 ± 5°C temp). The growing plants (at 2-5 leaves stage) were used in the experiments for inoculation of plant samples.

3.3.1.2 Preparation of inoculum

The symptoms on infected *C. gigantea* plants were observed and inoculation was done on healthy plants and the inoculum was prepared from infected leaves of *C. gigantea* plant which were previously collected and grown in CSIR-IHBT green house. The leaves were grinded in inoculation buffer (0.1 M potassium phosphate buffer pH 7.4 and 7.6) in a sterilized pre chilled mortar and pestle. This extract thus prepared was used for mechanical sap inoculation.

3.3.1.3 Mechanical sap inoculation

Mechanical sap inoculation was done on herbaceous indicator plants and for mechanical transmission studies, healthy seedlings of diagnostically susceptible host species at appropriate leaf stage were used. Carborundum (600 mesh) powder was generally used as a mechanical abrasive to make injuries on leaves of test plants (10 plants of each species used for mechanical sap inoculation) for an easy entry of virus.

(i) Mechanical Inoculation of Host plants (herbaceous indicator) from the original sample of *C. gigantea*

(a) Prepared 100 mM potassium phosphate buffer (7.4) from 1 M.
(b) Then different host plants like *Nicotiana* were taken for the inoculations. For this pestle mortar was kept on ice and used chilled.
(c) At first the leaves of healthy indicator plants were just rinsed with distilled water.
(d) Then buffer was spread on the leaves with the help of supporting finger from the below.
(e) A pinch of carborundum powder was spread for making injuries on the upper surface of the leaves.

(f) Then crushed the *calotropis* in 100 mM phosphate buffer and spread the suspension on the carborundum spread plants.

(g) The leaves were inoculated by usual rubbing method with the help of fore finger. two to three plants of each indicator plant species were kept as control and individual leaf of all the test plants was inoculated.

(h) These inoculated leaves were observed for the systemic infection. Finally inoculated leaves were washed thoroughly with distilled water immediately after inoculation to eliminate excess of inoculums and abrasive.

### 3.3.2 Virus Purification

A protocol was standardized for virus purification from infected *C. gigantea* leaf samples.

#### 3.3.2.1 Purification of Virus Like Particles (VLP)

(a) Approximately 100 g of diseased leaves were homogenized in 300 ml of cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4) in the 1:3 ratio (for 1g tissue, 3 ml of buffer).

(b) After that it was filtered through muslin cloth to remove debris.

(c) Triton X-100 in the (final concentration) of 2 per cent was added to the extract, slowly at room temperature and extracted for a minimum of 35 minutes, at 25°C.

(d) The extract was centrifuged at 12,000 g for 20 minutes, at 10°C for remaining removal of debris.

(e) The supernatant was mixed with polyethylene glycol (PEG 6000) to a final volume of 6 per cent (w/v) in the presence of 0.2 M NaCl and kept at 4°C for 4 hours.

(f) The turbid supernatant was again centrifuged at 12,000 rpm for 20 minutes at 4°C.
(g) The resulting pellets were resuspended overnight in 60 ml TN buffer (50 mM Tris HCl, pH 7.4 and 10 mM NaCl) and centrifuged again at 12,000 g for 10 minutes at 4°C. (Discard the pellet if any).

(h) The supernatant was ultracentrifuged at 1,50,000 g for 2 hours (at 4°C) to precipitate the virus particles. The pellet was resuspended in 600 µl of 50 mM Tris buffer (Tris HCl pH 7.0).

(i) The crude viral preparation obtained after this is called as Virus Like Particle (VLP).

3.3.2.2 Short Protocol for Virus Like Particle Preparation

For quick isolation of VLPs short protocol was standardized and it was slight modification of the method of Melcher et al. (2008).

(a) Plant material (10 g) was followed by addition of 75 ml of 0.1M ice cold sodium citrate pH 6.5 (Citrate buffer) and 630 µl of 0.25M iodoacetamide.

(b) After 10 minutes on ice samples were disrupted in blender and transferred to the falcon tubes.

(c) It was followed by centrifugation at 4,000 g for 45 minutes at 4°C

(d) The supernatant approximately 13.750 µl was transferred to fresh falcon tubes followed by the addition of 1.250 µl of 33.3 (v/v) per cent triton X-100.

(e) Now mixture was transferred to ultracentrifuge tubes under laid with 20 per cent sucrose cushion (sucrose solution prepared in 0.1M citrate buffer or sucrose-citrate cushion.

(f) Tubes were subjected to ultra centrifugation at 70,000 g for 45 minutes at room temperature in a Beckman Ti 42.2 rotor and supernatants were removed by aspiration.

(g) Pellets containing Virus like particles (VLP) were resuspended in 200 µl of 0.5X citrate buffer and the suspension was centrifuged for 10 minutes at 8000 g at 4°C.

(h) Again supernatant was subjected to ultracentrifugation over a sucrose citrate cushion at 1,50,000 g for 65 minutes at 4°C.
(i) Pellets containing virus like particles (VLPs) were resuspended in 250 µl of 0.1 M Tris HCl pH 7.5, 2.5 mM MgCl₂.

(j) Virus-like particles include the viral structural proteins and also envelope or capsid that play role in the assembly of virus like particles (VLPs).

3.3.3 Electron microscopy

For electron microscopy studies the prepared VLP was smeared on the copper grids and then staining was done by 2 per cent uranyl acetate. The imaging of sample was done by electron microscope device (Tecnai Twin G², 200 kV FEI, Netherlands) at 200 kV voltage.

3.3.4 Analysis of purified VLP on SDS-PAGE (Laemmli 1970)

Reagents

1. **30 per cent acryl amide / bisacrylamide**: Dissolved 87.6 g acryl amide and 2.4 g bisacrylamide in 250 ml water with gentle stirring and made to 300 ml with deionised water. The solution was filtered and stored at 4°C in the dark.

2. **1.5 M Tris-HCl, pH 8.8**: Dissolved 27.23 g Tris base in 80 ml deionised water and volume made up to 150 ml. The pH was adjusted to 8.8 using 6 N HCl. Solution was autoclaved.

3. **0.5 M Tris-HCl, pH 6.8**: Dissolved 27.23 g Tris base in 60 ml deionised water and volume made up to 100 ml. The pH was adjusted to 8.8 using 6 N HCl. Solution was autoclaved.

4. **10 per cent ammonium per sulfate (APS)**: Dissolved 0.1 g APS in 1ml of deionised water. Always prepared fresh.

5. **10 per cent sodium dodecyl sulfate (SDS)**: Dissolved 10 g SDS in 50 ml of deionised water. The volume was made up to 100 ml using deionised water.

6. **Tetra methylene diamine (TEMED)**

7. **Sample Buffer**: Added 3.55 ml deionised water in 1.25 ml 0.5 M Tris HCl (pH 6.8), 2.5 ml glycerol, 2.0 ml 10 per cent SDS, 0.2 ml, 0.5 per cent bromophenol blue. Added 50 µl β- Mercaptoethanol to 950 µl sample buffer prior to use. The sample solution was stored at room temperature.
8. **10 x Electrode (Running) buffer, pH 8.3:** Dissolved 30.3 g Tris base, 144.0 g Glycine and 10.0 g SDS in 500 ml deionised water and the volume was made up to 1000 ml using deionised water.

9. **Staining solution:** Dissolved Coomassie Brilliant Blue R-250 (625 mg), Methanol (125 ml), Glacial acetic acid (25 ml) in deionised water and volume was made up to 250 ml with deionised water.

10. **Destaining solution:** Dissolved Methanol (125 ml), Glacial acetic acid (50 ml) in deionised water and volume was made up to 500 ml with deionised water.

**Procedure**

Assembled the glass plates and spacers in the casting frame and checked for water leaks. The comb was placed into gel cassette for marking. The glass plate was marked 1 cm below comb teeth and this was the level up to which the resolving gel poured. The comb was then removed. The 12 per cent resolving gel prepared by combining all reagents except APS and TEMED. Degassed solution under vacuum for at least 15 minutes. The APS and TEMED were added to degassed solution. Gently swirl solution before pouring into gel mold and gel was allowed to polymerize. After polymerization, rinsed the gel surface completely with distilled water. The 5 per cent stacking gel was prepared as per Table 3.4 rest protocol as similar as resolving gel. Before pouring the stacking solution the water was allowed to drain from resolving gel by tilting the gel assembly. Gently the comb was inserted between spacers after pouring and it was allowed to polymerize. The gel assembly was removed from casting frame after polymerization and inserted into the electrode assembly. The running buffer was poured in the buffer tank and comb was carefully removed without perturbing the wells formed by teeth of comb. The 20 µl samples was loaded into the wells with pipette using gel loading tips along with 10 µl protein marker. The rest of the running buffer was poured into outer chamber of electrode assembly. The power was applied at 90V for running gel. When the dye front was near bottom of gel (about 1 cm from bottom), turns off the power supply and disconnects the electric leads. Removed the gel sandwich, disassembled the glass plates. The gel was placed in small tray containing water. Rinsed the gel for about 5 minutes with water. Immersed the gel in the coomassie dye staining solution until it is uniformly blue. This may take several hours; hence the gel was stained overnight. The following day, the gel was transferred into destaining solution. The successive changes of destaining solution were given till the coomassie dye was removed out of the gel and only the protein bands remain blue.
Table 3.4 Recipe for gel preparation using SDS-discontinuous system

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Resolving gel (12%)</th>
<th>Stacking gel (5%)</th>
<th>Reservoir buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 (%) Acryl amide-bis acrylamide</td>
<td>8.0</td>
<td>1.02</td>
<td>-</td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>-</td>
<td>1.50</td>
<td>-</td>
</tr>
<tr>
<td>0.25 M Tris, 1.92 glycine, 1% SDS, pH 8.3</td>
<td>-</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>10 (%) (w/v) Ammonium persulphate</td>
<td>0.2</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>10 (%) SDS</td>
<td>0.2</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.66</td>
<td>3.33</td>
<td>1800.0</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.010</td>
<td>0.006</td>
<td>-</td>
</tr>
<tr>
<td>Total (ml)</td>
<td>20.07</td>
<td>5.976</td>
<td>2000.0</td>
</tr>
</tbody>
</table>

3.3.5 MALDI TOF-TOF MS-MS analysis

For Matrix Assisted Laser Desorption Ionization Time of Flight-Time of Flight Mass spectrometry-Mass spectrometry (MALDI TOF-TOF Model Bruker Ultraflextreme) analysis first of all in gel digestion method is used.

(j) Ingel digestion

(a) Gel slice was cut from SDS-PAGE gel with help of sharp razor blade.
(b) To this gel slice 50 per cent acetonitrile (ACN) and 100 mM of ammonium bi carbonate (NH₄CO₃) was added.
(c) Gel slice was kept for 37°C or at room temperature for 30 minutes (repeat if needed).
(d) Again 50 per cent acetonitrile was added and kept for 5 minutes at room temperature.
(e) These steps were performed until the gel slices became white opaque in appearance.

(f) Then ACN was removed and dried gel slice was concentrated at vacuum dryer or DNA concentrator at low speed for 5 minutes.

(g) Then 20-40 µl trypsin (digestion buffer) was added per slice or according to the size of gel slice and it was kept for 60 minutes at room temp.

(h) Again 100 µl of digestion buffer was added to the gel slice and it was kept at 37°C for overnight.

(i) Next day 100 µl of milli Q water was added to this slice and it was vortexed for 30 to 60 minutes.

(j) Liquid was collected in an eppendorf tube and again gel slices were broken or scratched. The slices were then mixed with 0.1 per cent tri fluoroacetic acid (TFA), vortexed and pooled. This liquid solution was further used for MALDI TOF-TOF analysis.

(ii) Sample loading on MALDI plate

The prepared samples were loaded on the MALDI plate and were further analyzed in MALDI TOF-TOF.

3.3.6 Isolation of Nucleic Acid from VLP

The protocol for virus like particle- viral nucleic acid (VLP-VNA) was standardized.

\[
\begin{align*}
\text{VLP} & \quad 50 \mu l \\
\text{DNase (1:9)} & \quad 4 \mu l \\
\text{Buffer (Dnase I)} & \quad 6 \mu l \\
\text{Water to} & \quad 60 \mu l
\end{align*}
\]

(a) This recipe was kept at 37°C for 2 hours and to it 50 µl of proteinase K and 10 per cent SDS was added.
(b) This was incubated for 65°C for 2-3 hours and the total volume was increased to 400 µl with 18.2 milli Q water.

(c) Added equal volume of Chloroform:Isoamylalcohol (CI) and vortexed for 5 minutes at room temperature.

(d) Then centrifugation was done at 13,000 rpm for 10 minutes at 4°C and if there was contamination this step was again repeated.

(e) Took aqueous phase and added 3 fold volume of absolute ethanol to it.

(f) It was kept at -80°C for 30 minutes and again centrifuged at 13,000 rpm for 25 minutes at 4°C.

(g) To the pellet added 500 µl 80 per cent ethanol with the walls of eppendorf tube and it was incubated for 1 minute at room temperature.

(h) Spin at 13,000 rpm for 5 minutes at 4°C was given and supernatant was discarded and the pellet was resuspended in 15 µl milli Q water.

This virus Like Particle - Viral Nucleic acid (VLP-VNA) was then run on agarose gel for the checking of genomic nucleic acid quality.

3.3.6.1 Agarose gel electrophoresis Sambrook et al. (1989)

Gel electrophoresis was done by using submarine horizontal agarose slab gel. Agarose in different quantity was dissolved by using 1X TAE and it was boiled so that agarose is completely dissolved. The temperature of melted agarose was brought down to 50°C to little cooling and then it was poured into a gel casting tray. The slot containing comb was inserted so that well formation can be done. At this step complete precaution was taken so that no bubbles can be trapped in the comb wells when the gel solidifies. After agarose solidifies, the comb was removed and it was placed in the tank as like submarine way for electrophoresis. 2 µl of gel loading dye was mixed with DNA samples (about 500 ng) properly and the prepared samples were loaded onto the wells of the gel. A constant voltage of 5 V/cm was used to carry out electrophoresis. Ethidium bromide (EtBr; 1.0 µg/ml) was used for gel staining and stained gel was visualized using a UV trans-illuminator and it was photographed, documented using gel documentation system (Cell Biosciences, Alpha Innotech Corp., CA, USA).

Sample loading dye (6X): Bromophenol blue (0.25 per cent) and xylene cyanol; 15 per cent Ficoll 400; stored at room temperature
TAE (50X per liter): 57.1 ml glacial acetic acid; 242 g Tris base; and 100 ml EDTA (0.5 M, pH 8.0)

3.3.7 RNase A treatment to VLP genomic VNA

The total nucleic acids were incubated with DNase and protease-free RNase A (Fermentas, Maryland, USA) at 37°C for 30 minutes as described below:

- Total nucleic acids: 10 µl (2-3 µg)
- RNase A (10 mg/ml stock): 0.5 µl

After RNase A treatment the nucleic acids were electrophorised in 0.7 per cent agarose gel after mixing with loading dye for analyzing their integrity and quality. Quantification of nucleic acids was performed by using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA).

3.3.8 DNase and S1 nuclease treatment for checking of strandedness of Viral Nucleic Acid (VNA)

DNase I (RNase free) enzyme (0.5 µl) was added to 2 µl of VLP-VNA and a total volume of 20 µl was prepared. This was incubated for 37°C for 1 hour. Similarly S1 Nuclease (0.5 µl) was added to 2 µl of VLP-VNA and a total volume of 20 µl was prepared. This was also incubated for 37°C for 1 hour. Then this was checked on agarose gels for the visualization.

3.3.9 Polymerase Chain reaction (PCR) using Degenerate Oligonucleotide Primer (DOP-PCR)

For detection of virus from sample or VLPs, PCR was carried out in a Gene Amp. Thermocycler.

Reaction mixture:

- DNA: 1µl
- LA Buffer II (2X GC Buffer II): 12.5 µl
- 10 mM dNTPs: 0.75 µl
- DOP I Primer: 1 µl
- Enzyme (TAKARA LA Taq): 0.20 µl
- Water to: 25 µl
DOP-PCR Primers (DOP-1):

Primer: \[5'CCG\ A\ CTC\ GAG\ \text{NNNNNN}\ \text{ATG}\ \text{TGG}\ 3'

PCR was performed by using following cycling parameters: 96°C for 8 minutes; 8 cycles of 93°C for 1 minute, 30°C for 1 minute, 72°C for 3 minutes, 93°C for 1 minute; 28 cycles of 93°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes and 72°C for 8 minutes.

3.3.10 Cloning and sequencing (Sambrook et al. 1989)

3.3.10.1 Ligation reaction

Ligation is necessary in order to provide a compatible vector system to the gene of interest so as to further get transformed successfully in the host cells. Ligation of pure plasmids was done in TA cloning vector and it was set up in an eppendorf tube as follows:

- TA Vector (RBC) 20-50 ng
- Insert DNA 50-150 ng
- Buffer A 1 μl
- Buffer B 1 μl
- DNA ligase 1μl
- Water to 10 μl

The reaction mixture was incubated at 4°C overnight followed by storage at -20°C until transformation.

3.3.10.2 Preparation of competent cells and their transformation

Since DNA is a very hydrophilic molecule, it does not normally pass through a bacterial cell's membrane. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA. This was done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium. For the transformation, *E. coli* DH5α strain was used.
This procedure was performed under sterile conditions. During the preparation of competent cells, all the solutions were chilled, pipette tips also cooled and cells always kept on ice.

**Preparation of competent cells which can be stored at -70 ºC**

- E. coli cells (DH5α) were streaked on Luria Bertani (LB) plate and allowed cells to grow at 37ºC overnight.
- One colony was placed in 5 ml LB media and grown overnight at 37ºC.
- 250 ml LB was inoculated with 1 ml of overnight culture of *E.coli* and grown at 37 ºC on a shaker till the O.D. A₆₀₀ was around 0.5.
- Culture was cooled on ice immediately and cells were harvested by centrifugation at 6000 rpm for 5 minutes at 4ºC.
- Supernatant was removed carefully and traces of it were removed by inverting the centrifuge tube on paper towels.
- However, the tube was never removed from ice for longer time. The bacterial pellet was resuspended in 50-70 ml of ice-cold 0.1 M CaCl₂ (sterilized by filtration or autoclaving) and incubated on ice for 30 minutes.
- Cells were recovered by centrifugation as above, resuspended in 50-70 ml of 0.1 M MgCl₂ and incubated on ice for 30 minutes.
- The cells were finally recovered and suspended in 10 ml of 0.1 M CaCl₂ containing 10 per cent glycerol.
- Aliquots of 200 µl each were prepared and immediately stored at -70ºC.

**3.3.10.3 Transformation**

The process by which cells take up exogenous DNA from the outside is called transformation. The purpose of this technique is to introduce a foreign plasmid into bacteria and to use those bacteria to amplify the plasmid in order to make large quantities of it. Apparently, the treatment induces a transient state of “competence” in the recipient bacteria, during which they are able to take up DNAs derived from variety of sources.
Transformation of DH5α strains of *E. coli*

- Frozen competent cells were thawed on ice for 15-20 minutes.
- To these competent cells, ligated product was added and mixed gently and the mixture was incubated on ice for 30 minutes.
- The cells were given heat shock at 42°C for exactly 90 seconds and immediately transferred to ice for at least 5 minutes
- 800 µl of Luria broth was added and tube was incubated at 37°C for 1 hour in a rotary shaker set at 200 rpm.
- The cells were pelleted down and pelleted cells were resuspended in 100-150 µl of LB.
- Transformed cells were pipetted onto LB agar plate containing appropriate antibiotics for selection of positive clones containing plasmids and spread uniformly using sterilized glass beads or bent glass rod spreader. For selection of the recombinant plasmids by antibiotic selection, Ampicillin was used in LB agar plate in appropriate amounts. Plates were incubated upside down at 28°C for 12-16 hours till colonies are big enough for transfer.

3.3.10.5 Plasmid isolation from transformed cells

Transformed colonies were picked and inoculated on LA-ampicillin plates with the help of sterile toothpick and same was used for the inoculation in LB-Amp media. These plates and tubes were incubated overnight at 28°C.

**Principle**

Bacterial plasmids DNA are widely used as cloning vehicles in recombinant research. Method denatures high molecular weight chromosomal DNA while covalently closed circular DNA (cccDNA) remains double stranded.

(i) **Boiling miniprep method (Holmes and Quingley 1981)**

(a) 1.5 ml of the overnight grown culture was pelleted down in a microfuge by centrifugation at 14,000 rpm for 30 seconds.

(b) Supernatant was discarded and the pellet was resuspended in 110 µl of STET buffer.
(c) Then freshly prepared 10.0 µl of Lysozyme (10 mg/ml stock in 10 mM Tris pH 8.0) was added to the resuspended cells and thoroughly mixed and incubated at room temperature for 5 minutes.

(d) Heat shock was given for 42 seconds in boiling water followed by centrifugation at full speed for 20 minutes at room temperature.

(e) Pellet containing the cell debris was removed with the help of a sterile toothpick.

(f) DNA in the supernatant was precipitated by adding one volume of isopropanol (i.e. 120 µl).

(g) Precipitated DNA was collected immediately by centrifuging for 20 minutes at full speed (14,000 rpm).

(h) The supernatant was completely removed and the pellet was air dried to remove excess of isopropanol.

(i) DNA was resuspended in 50 µl nuclease free water

**STET buffer:** 8 per cent sucrose; 0.5 per cent triton X-100; 50 mM Tris HCl (pH 8) and 50 mM EDTA (pH 8). Add triton X-100 after autoclaving rest of components in solution.

3.3.10.5 Digestion of the plasmid DNA for checking positive clones

In order to find out positive clones among the transformed colonies plasmid were digested either with enzyme that was used for cloning into vector as follows:

Digestion mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (~1 µg)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td><em>Hind</em> III (10 U/µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Buffer H (Takara)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Water to</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
Procedure:

- The digestion mixture containing plasmid DNA, buffer H, enzyme *Hind* III and water was thoroughly mixed and centrifuged briefly.
- Mixture was incubated at 37°C for 3 hours.
- 2.5 volumes of cold ethanol was added in mixture and incubated at -80°C for 30 minutes.
- Precipitated nucleic acid was centrifuged at maximum speed rpm for 20 minutes at 4°C.
- The supernatant was removed, pellet was dried and resuspended in RNase free water and then checked on 1 per cent agarose gel.

3.3.10.6 Purification of plasmid DNA for sequencing

Sequencing requires ultra clean DNA and therefore plasmid DNA was re-extracted using commercially available plasmid isolation kits.

(i) **Plasmid isolation using commercial kits**

Plasmid was also isolated using NucleoSpin® Plasmid QuickPure kit (Macherey-Nagel) as per the manufacturer’s instructions.

3.3.10.7 Sequencing

Plasmid of recombinant clones was isolated using NucleoSpin® Plasmid QuickPure kit as per the manufacturer’s instructions. The sequences of the cloned viral DNA components were obtained by sequencing the recombinant plasmids using vector specific primers.

The sequencing PCR was performed as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid (200-500 ng)</td>
<td>1.0</td>
</tr>
<tr>
<td>5X sequencing buffer</td>
<td>1.0</td>
</tr>
<tr>
<td>Reaction mixture</td>
<td>1.0</td>
</tr>
<tr>
<td>Primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>
Sequencing PCR was performed by using following cycling parameters: 95°C for 1 minute; 30 cycles of 95°C for 10 seconds, 50°C for 40 seconds and 60°C for 4 minutes. The sample was washed with injection buffer for removing surplus dyes and taken for sequencing by ABI PRISM™, 310 Genetic analyzer.

3.3.10.8 Purification of the reaction mixture

At completion of the reaction, the mixture was purified either manually or using Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Billerica, MA, USA) as per manufacturer’s instructions. In manual cleanup, the volume of the reaction mixture was raised to 100 µl with (double distilled water) ddH₂O. To this, 10 µl 3M sodium acetate (pH 4.6) and 250 µl of 100 per cent ethanol were added followed by centrifugation at 14,000 rpm for 20 minutes. The pellet thus obtained was washed twice with 250 µl of 75 per cent ethanol and dried in air. The dried pellet was dissolved in 15 µl of template suppression reagent (TSR; Applied Biosystems), denatured by heating for 4 minutes at 5°C and placed on ice before loading in the sequencer.

3.3.10.9 SEQUENCE ANALYSIS USING BIOINFORMATICS TOOLS

Database searches for obtained sequences were performed using Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997) available at NCBI website (http://www.ncbi.nlm.nih.gov/blast). The program BLASTP was used to search the amino acid sequence database. Phylogenetic analysis was performed by neighbor-joining method selecting 1000 bootstrap replicates, using clustalW program (Thompson et al. 1994) version 1.83 available online (http://www.ddbj.nig.ac.jp) and trees were viewed and edited using TreeView software version 1.6.6 (Page 1996). Multiple sequence alignments were (MSA) performed using MultAlin program available online (Corpet 1988). MSA were also performed using ClustalW program (Thompson et al. 1994) included in MEGA5 software (Tamura et al. 2007), available at http://www.megasoftware.net. Phylogenetic analysis was inferred using neighbor-joining method with 1000 bootstrap replicates and evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) in MEGA5 software. Pairwise sequence alignment scores (percentage identities) were determined using ClustalW program version 1.83 available online (http://www.ddbj.nig.ac.jp). Genome maps were constructed using BVTech Plasmid software (http://www.biovisualtech.com/).
3.3.4 Analysis using primers designed on the basis of available sequence

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Primer</th>
<th>DNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>PBCV-F-1</td>
<td>VLP</td>
<td>TACCGAGCTCGCCCTATA</td>
</tr>
<tr>
<td>2.</td>
<td>PBCV-R-1</td>
<td>VLP</td>
<td>AATAGCGTATCAGGAGCG</td>
</tr>
<tr>
<td>3.</td>
<td>NPV-F-1</td>
<td>VLP</td>
<td>GATTCAGTCGTCACTGAT</td>
</tr>
<tr>
<td>4.</td>
<td>NPV-R-1</td>
<td>VLP</td>
<td>GGATCCCCCATGCTGGATG</td>
</tr>
<tr>
<td>5.</td>
<td>NPV-25-F</td>
<td>VLP</td>
<td>TAGGTTTCGTTCCCTCACC</td>
</tr>
<tr>
<td>6.</td>
<td>NPV-25-F</td>
<td>VLP</td>
<td>GGGGATGTGGATAAAAAC</td>
</tr>
</tbody>
</table>

The recipe for PCR reaction:

- **PCR Reaction**
  - DNA: 3 µl
  - Forward primer: 0.5 µl
  - Reverse primer: 0.5 µl
  - High fidelity buffer: 5 µl
  - 10mM dNTPs: 1 µl
  - Taq Pol: 0.25 µl
  - Water: 13.75 µl

**PCR conditions**: 98°C - 1 minute, 30 cycles of 98°C - 20 seconds, 48°C - 30 seconds, 72°C - 2 minutes, 72°C - 10 minutes

These primers were then used for the PCR reaction at gradient of different annealing temperature viz. 46°C, 48°C and 50°C. In this case different bands were observed with respect to marker, these bands were cut, eluted, transformed and cloned in DH5α strain. Then different recombinant clones were observed, they were restricted digested and cloned in TA cloning vector and sequenced.