Chapter 3

3.0 Basic criteria for selection of sites

- Locations were representative of the potato growing region of the state with different characteristics that would satisfy the entire growing region
- Locations were easily accessed by road
- Each location was within the altitude range of 1500-2700 m amsl with about 100 to 200 m difference between each
- Some of the locations selected were close to the state farms

The locations, their altitudes, latitudes and longitudes for the different states were identified with the help of Global Positioning System (GPS, Garmin-60) are given below.

3.1 Identified sites for the studies

3.1.1 Meghalaya

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>M01 (CPRS)</td>
<td>N25 32.717</td>
<td>E91 50.743</td>
<td>1787 m</td>
</tr>
<tr>
<td>M02 (Mylliem)</td>
<td>N25 30.392</td>
<td>E91 49.381</td>
<td>1675 m</td>
</tr>
<tr>
<td>M04 (Shillong Peak)</td>
<td>N25 31.997</td>
<td>E91 51.216</td>
<td>1919 m</td>
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<tr>
<td>M05 (Laitkor Mawri)</td>
<td>N25 32.197</td>
<td>E91 54.085</td>
<td>1830 m</td>
</tr>
<tr>
<td>M06 (Mawjrong)</td>
<td>N25 26.191</td>
<td>E91 48.047</td>
<td>1864 m</td>
</tr>
<tr>
<td>M07 (Nongkynrih)</td>
<td>N25 28.004</td>
<td>E91 53.602</td>
<td>1779 m</td>
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3.1.2 Sikkim

<table>
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<tbody>
<tr>
<td>S01 (Rawangla Potato Seed Farm)</td>
<td>N27 17.439</td>
<td>E88 21.254</td>
<td>1920 m</td>
</tr>
<tr>
<td>S02 (New Sada)</td>
<td>N27 17.241</td>
<td>E88 21.168</td>
<td>1927 m</td>
</tr>
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<td>S03 (Bakkhim)</td>
<td>N27 16.907</td>
<td>E88 20.542</td>
<td>1808 m</td>
</tr>
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<td>S04 (Okhrey)</td>
<td>N27 08.704</td>
<td>E88 06.076</td>
<td>2324 m</td>
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<tr>
<td>S05 (Ribdi)</td>
<td>N27 10.064</td>
<td>E88 05.745</td>
<td>2358 m</td>
</tr>
<tr>
<td>S06 (Hiley Potato Seed Farm)</td>
<td>N27 11.055</td>
<td>E88 07.303</td>
<td>2761 m</td>
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23
3.1.3 Arunachal Pradesh

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<tr>
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<tbody>
<tr>
<td>AP 01 (Upper Wanghoo village)</td>
<td>N27 14.459</td>
<td>E92 25.286</td>
<td>2173 m</td>
</tr>
<tr>
<td>AP 02 (Lower Wanghoo village)</td>
<td>N27 14.642</td>
<td>E92 25.042</td>
<td>1968 m</td>
</tr>
<tr>
<td>AP 03 (Pedung village)</td>
<td>N27 15.381</td>
<td>E92 24.508</td>
<td>2351 m</td>
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<td>AP 04 (Ramling)</td>
<td>N27 11.878</td>
<td>E92 27.934</td>
<td>1721 m</td>
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<tr>
<td>AP 05 (Warjung)</td>
<td>N27 17.421</td>
<td>E92 27.934</td>
<td>2013 m</td>
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3.1.4 Nagaland

<table>
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<tbody>
<tr>
<td>N01 (Kigwema)</td>
<td>N25 36.544</td>
<td>E94 07.637</td>
<td>1533 m</td>
</tr>
<tr>
<td>N02 (Lower Ukule Kigwema)</td>
<td>N25 35.367</td>
<td>E94 07.560</td>
<td>1635 m</td>
</tr>
<tr>
<td>N03 (Khuzama)</td>
<td>N25 32.088</td>
<td>E94 08.270</td>
<td>1677 m</td>
</tr>
<tr>
<td>N04 (Khuzama NH 39)</td>
<td>N25 35.050</td>
<td>E94 08.080</td>
<td>1668 m</td>
</tr>
<tr>
<td>N05 (Upper Ukule Kigwema)</td>
<td>N25 35.546</td>
<td>E94 06.546</td>
<td>2057 m</td>
</tr>
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</table>

3.2 Aphid monitoring

Survey was carried out in the selected sites in four NE hill states viz. Meghalaya, Sikkim, Arunachal Pradesh and Nagaland in main (Summer) cropping seasons during 2008-2011 and in Meghalaya under autumn season during 2008-2011. A weekly aphid, *M. persicae* (Sulzer) count were taken in the un-sprayed crops at all the locations and simultaneously from sprayed crops at 2-3 locations, following 100 leaves count methods (Simpson, 1940; Raman, 1984), consists of counting aphids on equal number of leaves selected at random from top, middle and bottom of 33 plants and one bottom leaf from the 34th plant. Populations were counted every week throughout the period of crop growth. Beside aphid count on leaf, yellow sticky traps (Braodbent *et al*., 1948) coated with a grease-banding preparation about 1 mm thick were used to trap winged aphids, mounted on the post with its top 1.5 m from the ground and water pan traps (Moericke, 1951) painted yellow, half filled with water with some detergent to trap the winged aphids. The traps were placed within the experimental potato fields at the level of the crop and adjusted with the crop growth. The trapped aphids were counted and removed at week intervals.
3.3 Degeneration Study

The experiment conducted at two different locations viz. CPRS, Shillong and Kohima, Nagaland representing major potato growing states in NE Hills during 2008-11, using predominant varieties of the region to study the rate of degeneration and to find out the optimum period up to which the seed stocks may be used without replacement and reduction in yield when potato seed is planted in the subsequent generations. The data on total viral incidence (%) of the three virus diseases like Mild Mosaic (MM), Severe Mosaic (SM) and Potato Leaf Roll Virus (PLRV) observed thrice at 50, 65 and 80 days after planting for both the varieties popularly grown in the states, in subsequent generations at both the locations after crop was exposed to natural environment.

Two sets of field experiments were conducted.

i) For direct evaluation of the varieties for prevalence of infection/infiltration of viruses following their exposure in field for different periods.

ii) For assessment of the reduction in their yield brought after 3-4 years of their cropping or field exposure.

The experiments were conducted during 2008-11 at CPRS, Shillong in Meghalaya and Kohima in Nagaland, the important potato producing states in North Eastern Hills of India. Two popularly grown varieties in the region were included in the experiments were K. Jyoti and K. Giriraj.

The trials were planted in Randomized Block Design (RBD) with four replications in 2X3m plots with tuber planted at 60x20 cm inter and intra row distances. There were two treatments in the first year of the experiments which are as follows.

T-1 Crop planted as ware crop with no systemic insecticides and only fungicide sprays were given at 5-7 days interval against late blight and crop was harvested at maturity (Farmers’ system).

T-2 Crop planted with seed plot technique, i.e. soil application of systemic insecticide (Phorate 10 G @ 15 kg/ha) at planting + two sprays with systemic insecticides i.e. Imidacloprid @0.025% conc.(2.5ml/10 lit of water) at 15 days interval of the appearance on 1-2 aphids/100 leaves in the crop + fungicide (Curzate M-8 @ 3g/l of water alternating with Mancozeb @ 2.5g/l of water) against late blight 6-7 times starting from 100% plants emergence thereafter at 7-10 days intervals and rouging out the viral infected plants and off type variety to
maintain the trueness to type of the variety, haulms cutting when aphid population reaches initial level of 20 aphids per 100 compound leaves.

In the subsequent years the trials were repeated wherein corresponding treatments were planted with seeds drawn from previous treatments (2 tubers each from individually harvested plants/respective treatment plot, bulked and stored).

Besides, an additional treatment i.e., T-3 and T-4 planting of fresh breeder seed every year along with the application of systemic insecticides as in T-2.

3.4 Transmission studies

3.4.1 Maintenance of virus inoculum

3.4.2 Cultivation of plants: The plants used in experiments were grown in clay pots sterilized by rinsing with 4% formalin solution filled with a mixture of autoclaved soil, sand and compost in the ratio of 2:2:1.

3.4.3 Virus culture: Young leaves of naturally infected potato plant showing the typical viral symptoms were macerated in a pre-chilled mortar and pestle with phosphate buffer (0.1M, pH 7.0). The slurry thus obtained was squeezed with muslin cloth and centrifuged at low speed (5,000 rpm) for 10 minutes. The supernatant was used as an inoculum to inoculate healthy potato plants as well as test plants. To ensure the biological purity of virus and to avoid any possible contaminations, the sap obtained from the diseased potato plant showing typical symptoms was inoculated to *Chenopodium amaranticolor* (Coste & Reyn), *C. quinova* (Willd), *C. murale* (L.) a local lesion host of the virus. The lesions developed after 4-5 days were excised singly and macerated on a glass slide separately with the help of a glass spatula in a drop of phosphate buffer (0.1M, pH 7.0) and then inoculated separately to young potato plants for PVY infection. After symptoms developed, only one out of the inoculated plants was taken for further experiments to maintain pure culture of the virus.

3.5 Transmission

Different methods of transmission were employed to ascertain the spread of virus in nature. Standard methods and some modifications were carried out for the transmission studies.
3.5.1 By Sap

Three to four fully expanded leaves of the plants were used for mechanical inoculation of the virus. The leaves were pre-dusted uniformly with carborundum (500 mesh, MP Biomedicals, LLC, France) and standard inoculum prepared in 0.1M phosphate buffer at pH 7.0 was applied gently but firmly on the upper surface of the leaves with the help of forefinger by keeping the other hand beneath the leaf to be inoculated. The inoculated leaves were rinsed with gentle stream of water before the inoculum on the surface of the leaves dried up.

3.5.2 Biological Transmission

Attempts were made to find out the mode of transmission of virus in the field. Therefore, aphids, *M. persicae* (Sulzer) and *A. gossypii* (Glover), and dodder plant (*Cuscuta reflexa* Roxb.) were used to study the transmission.

3.5.3 By Aphids

3.5.3.1 Raising of virus free aphids

Viviparous adult aphids were starved for 4-6 hr at room temperature and then placed upon a detected leaf of appropriate healthy host plant in a petridish. The atmosphere inside the petridish was made humid by covering the inner surface of the petridish with wet filter paper. Young nymphs produced by such aphids served as the parents for initiating the colony on healthy plants maintained in the cages of 90 x 90 x 90 cm. Fresh young colonies were maintained by transferring them regularly at every 2-5 days interval to young healthy plants.

To ascertain the mode of transmission by the aphids i.e. either persistent or non-persistent following procedures were employed.

3.5.4 Non-Persistent transmission

The nymphs collected were starved for 1-2 hr in a petridish having the inner surface covered with a net piece of filter paper before allowing them an acquisition feeding period for 1-5 min on the leaf of diseased plant. After this, the nymphs were transferred to healthy seedlings of test plant. The seedlings were covered with cages for an inoculation feeding period up to 24 hr. The aphids were killed by spraying the insecticide after the end of inoculation feeding period and the plants were kept in an insect proof glass house to develop the symptoms.
Pre-acquisition starvation period 1-2 hr.
Acquisition access period 1-5 min
Inoculation access period 24 hr
Number of aphids/plant 10

3.5.5 Persistent transmission

The nymphs collected from the fresh healthy colony, with the help of moistened tip of a Camel’s hair brush type A, No. 1, in batches of 8-10 were allowed up to 24 hr acquisition feeding on diseased leaves followed by an inoculation feeding period of 48 hr on healthy seedlings of test plants without being given any pre-acquisition starvation period. The seedlings were covered with cages having wooden frames. The top and two sides of the cage were closed by glass and the remaining sides were closed by wire gauze. The plants were kept on a zinc tray and the bottom of the tray was covered with the layer of moist sand to prevent the passing of the aphids through chinks between the strong and the rim of the cage.

The inoculation feeding was terminated by spraying the plant, with 0.2% cypermethrine (insecticide). These test plants were kept in an insect proof glass house to develop the symptoms.

Acquisition access period 24 h
Inoculation access period 48 h
Number of aphids plant 10

3.6 By dodder:

Dodder plant (Cuscuta reflexa Roxb.) was trained on infected S. tuberosum (L.) and after that 10-15 days established on healthy N. tabacum (L.) and P. floridana (Rydb.), for different viruses studied. The stem portion of dodder was placed and allowed to train on healthy N. tabacum (L.) and P. floridana (Rydb.) which was placed near the infected ones having dodder established on it. The plant was kept in an insect proof glass house for observation and development of symptoms.
3.7 Host range and symptomatology

Several species of plants belonging to different families were screened for the susceptibility of the virus by aphid species. At least 3 plants of each species/cultivar at 4-6 leaf stage were inoculated and the same numbers of plants were left as control. Plants were observed till two months after inoculation for symptoms development, sequence and severity of symptoms. Back inoculations from all plants including those which did not show any visible symptoms were made to work out the latent infection, if any.

3.7.1 Selection of local lesion host

The standard inoculum was inoculated on various local lesion hosts like *C. amaranticolor* (Coste & Reyn.), *C. quinova* (Willd.), *C. murale* (L.) for host range studies. The most suitable of them which reacted with clear, discrete, and good number of local lesions were selected.

3.8 Double-Antibody Sandwich-ELISA (DAS-ELISA, ADGEN Phytodiagnostics)

The infected leaf samples collected randomly from farmers’ fields and the sprouts from freshly harvested tubers (treated with GA-3 to allow early sprouting) (2 tubers/treatment/replication/variety) from degeneration trail conducted at the locations were assayed through DAS-ELISA using antibodies (Ab) which were bound to the surface of micro titre plate (Greiner, Germany) to capture the antigen of interests. A specific antibody-enzyme conjugate was then used to detect the trapped antigens.

3.8.1 Reagents required for the assay

Coating Buffer (carbonate buffer)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Carbonate</td>
<td>1.59 g</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>2.93 g</td>
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</tbody>
</table>

Made one litre with Double Distilled water (DDW) and maintained the pH at 9.6.

Phosphate buffered saline

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Sodium Chloride</td>
<td>8.0g</td>
</tr>
<tr>
<td>Potassium di-hydrogen ortho-phosphate</td>
<td>0.2g</td>
</tr>
<tr>
<td>Di Sodium Hygrogen ortho-phosphate</td>
<td>2.9g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2g</td>
</tr>
</tbody>
</table>

Made up to one litre with DDW and pH maintained at 7.4.
**Wash buffer (PBS+Tween 20)**

Phosphate buffer saline  
Tween 20

**General Extraction Buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Polyvinylpyrrolidone (PVP)</td>
<td>20g</td>
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<tr>
<td>Ovalbumin</td>
<td>2g</td>
</tr>
<tr>
<td>Sodium sulphite (anhydrous)</td>
<td>1.3g</td>
</tr>
<tr>
<td>Sodium azide</td>
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</tr>
<tr>
<td>Tween 20</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>8g</td>
</tr>
<tr>
<td>Potassium di-Hydrogen Ortho-phosphate</td>
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</tr>
<tr>
<td>Di Sodium Hydogen ortho-phosphate</td>
<td>2.9g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2g</td>
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</table>

**Conjugate Buffer**

<table>
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<tr>
<td>Bovine serum albumin</td>
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<tr>
<td>PBST</td>
<td>100ml</td>
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**Substrate buffer (Diethanolamine buffer 1M)**

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<th>Amount</th>
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<tbody>
<tr>
<td>Diethanolamine</td>
<td>97 ml</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.2 g</td>
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Made 1 litre with DDW and adjusted pH at 9.8. pNPP was added to the substrate buffer at 1mg/ml to make up to the substrate for alkaline phosphatase.
3.8.2 Procedure

1. Diluted coating antibody in coating buffer (Agdia, U.S.A.) and added 100-200μl to the required number of wells.

2. Wrapped the micro titre plates (Greiner, Germany) tightly in cling film or in a plastic box with some damp paper towel and closed the box. Incubated the plates at 37°C for 3 hr.

3. Washed the plates at least three to five times with Phosphate buffer saline + Tween 20 in automatic washer (Tecan, U.S.A.). To do so filled the wells of the plates with PBST and inverted to remove the buffer. Repeated twice and made the plates dry on paper towels.

4. Extracted the sample by grinding 1 g of tissue with 10 ml of general extraction buffer in a mortar and pestle. Then filtered the sample through a layer of muslin cloth.

5. Added 100-200 μl of each sample, positive and negative control to the coated wells.

6. Wrapped the plates in a cling film and incubated at 4°C overnight.

7. Washed the plates as described above at least 3-5 times to remove the crude sap stuck to the wall of micro titre plates.

8. Diluted the antibody-enzyme conjugate (Agdia, U.S.A) in conjugate buffer and added 150-200 μl to each test wells.

9. Wrapped as described above and incubated at 37°C for 3 hr.

10. Washed the plates at least 3-5 times as above and an extra wash was included at this stage to ensure that all unbound antibody-enzyme conjugate is removed from the wells.

11. Prepared the substrate just before use. Added pNPP at 1mg/ml to substrate buffer.

12. Added 100-200 μl prepared substrate to each well.

13. Wrapped the plates as described above and incubated in the dark room at room temperature for 10-15 minute for developing the yellow colour.

14. Read the absorbance using ELISA reader at 405 nm (Tecan, U.S.A).
3.9 Purification

3.9.1 Selection of propagation host

To select the appropriate plant species/cultivars for maximum virus concentration, various systemic hosts were selected and inoculated with standard inoculums (extract of young infected apical leaves). For PVY and PLRV transmission *Nicotiana tabacum* (L.) cv. Samsun and *P. floridana* (Rydb.) were used as propagation host. Leaves showing typical disease symptoms, 2-3 weeks after inoculation, were collected and used for purification of the viruses.

3.9.2 Clarification of extract

Clarification was done by addition chloroform as organic solvent. The requisite amount of solvent was mixed and the mixture was kept at 4°C for 1/2 hr. The aqueous layer was separated by low speed centrifugation (10,000 rpm for 20 min) and then assayed on local lesion host.

3.9.3 Procedure for purification of PVY (Moghal and Francki, 1976)

1. Harvested symptomatic tissue, thoroughly cooled at 4°C and weighed.
2. Homogenized tissue in cold grinding buffer (1 gram tissue: 2 ml buffer) in a pre-cooled blender for ~ 1 minute. Added one half volume each of chloroform and carbon tetrachloride (e.g. 0.5 ml per gram tissue) and blended another minute.
3. Transferred the homogenate into 250 ml bottles, Centrifuged in a JA14 rotor at 7,000 rpm for 15 minutes at 4°C.
4. Pippet the upper aqueous phase off using a 25 ml glass pippet and without disturbing the white crud at the interface filtered the extract through Miracloth into a pre-weighed beaker on ice. Weighed the supernatant.
5. Placed solution on a stir plate and while stirring added NaCl to 1.75% and PEG 8000 to 4% (w/w). Added PEG slowly to ensure it is dispersed well and avoiding clumping. Stirred at 4°C for 1 hour.
6. Transferred the solution to pre-cooled 250 ml polypropylene bottles and centrifuged in a JA14 rotor at 10,000 rpm for 15 minutes at 4°C. Pour off the supernatant without dislodging the pellet and drain, saved the pellet.
7. Added one-tenth the original volume of cold PEG re-suspension buffer (0.5M Borate, pH 8.0 with 0.25% Triton X 100). Dislodged the pellet with a plastic Pasteur pipette, added a stir bar and stirred on a stir plate at 4°C for 1.5 - 2 hours.
8. Transferred the solutions to 50 ml polypropylene Oakridge tubes to clarify. Centrifuged in a JA20 rotor at 10,000 rpm for 15 minutes. Gently poured
off the supernatant (to be saved) and avoid disrupting the pellet (to be discarded).

9. Transferred the solutions to polycarbonate bottles for the Ti 50.2 rotor. Centrifuged at 32,000 rpm for 1.5 hours. Gently poured off the supernatant (to be discarded) and avoid disrupting the pellet (to be saved). Noted the size and appearance.

10. Added a small volume (1 ml or more) of virus buffer (50 mM Boric Acid, pH 8.0) to each bottle. Gently dislodged the pellet using a pipette tip from a P1000 pipeter. Placed on a shaker at 4°C for 2 hours or overnight.

11. Transferred the virus solution to a microfuge tube. Transferred the supernatant to a labeled microcentrifuge tube. This is a relatively crude purification.

12. Measured the absorbance values at 240, 260, 280 and 320 nm.

3.9.4 Procedure for the purification of PLRV (U. Jayasinghe and J. Rocha)

1. Ground 100 g of previously frozen (-70°C) infected tissue of *P. floridana* in a meat grinder and continuously added liquid nitrogen to maintain the tissue in powder form.

2. To the powder added 200 ml of 0.1 M trisodium phosphate, pH 6.0 containing 10 mM sodium EDTA, 0.1% thioglycolic acid and 1.5% celluclast. Stirred until completely homogenized and incubated overnight at room temperature.

3. Clarified the homogenate by centrifugation (9,000 g) for 20 min at 15°C. Saved the supernatant and re-suspended the precipitate in 10 ml of buffer solution used in step 2. Clarified again by centrifugation, discarded the precipitate and mixed the supernatants.

4. Added ¼ volume of 1:1 chloroform to butanol mixture, stirred for 5-10 min, and clarified by centrifugation as before. Discarded the precipitate.

5. Precipitated the virus by adding 8% polyethylene glycol (PEG 8000) and 0.4 M NaCL and stirred at 4°C for 1 hr.

6. Collected the precipitate by centrifugation (9,000 g, 1 hr) and re-suspended the pellet by agitation for 2-3 hr in 1/5 initial volume of 10 mM sodium phosphate at pH 7.6.

7. Clarified the centrifugation for 10 min, saved the supernatant, and re-suspended the pellet in 1/10 of the volume used in step 6. Clarified again and mixed both solutions.
8. Further purified the virus by centrifugation at 15,000 g for 3 hr through a sucrose cushion (20%) prepared in 10 mM sodium phosphate, and by rate zonal centrifugation in density gradient.

3.10 Density gradient centrifugation

1. Linear sucrose gradient columns were prepared by the method of Brakke (1960) using 40, 30, 20 and 10% sucrose solutions prepared in extraction buffer.

2. Gradient columns were prepared by layering 7, 7, 7 and 3 ml of 40, 30, 20 and 10% sucrose solutions respectively in 1x3” tubes.

3. The heaviest solution was layered first and solutions of decreasing concentration were layered on top of each other. The tubes were kept overnight in a refrigerator at 4°C to get a linear gradient.

4. Next day, 2 ml freshly prepared partially purified virus suspension was loaded on each tube with the help of LKB-Varioperox pump and centrifuged at 24,000 rpm in swing bucket rotor (SW 24.1) for 2 hr in a Beckman L7-65 R ultracentrifuge.

5. The tubes were taken out after centrifugation and examined in a dark room by projecting a narrow beam of light from the top.

6. The light scattering band that is the virus zone was removed from the tubes by a 20 gauge 10 cm long needle bent twice at right angles and attached to a hypodermic syringe.

7. The sample thus obtained was diluted with phosphate buffer (0.1M, pH 7.0) and the virus pelleted down by high speed centrifugation (30,000 rpm for 2 hr).

8. The pellet obtained was re-suspended in phosphate buffer (0.1M, pH 7.0) and centrifuged at low speed (5,000 rpm for 10 min).

9. The supernatant, thus obtained was purified virus preparation and was inoculated on *C. amaranticolor* (Coste & Reyn.) for determining the infectivity of the virus.

3.10.1 UV absorption spectra

The purified virus preparation obtained after density gradient centrifugation was screened in a CE-594 double beam Spectrophotometer after diluting it about 25 folds. Absorbance (A) of samples was recorded in UV range. Values of
$A_{260}/A_{280}$ and $A_{280}/A_{260}$ were determined to get the appropriate percentage of viral nucleic acid by comparing the data with standard (Gibbs and Harrison, 1976).

### 3.11 Electron Microscopy (EM)

As Roberts (1986) stated, “electron microscopy has two great advantages namely, the speed with which results can be obtained, and the convincing, if not unequivocal, nature of visual evidence”. Recent development in electron microscopy, such as immunosorbent electron microscopy (ISEM) (Milne and Lesemann, 1984), increased sensitivity and provided the diagnosticians with a practical tool for virus detection. The grids were prepared by different methods to ascertain the particle morphology.

#### 3.11.1 Droplet method (Shadow casting):

A small drop of the clarified virus preparation has placed on a collodion created carbon backed filmed copper grid. After two minutes, the excess of samples was drained by touching the grid edge with Whatman No. 1 filter paper. The grid was then stained with 2% (w/v) aqueous uranyl acetate solution for 90 sec. The samples were alternatively stained with 2% (w/v) phosphotungstic acid (PTA) and prepared grid was screened under a JEOL-JEM100S electron microscope.

#### 3.11.2 Leaf dip method:

A cut surface of virus containing leaf was dipped for 10 seconds into a drop of distilled water on a film coated grid. After several minutes when the particles became attached to the film, the excess material was removed and the specimen was stained. Green fluid was separated from the plant debris with the help of Pasteur pipette and put on the grid. After 1 minute the excess fluid was removed with a filter paper and specimen was examined.

### 3.12 Immuno Sorbent Electron Microscopy (ISEM)

A combination of electron microscopy and serology is used as an effective and widely applicable diagnostic tool for virus detection. In this technique virus and antiserum is reacted together.

#### 3.12.1 Trapping

Collodion film covered grids were coated with antiserum to PVY and PLRV by floating them on micro drops of the 1000 fold diluted antiserum for 5 minutes at room temperature. Grids were washed, drained and then floated on micro drops of the clarified virus extract placed over parafilm fixed on glass slides.
kept under humid petri-dish. They were incubated at 37 °C for 1 hr followed by washing, draining, and staining with 2% aqueous uranyl acetate. The grids were then observed under transmission electron microscope (TEM, JEOL-100S).

3.12.2 Decoration/Clumping

To confirm the virus through decoration and clumping in case of PVY and PLRV was performed. For decoration, grids with trapped virions (as described under trapping), before staining, were again floated on a micro drop of diluted antisera and incubated for 1 hr at 37 °C. Then, the grid was washed, drained, stained in the 2% aqueous uranyl acetate, drained and air dried before examination under TEM.

3.13 Polymerase Chain Reaction (PCR)

In recent years, PCR has been developed as a diagnostic method based on the specific synthesis by the enzyme polymerase of DNA sequences that are located between two primers of known sequence that are complementary to a region of the target DNA (Saiki et al., 1985; Querci, 1993). The combination of reverse transcription and PCR makes amplification of viral RNA possible, thus allowing the detection of RNA-containing viruses. PCR is highly sensitive, versatile and specific.

3.13.1 Isolation of total RNA

RNeasy Plant Mini kit (Qiagen) was used for total RNA extraction from virus infected leaves.

3.13.2 RNeasy plant mini kit (Qiagen):

The total RNA isolation was carried out by following guidelines provided by the manufacturer of the kit. A brief description of the same is as follows.

1. PVY/PLRV infected leaf tissue was ground separately to a fine powder in liquid nitrogen using a mortar and pestle.
2. RLT buffer (450 μL) was added to a maximum of 100 mg of tissue powder in a microfuge tube; 10μl β- mercaptoethanol was added per 1.0 ml RLT buffer before use. The tubes were vortexed vigorously and incubated at 56 °C for 3-4 minutes.
3. Mixture was transferred to a QUIA shredder spin column (lilac) sitting in a 2.0 ml collection tube, and centrifuged for 2 min at 8000 x g. The flow-through fraction from QUIA shredder was transferred to a new microfuge tube without disturbing the pellet.
4. Ethanol (0.5 volumes of 96-100%) was added to the cleared lysate and mixed well by pipetting.
5. Clear lysate of PVY/PLRV was transferred (usually 675 μL including any precipitate which might have formed) onto an RNeasy mini spin column (pink) sitting in a 2.0 ml collection tube. Centrifuged for 15 sec at 8000 x g. The flow-through was discarded.
6. RW1 buffer (700μL) was added onto the RNeasy column and centrifuged for 15 sec at 8000 x g to wash. The flow-through and the collection tube were discarded.
7. RNeasy column was transferred into a new 2.0 ml collection tube and 500 μL of RPE buffer was added onto the RNeasy column and centrifuged for 15 sec at 8000 x g. The flow through was discarded but re-used the collection tube in step h.
8. RPE buffer (500 μL) was added to the RNeasy column and centrifuged for 2 min at maximum speed to dry the RNeasy membrane.
9. RNeasy column was transferred into a new 1.5 ml collection tube and 30-50 μL of RNase free water was added directly onto the RNeasy membrane. Centrifuged for 1 min at 8000 x g to elute RNA.

3.13.3 Reverse transcriptase-polymerase chain reaction (RT-PCR)
3.13.4 Primer designing

The designing of coat protein specific primer of PVY and PLRV was done by alignment of nucleotide sequences available from the NCBI (National Centre for Biotechnology Information, U.S.A) nucleotide database.

**Primers used:** PVY: 5'-ACGGTGGCATCAGTACACAA-3' Forward
5'-TGCTACGACAGAATCGCAAC-3' Reverse
PLRV: 5'-CTAACAGAGTTCAGCCAGTGGTTA-3' Forward
5'-CGGTATCTGAAGATTCTTCCATTTCCATTT-3' Reverse
3.13.5 Reverse transcription of PVY and PLRV

Reverse transcription reaction was carried out for both PVY and PLRV separately using the Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermantas).

3.13.5.1 Procedure

The reaction volume for reverse transcription was set as follows in a PCR tube (0.2 ml)

<table>
<thead>
<tr>
<th>Contents</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x M-MuLV RT buffer*</td>
<td>4.0 µL</td>
</tr>
<tr>
<td>dNTP mix (10 mM)</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>RNase inhibitor (20U/µL)</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Total RNA</td>
<td>3.0 µL</td>
</tr>
<tr>
<td>Specific CP downstream primer</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>9.0 µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20.0 µL</td>
</tr>
</tbody>
</table>

* (10mM Tris-HCl, 50mM KCl, 0.1% Triton® X-100)

The above mixture was incubated at 25°C for 5 min, followed by 37°C for 60 min, and the reaction was stopped by heating at 75°C for 10 min in a thermal cycler.

3.13.6 PCR amplification:

1. Dilute the cDNA generated with the first strand cDNA reaction 1:1000 in nuclease-free water.
2. Gently vortex and briefly centrifuge all PCR reagents after thawing.
3. Place a thin-walled PCR tube on ice and add the following reagents:

   | cDNA from control RT reaction 1:1000 | 2 µl |
   | 10X PCR buffer                       | 5 µl |
   | 10 mM dNTP Mix                      | 1 µl (0.2 mM each) |
   | 25 mM MgCl₂                         | 3 µl |
   | Forward primer                      | 1.5 µl |
   | Reverse primer                      | 1.5 µl |
   | *Ta* DNA Polymerase (5 u/µl)        | 0.5 µl |
   | Nuclease-free water                 | 35.5 µl |

   **Total volume** 50 µl
4. Perform PCR in a thermal cycler with a heated lid or overlay with 25 μl of mineral oil.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58</td>
<td>30 sec</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
<td></td>
</tr>
</tbody>
</table>

5. Load 5-10 μl of the RT-PCR product on 1% agarose gel.

3.13.7 Agarose gel electrophoresis (Sambrook et al. 1989)

Agarose gel electrophoresis was used to visualize the PCR products.

Reagents:

**Ethidium bromide stock solution (5 mg/ml)**

250 mg Ethidium bromide

45 ml double distilled water

Final volume made to 50 ml by adding double distilled water and stored at 2-8°C in brown bottle.

**50X TAE buffer (1000 ml)**

242 g Tris Base

57.1 Glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Final volume made to 1000 ml by adding double distilled water and autoclaved.

**6X Gel loading dye**

30% (v/v) glycerol

0.25% (w/v) Xylene cyanol FF

0.25% (w/v) Bromophenol blue
3.13.7.1 Preparation of agarose gel

Agarose as per the requirement was added to 1X TAE buffer and solubilised by heating in a microwave oven and cooled to 60°C. Ethidium bromide to a concentration of 0.5 μg/ml was added mixed by gentle swirling and poured in to the gel casting tray.

3.13.7.2 Sample preparation for agarose gel electrophoresis

After completion of amplification, the PCR products (10μL) of PVY/PLRV were mixed with the gel loading dye in a ratio of 5:1 and slowly transferred in to the wells of 1 % agarose gel submerged in 1X TAE.

3.13.7.3 Electrophoresis

The samples loaded in the wells of agarose gel were electrophoresed at a constant voltage of 100 V till the required migration of tracking dye was achieved. DNA ladder (1kb DNA ladder, Fermentas) was loaded in one well for size comparison. After the run, gel was viewed under UV (Ultra violet) transilluminator and photographed in Biovis gel documentation system.