Chapter - III

Results
emerging leaves and gradually these chlorotic spots developed in size to form large chlorotic areas there giving mosaic appearance (Morden variety MHSF-8, MHSF-17 and Cargil variety). After ten days necrotic symptoms were observed on the leaves as necrotic rings, necrosis spread along the veins and leaf lamina (Fig.1). Systemic necrosis was observed along the stem, (Fig.2) petioles of the leaves and the pedicle of the floral head. Concentric necrotic rings were observed on the calyx (Fig.3) and spots on the petals of the ray florets in the infected plants (MHSF-8 and Cargil variety). Finally necrosis (death) of the plant occurs. Early infected plants show severe necrotic symptoms and chlorosis of the plant. Plants were stunted and demolished due to necrosis.

Disease symptoms ranging from chlorosis, mottling, stunting and wilting due to severe necrosis of leaf and stem tissues. However, symptoms varied with in a host species according to the condition and age of the plant.

The early infected plant produced no seeds, the floral heads were shrivelled and floral breakings of floral head were observed.

Host Range

In the host range studies plant species belong to families Chenopodiaceae, Solanaceae, Amaranthaceae, Cucurbitaceae, Asteraceae, Leguminosae were tested. Six plants of each species were used for inoculation. Reactions of various host plants to the virus were observed. The results showed that the virus has a narrow host range.
Symptoms on Sunflower

Fig. 1: Infected sunflower leaf showing chlorotic spots, rings and necrosis
Fig. 2: (a) healthy stem (b) infected stem of sunflower showing necrosis
Fig. 3: Infected sunflower floral head showing necrosis and necrotic rings
producing chlorotic and necrotic local lesions on members of the family Chenopodiceae i.e., *Chenopodium amaranticolor* (Fig.4), local chlorotic lesions on *Damra* metal (Fig.5), local chlorotic spots were observed initially later turn to chlorotic to necrotic ring spots on inoculated leaves and turns systematic mosaic on developing leaves on *Gompherenia globosa* (Fig.5a,5b), tar like symptoms produced on the leaves of *Catheranthus rosea* (Fig.7), chlorotic spots on *Compea* CV 152 were observed later turned to chlorotic ring spots and finally necrotic ring spots were observed (Fig.8). Chlorotic symptoms produced on inoculated leaves of *Petunia hybrida*. Brown spots produced on the inoculated leaves of *Cassia tora*, Cucumber long plants produced systematic symptoms like bronzing of leaves and stunting of plant and the tip of plant dying back. *Watermelon* produced systemic symptoms like mosaic, necrosis along the stem and leaves and finally the terminal bud died back due to necrosis (Sreenivasulu et al., 1991., Brunt et al., 1990., 1996).

The following plants neither showed any symptoms nor virus recovered from them- *Arachis hypogaea, Cajanus cajan, Cassia occidentalis, Celosia argentea, Commelina, Cynopsis tetragonoloba, Cucumis melo, Cucumis pepo, Cucumis moschata, Dolichos lablab, Glycine max, Hibiscus asculntus, Luffa acutangula, Luffa cylindrica, Lycopersicon esculentum, Mimordica charantia, N. tobaicum Vs Harrison special, Phaseolus aequus, P. mungo, P. vulgaris, Solanum melangena, Tridax and Tagetes.*
Host Range

Fig. 4: Infected *Chenopodium amaranticolor* leaf showing chlorotic and necrotic spots

Fig. 5: Infected *Datura metel* leaf showing chlorotic spots
Fig. 6a: Infected *Comphrena globosa* leaves showing chlorotic spots, mosaic symptoms

Fig. 6b: and chlorotic rings
Fig. 7: Infected *Catherineus rosea* leaf showing tar-like symptoms

Fig. 8: Infected Cowpea cv. 152 leaf showing chlorotic and necrotic ring spot symptoms
Mechanical transmission

8 to 10 days old sunflower seedlings with primary leaves were more susceptible to virus infection. All the varieties tested are susceptible to the virus but the symptoms were different in different varieties. In Morden variety the infected plants showed systemic mosaic, later stage the plant showed necrosis, but in the case of MHSP-8, MHSP-18 the early symptoms were observed as mosaic and followed by necrotic ringspots and necrosis was observed on developing leaves. In KBSH-1, initially the symptoms were chlorotic mosaic and later turned to necrotic spots along the leaves and the tissues died back. Necrotic local lesions were observed on inoculated leaves of cargil variety and very severe systemic necrosis was observed on the newly emerging leaves.

Seventy percent of the inoculated sunflower plants showed characteristic chlorotic spots, which later turned to mosaic, necrosis on the leaf lamina, along the stems, petiole and also on floral heads of the plant. Sometimes the floral heads died back due to severe necrosis. The systemic infection was observed after 7 to 14 days after inoculation. Symptoms varied with age of plants and climatic conditions.

Varietal reaction

In varietal reaction the following are the different varieties of sunflower seeds used to study the resistance for sunflower tospovirus. The symptoms are given below in Table 7.
Table 7: Symptoms on different Sunflower cultivars by Varietal reaction

<table>
<thead>
<tr>
<th>SL. No.</th>
<th>Variety</th>
<th>Symptoms</th>
<th>SL. No.</th>
<th>Variety</th>
<th>Symptoms</th>
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<tbody>
<tr>
<td>1.</td>
<td>Morden</td>
<td>CS, M, SN</td>
<td>21.</td>
<td>CMS 7-1 A X</td>
<td>NS, NR, SN</td>
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<td></td>
<td></td>
<td></td>
<td>22.</td>
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<td>2.</td>
<td>KBSH 1</td>
<td>CS, SM, N</td>
<td>23.</td>
<td>CMS 86 A X RHA 1B-20</td>
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</tr>
<tr>
<td>3.</td>
<td>MHSF 8</td>
<td>NS, NR, SN</td>
<td>24.</td>
<td>CMS 86 A X RHA single head</td>
<td>CS, NS, SN</td>
</tr>
<tr>
<td>4.</td>
<td>MHSF 17</td>
<td>CS, NS, RN, SN</td>
<td>25.</td>
<td>CMS 86 A X RHA 271-1</td>
<td>CS, NS</td>
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<tr>
<td>5.</td>
<td>Cargil</td>
<td>NS, M, SN</td>
<td>26.</td>
<td>CMS 86 A X RHA 274</td>
<td>CS, LD</td>
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<tr>
<td>6.</td>
<td>CMS 7-1A</td>
<td>CS, MM</td>
<td>27.</td>
<td>CMS 26 A X 297 R</td>
<td>CS, NS, SN</td>
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<td>7.</td>
<td>CMS 86 A</td>
<td>CS, M, SN</td>
<td>28.</td>
<td>CMS 234 A X RHA 1B-5</td>
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<td>8.</td>
<td>CMS 234 A</td>
<td>NS, MM, M</td>
<td>29.</td>
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<td>CS, NS</td>
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<td>9.</td>
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<td>NS, M</td>
<td>30.</td>
<td>CMS 234 A X RHA single head</td>
<td>NS, SN</td>
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<td>10.</td>
<td>RHA 1B-5</td>
<td>CS, SM, N</td>
<td>31.</td>
<td>CMS 234 A X RHA 271-1</td>
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<td>11.</td>
<td>RHA 1B-20</td>
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<td>32.</td>
<td>CMS 234 A X RHA 274</td>
<td>CS, NS</td>
</tr>
<tr>
<td>12.</td>
<td>RHA single head</td>
<td>CS, M, LD</td>
<td>33.</td>
<td>CMS 234 A X RHA 297 R</td>
<td>NS, M, SN</td>
</tr>
<tr>
<td>13.</td>
<td>RHA 271-1</td>
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<td>34.</td>
<td>CMS 234 A X RHA 1B-5</td>
<td>CS, NR, SN</td>
</tr>
<tr>
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<td>RHA 274</td>
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<td>NS, NR, SN</td>
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<td>15.</td>
<td>297 R</td>
<td>NS, SN</td>
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<td>CS, M, SM</td>
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<td>CS, MM</td>
</tr>
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<td>18.</td>
<td>CMS 7-1A X RHA single head</td>
<td>CS, MM</td>
<td>39.</td>
<td>CMS 805 A X 297-R</td>
<td>CS, M, SN</td>
</tr>
<tr>
<td>19.</td>
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<td>CS, MM, SM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>CMS 7-1 A X RHA 274</td>
<td>CS, NS, SN</td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>
Out of the thirty nine varieties of sunflower seeds only two varieties CMS 234 A X RHA 1B-5 and CMS 234 A X RHA 271-1 did not show any symptoms. All the samples were screened by DAC-ELISA using polyclonal antibodies produced against sunflower tospovirus. In ELISA all the samples reacted positively including the two varieties which does not show any symptoms visually.

**Purification of Virus**

The virus was successfully purified from infected sunflower leaves by extracting in 0.1M of sodium phosphate buffer pH 7.0 containing 0.2% of 2-mercaptoethanol, 0.02% of sodium sulphate and 225 mg of DIBCA was added to the extraction buffer before extraction of the virus. Clarification of sap was done with 1% nonidet P-40, concentration of virus was done by high speed pelleting and sucrose gradients centrifugation of concentrated virus, single light scattering zone was observed in the density gradient tube at the height of 4 to 4.3 cm from the bottom of the tube (Fig.9). Purified virus was proved to be infectious on sunflower, *Gomphrena globosa* and *C. amaranthicolor* when inoculated on healthy plants.
The virus culture was maintained and propagated on sunflower and *Gomphrena globosa*.

The purified virus obtained from sunflower leaves was scanned in UV Vis spectrophotometer from 220 nm to 300 nm (Fig.10). The virus has maximum absorption at 258.2 nm and minimum absorption at 240 nm. $A_{260}/A_{280}$ and $A_{max}/A_{min}$ ratios were 1.547 and 1.419, respectively. The virus yield was ranged from 37 mg / 100g freshly infected sunflower leaves.

In DAC-ELISA almost all the samples from sunflower, *C. amaranthicolour*, *Cowpea*, *Gomphrena globosa*, *Catheranthus rosea*, *Datura metal*, *Petunia hybrida* are indexed with different antisera to CMV isolates, TSWV-T, (Dept. of Virology, S.V.University, Tirupati), PBNV, PSNV (ICRISAT, Hyderabad) INSV (IIHR, Bangalore) IYSV (Dr.Kromelink, Netherlands) and also with the homologous antisera. In ELISA all the samples reacted positively with sunflower mosaic homologous antisera and also with PBNV antisera and negative reaction with INSV, TSWV-T, IYSV and CMV antisera. There was slight healthy background reaction with homologous antisera.

Molecular weight of virus coat protein was determined by SDS-PAGE. The virus when analysed soon after purification resolved as 4 major polypeptide of Mw. 31 kDa, 52 kDa, 58 kDa and 331 kDa (Fig.11).

The RNA isolated from purified virus was run in 1% MOPS gel electrophoresis, the RNA resolved as three bands which had the molecular size similar to those of reported tospoviruses. The three bands were estimated having the following molecular weights L-RNA-9.0Kb, M-RNA-5.0Kb and S-RNA-3.5Kb (Fig.12).
Fig. 9: Light scattering zone after density gradient centrifugation
Fig. 10: U.V. absorption spectrum of the purified virus
Fig. 11: SDS-PAGE analysis of viral proteins
Lane.1: purified virus
Lane.2: Protein markers (GIBCO-BRL)

Fig. 12: MOPS gel electrophoresis of nucleic acid
Lane.1: RNA markers (GIBCO-BRL)
Lane.2: Isolated RNA from purified virus

Fig. 13: Agarose gel electrophoresis of dsRNA
Lane.1: DNA double digest markers
Lane.2: ds RNA isolated from infected leaves of sunflower
Lane.3: ds RNA isolated from healthy sunflower leaves
The molecular weight of dsRNA isolated from infected sunflower was determined by running the preparations in 2% agarose gel electrophoresis.

In infected sunflower leaves dsRNA was resolved as three bands of different molecular weight corresponding to L-RNA, M-RNA and S-RNA similar to the tospoviral genome and no bands were observed in healthy leaf samples (Fig.13).

After four successive injections of the purified virus, the test bleed was done by cutting the vein on ear of the rabbit and the serum was collected. Antisera was collected weekly and the titer was detected by performing DAC-ELISA. The positive reaction up to 1/5000 titre of antisera of IVth bleed was observed, but in case of 1st bleed reaction was observed up to 1/2000 dilution.

The reaction was observed strong with purified virus as compared with partially purified virus and slight back ground reaction was observed in healthy samples. No colour was observed in buffer control and in 11 and 12 row PBNV antisera was checked for positive control.

In agarose double diffusion test a precipitin line was observed with homologous antisera and with PBNV antisera (Fig.14).

In Electron Microscopy a cluster of particles were observed in purified preparation stained with uranylacetate. The particles are irregular in shape around 80-90 nm in diameter (Fig.15). This is the characteristic feature of the genus Toospovirus.

In leaf sections also virus particles were seen along with viroplasm. The virus particles were distributed in cytoplasm and endoplasmic reticulum areas (Fig.16).
Fig. 14: Agarose gel double diffusion test
Central well: purified virus
Peripheral wells (IVth bleed): 1. 1/1000 dilution 2. 1/2000 dilution
3. 1/3000 dilution 4. 1/4000 dilution 5. 1/10 dilution crude antiserum
6. PBNV antiserum

Fig. 15: Electron microscopy of purified virus showing a cluster of
unusual shaped particles

Fig. 16: Electron microscopy of infected leaf section showing virus
particles and viroplasms
In Electro blot Immuno assay all the bands transferred from gel to PVDF membrane and reacted strongly with homologous antiserum and with PBNV antiserum. The band corresponding to the nucleocapsid protein was reacted strongly with PBNV antiserum produced against recombinant N protein of virus (ICRISAT, Hyderabad) (Fig. 17).

In RT-PCR reaction, the RNA was isolated from purified virus and gel checked in the MOPS agarose gel. The RNA isolated from purified virus resolved as three bands in MOPS gel electrophoresis. The three bands were considered corresponding to L, M and S RNA of the Tospovirus.

The 1st strand cDNA was synthesised using the NREV tospo primer 5' ATG TCT AAC GTY AAG CAR CTC AC 3' using Superscript RT (Gibco-BRL) in the presence of Trehalose. Here trehalose was used to increase the activity of superscript RT (Piero et al., 1998). After completion of RT, IIind strand cDNA synthesis was done by using the product of 1st strand cDNA.

Synthesis of IIind strand cDNA

25 :1 of the 1st strand cDNA reaction was diluted to 40 :1 with sterile distilled water and from that 33 :1 was used to synthesis IIind strand cDNA in the presence 10 mM dNTPS, RNase H 1 :1, 0.1 mM DTT, Ecoli DNA ligase 1 U, Ecoli DNA Pol I 2 U, 1st buffer and 5x IIind strand buffer. The eppendorf tube containing the mixture was incubated at 16°C for 3hrs. The IIind strand cDNA was diluted and
phenol: chloroform extraction followed by ethanol precipitation was conducted. The final pellet of $IP^d$ strand cDNA was dissolved in 25 μl of sterile distilled water and used for further PCR amplification.

**Polymerase chain reaction**

In PCR the $IP^d$ strand cDNA was used as template for amplification of the N gene of S RNA. The PCR reaction was set for 25 μl in separate tubes (4 tubes) by using 4 picomoles of NREV tspo primer and 10 picomoles of NFOR Tospo primer, 10 mM dNTPs, 10x chelating buffer, 10 mM MgCl₂ and 2 U of rt₇th Polymerase (Perkin Elmer). After completion of PCR, 10 μl of the product was gel checked along with 1 kbp DNA ladder. A single band of 780 bps was observed in the gel (Fig.18). No band was observed in control, i.e without template. The remaining PCR product was loaded in 1% agarose gel and the PCR product (DNA) was eluted.

**Cloning of the PCR product**

The eluted DNA (PCR product) N-gene of viral S-RNA was cloned into pGEM-T easy vector (Promega cloning kit). After ligation the ligation mixture with insert and vector was spread on to LB agar Amp plates with X-gal and IPTG for blue white selection of the clones. The plates were incubated at 37°C for overnight, a number of blue and white colonies were observed. Individual white colonies were screened for viral DNA insert at the same time blue colonies were also screened as control.
**Fig. 17:** Immuno electroblot analysis of virus coat protein
Lane 1. Homologous antiserum
Lane 2. PBNV antiserum

**Fig. 18:** Analysis of RT-PCR product by agarose gel electrophoresis
Lane 1. DNA ladder (GIBCO-BRL) marker
Lane 2. RT-PCR product
Quick screening

The white colonies along with control blue colonies were inoculated into 1ml LB Amp, and incubated at 37°C for 8 hr in incubator-shaker. After that the bacterial culture was pelleted by spinning at 5000 rpm for 5 min. 45 :1 of loading dye (6% Sucrose, 0.1% Bromophenol blue in TE 10 mM) and 25 :1 of water saturated phenol were added to the pellet and vortexed to dissolve the pellet. The sample was spun at 10 k for 5 min and loaded the supernatant on to 0.8% agarose gel. The gel was run at 80 volts for 1 hr and stained with ethidiumbromide for ten min and observed on the UV-transilluminator. The clones showing mobility shift when compared with the control (blue colony) were selected for further work.

Medi Prep. preparation

Two clones were selected for mediprep preparation and both of them were inoculated into 50 ml LB Amp medium and incubated at 37°C in incubator/shaker for 8 hr. After 8 hr the plasmid DNA was extracted by using the procedure of Brinbrom Dolly method. The extracted plasmid DNA was gel checked for the concentration and purity of the plasmid DNA. This plasmid DNA is used for further restriction enzyme analysis.

Digestion of clone

The plasmid DNA was digested using the enzyme EcoRI, which released the fragment from the vector. The digestion was set as follows: 6 :1 of plasmid DNA, 1.5 :1 EcoRI enzyme, buffer 2 :1 and 10.5 :1 distilled water (total reaction 20 :1) and the digestion mixture was incubated by water bath at 37°C for 3 hr.
After the digestion the product was gel checked along with marker, uncut and cut (Fig.19). A fragment of 780 bps from the digested product was released and indicating that the vector contained viral DNA fragment.

**Northern Blotting**

The RNA was isolated from the purified virus and the total RNA from virus infected sunflower leaves and healthy leaves was extracted. The RNA samples were loaded in 1% MOPS agarose gel and run at 80 volts for 1 1⁄4 hr and after staining the bands were transferred to PVDF membrane (Millipore). This blot was used for Northern hybridisation

**Northern blot hybridisation**

After transfer of RNA to the PVDF membrane, it was washed with 10X SSPE buffer twice and air-dried. The membrane was transferred to UV-transilluminator by facing the transferred side exposed to UV light for 5 minutes. The bands were cross linked in the presence of UV light.

The prehybridization solution was prepared and to this the UV cross linked PVDF membrane was kept in a polybag and incubated at 42°C for four hr in shaking water bath. Mean while the probe was prepared using random primer labelling kit (Mega primer labelling kit, Amersham). 50 :1 of reaction mixture contained, 3 :1 of template (PCR product of N gene of S RNA), 5 :1 of Random primer (GIBCO BRL), 10 :1 of labelling buffer, 4 :1 of K23 p dCTP, 2 :1 of Klenow and 26 :1 of distilled water. The reaction mixture was incubated for 1 hr at 37°C. After one hour equal volume of stopping solution (50 :1 of Orange-G/Dextran blue) was added to
the probe and passed through the Sephadex G-50 column. The blue, intermediate and orange fractions were eluted using column buffer into different eppendorf tubes.

The blue fraction consisted of probe was denatured at 80°C for 3 minutes and added to the prehybridization mix and incubated at 42°C on shaking water bath overnight.

After hybridization the PVDF membrane was washed twice with 2X SSPE buffer. The blot was exposed to X-ray film (Kodak) overnight at -80°C, then the film was developed. Bands corresponding to S RNA of the virus and infected total RNA sample band were hybridised and no band was seen in healthy sample (Fig.20).

Southern Dot blot hybridization

The isolated plasmid DNA of the clones and PCR products loaded on to the PVDF membrane as small dots by using Dot blot apparatus (Millipore). The membrane along with spots of sample was vacuum dried and the membrane was processed same as the Northern hybridisation, except the prehybridization step. Here the prehybridization was done at 60°C for overnight.

After hybridization the membrane was washed twice with 2X SSPE buffer and exposed to X-ray film overnight at -80°C and the film was developed. In X-ray film the positive clones and PCR product were hybridised (Fig.21), and no hybridization was observed with healthy samples.

This confirms that the clones are authentic.
Fig. 19: Restriction enzyme analysis of the clone
Lane 1. DNA ladder (GIBCO-BRL) marker
Lane 2. Uncut plasmid
Lane 3. Cut plasmid showing 779 bp fragment resealed by EcoRI enzyme

Fig. 20: Northern hybridisation
Lane 1. Isolated RNA from purified virus
Lane 2. Total RNA isolated from healthy leaves of sunflower
Lane 3. Total RNA isolated from healthy leaves of sunflower
Lane 4. Total RNA isolated from infected leaves of sunflower

Fig. 21: Southern dot blot hybridisation
1. PCR product
2. Total RNA isolated from infected leaves of sunflower
3. Plasmid DNA isolated from clone 5
4. Plasmid DNA isolated from clone 7
5. Plasmid DNA isolated from clone 9
6. Total RNA isolated from healthy leaves of sunflower
The above results revealed that the virus-infecting sunflower was serologically related to PBNV of serogroup IV of tospoviruses.