MATERIALS AND METHODS
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RAISING AND MAINTENANCE OF TEST PLANTS

Potting medium

A mixture of sand, soil and compost was prepared in the ratio of 1:2:1 for growing the test plants. The soil mixture was sterilized by autoclaving at 20 lb pressure for one hour. The pots were sterilized by rinsing with 5 per cent solution of formaline and filled with the sterilized soil mixture autoclaved 24 hours earlier.

Cultivation of test plants

The seedlings were raised in shallow pots and transplanted singly in clay pots of 4" and 6" diameter at 2-3 leaf stage, when they were about 2 weeks old. Plants belonging to the family Cucurbitaceae and Leguminosae were raised by direct sowing in 6" pots. For inoculation the plants were used two weeks after transplantation and kept in an insect proof glasshouse at a temperature of 20-30°C.

Culture of virus isolate

Leaves of young, naturally infected plants of turnip (Brassica rapa L.) showing mosaic, downward curling of the leaves, shortening of roots and some floral abnormalities were macerated
using mortar and pestle with simultaneous addition of phosphate buffer (0.1M, pH 7.0). The macerate was filtered through two layers of cheese cloth and centrifuged at 6,000 rpm for 5 min. Culture of turnip mosaic virus was maintained by sap inoculation on a group of healthy and uniform plants of *Nicotiana glutinosa* at 4-5 leaf stage using sap prepared as above, by single lesion inoculation. Inoculations were made by weekly mechanical inoculation to healthy plants to increase culture. Periodic checks were made on assay host, *Chenopodium amaranticolor* to ensure biological purity of the virus.

**DISEASE INCIDENCE**

Turnip (*Brassica rapa* L.) grown in and around Aligarh, U.P., India were surveyed during October, 1983 to March, 1984. Disease incidence was calculated by the following formula:

\[
\text{Disease incidence} = \frac{\text{Number of plants showing symptoms}}{\text{Total number of plants observed}} \times 100
\]

**TRANSMISSION**

Different methods of transmission as mechanical, aphids, dodder, soil and seeds were made to ascertain the spread of virus in nature. Modification, if any, are described in the text at appropriate places.
By sap inoculation

Preparation of standard inoculum

Young infected leaves of turnip (*Brassica rapa* L.) showing prominent symptoms were macerated with requisite amount of phosphate buffer (0.1M pH 7.0). The slurry was squeezed through double folds of muslin cloth. Sap was centrifuged at 6,000 rpm for 5 min and the supernatant thus obtained has been termed as Standard Inoculum (SI) in further tests. For each gram of infected leaf material, 1 ml of the buffer was used.

Inoculation

Inoculations were made by rubbing the forefinger, dipped in the Standard Inoculum (SI), on the upper surface of leaves of the test plants predusted with carborundum (500 mesh). Two or three lower most leaves were inoculated and were rinsed by a gentle stream of water soon after inoculation.

By aphids

Rearing of aphids

Colonies of virus free aphids were raised on suitable plant in cages having wooden frame. The top and the two sides of the cage were closed by glass and the remaining sides were closed by wire gauze. A fluorescent tube was fitted in the cage to
provide artificial light to the plants and to keep the aphids under long day conditions to get the apterous (wingless) aphids. The plants were kept on a zinc tray and the bottom of the tray was covered with a layer of wet sand to prevent the passing of the aphids through spaces between the tray and the rim of the cage. Each new colony of the aphids was started by placing about ten aphids on a fresh plant. Viviparous adults were starved for about 2 h at room temperature in a Petri dish and then placed on a detached leaf of appropriate healthy host plant in a Petri dish. The atmosphere inside the Petri dish was made humid by covering the inner surface of the Petri dish with wet filter paper. Newly born nymphs were transferred to a fresh and healthy plant.

Transmission

The nymphs were collected carefully from the healthy colony by the moistened tip of camel hair brush type A, No. 1. To ascertain the nature of transmission i.e., either in non-persistent or in persistent manner following procedures were followed:

Non-persistent

1. Pre-acquisition starvation period 2 to 3 h
2. Acquisition access period 1 to 2 min
3. Inoculation access period 24 h
4. Number of aphids/plant 5 to 10
The nymphs were starved for 2-3 h in a Petri dish having the inner surface covered with a wet piece of filter paper before an acquisition access period of 1 to 2 min on the leaf of the diseased plant. After allowing acquisition feeding time, the nymphs in the batches of 10 were transferred to each healthy seedling and the plants were covered with Lenz caging cages for an inoculation access period of 24 h. The nymphs after the end of inoculation access were killed by spraying with 0.2 per cent Dimecron (insecticide) and the plants were kept under insect proof glasshouse for the development of symptoms. Back inoculations from each plant were made to an appropriate local lesion host, i.e. Chenopodium amaranticolor.

Persistent

1. Acquisition access period
   6 to 24 h
2. Inoculation access period
   48 h
3. Number of aphids/plant
   5 to 10

In this process nymphs without being given any pre-acquisition starvation were allowed for 6-24 h acquisition access on diseased leaves followed by an inoculation access period of 48 h on indicator plants. The nymphs after the end of inoculation access were killed by spraying with 0.2 per cent Dimecron (insecticide) and the plants were kept under insect proof glasshouse for the development of symptoms. Back inoculations from each plant were made to C. amaranticolor.
For aphid transmission, four different aphid spp. were used in the tests and were bred on appropriate hosts as mentioned below:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Aphid species</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Aphis gossypii</em> Glov.</td>
<td><em>Cucumus sativus</em></td>
</tr>
<tr>
<td>2</td>
<td><em>A. fabae</em> Theobald</td>
<td><em>Solanum nigrum</em></td>
</tr>
<tr>
<td>3</td>
<td><em>Brevicoryne brassicae</em> L.</td>
<td><em>Brassica oleracea</em> var. <em>capitata</em></td>
</tr>
<tr>
<td>4</td>
<td><em>Myzus persicae</em> Sulz.</td>
<td><em>Nicotiana tabacum</em> var. <em>White Burley</em></td>
</tr>
</tbody>
</table>

By soil

Soil was collected from and around the naturally infected turnip (*Brassica rapa* L.) plants and roots, debris and stones etc. were removed. The soil was divided into two parts: one part was autoclaved at 15 lb pressure for 30 min which served as control and the remaining other part left as such. Healthy seedlings susceptible to turnip mosaic virus were transplanted in autoclaved (control) and unautoclaved soil separately contained in pots. The plants were transferred to an insect proof glasshouse and symptoms were observed till a period of two months after transplantation. Back inoculations from each plant were made to *C. amaranticolor* to ascertain the presence of virus in them.
By seed

To determine the seed transmission of virus, the experiments were carried out in the following method:

Sowing-on method

Seeds were collected from infected and healthy plants and were sown separately in autoclaved soil in an insect proof glasshouse. After seedling emergence, the plants were observed till six weeks and were sprayed with 0.2 per cent Dimecron (insecticide) at weekly intervals to prevent insect infestation.

Infectivity test method

Leaf tissues of such plants, grown as above, were macerated in suitable buffer and the sap obtained was inoculated manually on diagnostic host of the virus.

By dodder

Seeds of the dodder (Cuscuta reflexa L.) were sown in a Petri dish. After germination the dodder seedlings were trained on N. glutinosa plants which were inoculated after the dodder had established on them. After one week of inoculation of N. glutinosa, the stems of dodder were placed and allowed to train on healthy N. glutinosa plants placed near the infected N. glutinosa plants.
having dodder established on them. Presence of virus in dodder inoculated N. glutinosa plants was assessed on test plants.

**HOST RANGE AND SYMPTOMATOLOGY**

Several species of plants belonging to different families were screened for the susceptibility to the virus under study by mechanical transmission. For each gram of the material 1 ml of the buffer was used. At least three plants of each species/cultivar were inoculated with SI and the same number of plants were left as control. Test plants used during host-range studies were young seedlings at 3-4 leaf stage. Plants were observed till two months after inoculation for development, sequence and severity of symptoms. Standard inoculum from plants with apparently no symptoms was prepared and inoculated on C. amaranticolor to work out the latent infection, if any.

**BIO-PHYSICAL PROPERTIES**

**Dilution end point (DEP)**

Young leaves of N. glutinosa inoculated 10-15 days earlier were macerated in a mortar with pestle and the sap was obtained by squeezing the macerate through two layers of cheese cloth. Ten fold dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were made of the sap by the addition of 0.1M phosphate buffer pH 7.5. From each dilution three plants of C. amaranticolor bear-
ing leaves of almost equal size were inoculated manually using carborundum 500 mesh as an abrasive. The leaves of the plants were washed by a gentle stream of water immediately after inoculation. Local lesions developed on the inoculated leaves were counted after one week.

Thermal inactivation point (TIP)

The sap was obtained by macerating the young leaves of \textit{N. glutinosa} inoculated 10-15 days earlier, in a mortar with pestle. The macerate was filtered through two layers of muslin cloth. The sap thus obtained was divided into 10 aliquots of 5 ml each and were kept separately in glass vials. The glass vials were held in water bath in such a way that the sap level was slightly below the level of the water in the bath. The different aliquots were heated at 40, 45, 50, 55, 60, 65, 70 and 75°C for ten minutes and then cooled by dipping in cold water, immediately after the treatment. Each aliquot was inoculated to three \textit{C. amaranticolor} plants and local lesions produced on the inoculated leaves were counted after one week.

Longevity in vitro (LIV)

Young leaves of the \textit{N. glutinosa} inoculated 10-15 days earlier were homogenized in a mortar with pestle and the sap was obtained by filtering the pulp through two layers of cheese cloth. Two aliquots of the sap were made and one of them was stored at
4°C, while another at room temperature (20 ± 5°C). Three
*C. amaranticolor* plants of the same age bearing leaves of almost
equal size were inoculated with sap from each aliquot, separately
at different intervals up to several days. Inoculations were made
manually using carborundum 500 mesh as an abrasive and the inocu-
lated leaves were washed by a gentle stream of water soon after
inoculation. Local lesions developed on inoculated leaves were
counted after one week.

**PURIFICATION**

**Selection of propagation host**

To ascertain the appropriate plant species/cultivar for
maximum virus concentration, various systemic hosts were selected
and mechanically inoculated with standard inoculum (SI). Fifteen
plants of each species were inoculated with SI using carborundum
500 mesh. The inoculated plants were assayed for active virus on
*C. amaranticolor*.

**Selection of local lesion host**

Various local lesion host were considered. The most
suitable of them which reacted with clear, discrete and good
number of local lesions was selected.
Standardization of extraction procedure

Effect of buffers

Various buffers (acetate, borate, citrate and phosphate) at different pH and molarities were tested to work out the most suitable buffer for maintaining the virus infectivity.

Sap obtained by macerating the young infected *N. glutinosa* leaves in each of the buffers mentioned above, separately was inoculated on local lesion host. A buffer at the pH and molarity in which virus infectivity was comparatively higher was selected and used regularly for extracting the virus under study.

Different kinds of buffers were prepared by the method detailed by Gomori (1955) and pH of buffers was measured by ELICO pH meter model LI-10.

Concentration of the virus in different parts of the host

*N. glutinosa* plants inoculated 10-15 days earlier were uprooted carefully and washed. The plants were placed on blotting paper and dried, and the root, shoot and leaf tissues were cut into pieces separately. Equal amounts of root, shoot and leaf tissues were homogenized separately in mortar with a pestle with 0.1M phosphate buffer pH 7.5. Each homogenate was filtered through two layers of cheese cloth and three *C. amaranticolor*
plants were inoculated with each sample. The inoculation was done manually using carborundum 500 mesh as an abrasive. The leaves were rinsed with a gentle stream of water soon after inoculation. The local lesions developed on inoculated leaves were recorded for comparison after one week of inoculation.

**Effect of additives**

Various additives (reducing and chelating agents) were tested to find out their effect on virus infectivity. Following additives obtained from sources mentioned below were used.

1. Ethylenediamine tetra acetic acid (EDTA) BDH (India) Pvt. Ltd., India
2. 2-Mercapto-ethanol Sigma Chemical Co. Ltd., U.S.A.
3. Thioglycollic acid Polypharm Pvt. Ltd., India
4. Sodium sulphite AR BDH (India) Pvt. Ltd., India

**Clarification of extract**

**By organic solvents**

Inoculum was treated with organic solvents (butanol, carbon tetrachloride and chloroform) either separately or in combination for removal of extraneous material from crude sap obtained from infected plant tissues. The requisite amount of solvent was mixed and the mixture kept for 15 min. The aqueous layer was separated by low speed centrifugation (6,000 rpm for 10 min) and the active virus content was assayed on C. amaranti-color plants.
Precipitation of the virus

Polyethylene glycol (MW 6,000) was tried using it in 2, 4, 6 and 8 per cent separately, along with or without one per cent sodium chloride (NaCl). After the addition of requisite amount of PEG and NaCl to the clarified extract, the mixture was stirred on a stirrer for 2 h to allow precipitation. Thereafter, the mixture was centrifuged at 10,000 rpm for 20 min to collect the precipitate (Pellet).

The pellet obtained in each case was dissolved in extraction buffer and centrifuged at low speed (6,000 rpm for 10 min). Supernatant was collected and assayed for virus infectivity on local lesion host, C. amaranticolor.

Differential centrifugation

The technique of differential centrifugation was applied to sediment the virus particles into a pellet form with active infectivity. Ultracentrifugation of different samples was done in model L5-50 Beckman preparative ultracentrifuge class H using rotor type 50. Normally high speed centrifugation was done at 40,000 rpm (97,000 g) for 90 or 120 min. The pellet thus obtained was dissolved in same buffer and centrifuged at low speed (6,000 rpm for 10 min). Infectivity of different samples was determined by assaying on local lesion host.
DENSITY GRADIENT CENTRIFUGATION.

Gradient columns were prepared by the method of Brakke (1960). Linear sucrose gradient columns were prepared using 40, 30, 20 and 10 per cent sucrose solutions prepared in 0.1M phosphate buffer pH 7.5. 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.

Next day 1 ml freshly prepared partially purified virus suspension was layered on each tube with the help of LKB-varioperex pump and centrifuged at 40,000 rpm in swinging-bucket rotor (SW 50L) for 90 min in a Beckman L5-50 ultracentrifuge. The tubes were taken out and were examined in a dark room by projecting a narrow beam of light down the tube from the top. The band (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.

The samples thus obtained were examined in SP8-100 Pye Unicam UV/Visible Spectrophotometer and assayed on local lesion host to determine the infectivity.

BUOYANT DENSITY

Appropriate amount of partially purified virus was mixed with cesium chloride (CsCl) and the mixture was centrifuged at 40,000 rpm (97,000 g) for 12 h in a Beckman L5-50 preparative
ultracentrifuge using a S.50 rotor. The band (viral zone) was collected and examined spectrophotometrically in SP8-100 Pye Unicam UV/Visible Spectrophotometer.

The refractive indices of virus samples were measured with an Abbe-type Refractometer at room temperature and the values were converted into buoyant density (B.D.) by the equation described by Szybalski (1968).

**ANALYTICAL CENTRIFUGATION**

Sedimentation coefficient studies of the virus were carried out by employing analytical ultracentrifuge, Beckman Model E equipped with Schlieren optics and rotor type An-D SER NO. 2402 with the single sector cell. Drive was supplied a constant acceleration. Virus suspension was in 0.1M phosphate buffer pH 7.5. The centrifuge was run at 17,000 rpm. Photograph timer was set at intervals of 4 min. Boundary curve was visually observed before starting timer. All runs were made at 20°C. Sedimentation rate (s-rate) was calculated by applying the following formula.

\[ s = \frac{1}{w^2 r} \frac{dr}{dt} \]

\[ w = 2 \pi (\text{rpm})/60 \]

\[ s = \text{Sedimentation rate} \]

\[ w = \text{Angular velocity} \]

\[ r = \text{Position of boundary} \]

\[ t = \text{Time difference} \]
UV-ABSORPTION SPECTRUM

The purified virus preparations obtained after differential centrifugation were scanned in SP8-100 Pye Unicam UV/Visible Spectrophotometer. Absorbance (A) of samples was recorded in UV range (220 nm to 300 nm). Correction for light scattering, however, was not done. Values of A min, A max, A260/A280 and A280/A260 were determined to get the approximate percentage of nucleic acid by comparing the data with standard graph (Gibbs and Harrison, 1976). Spectral curve (absorbance vs. wave length) were recorded using an automatic recorder.

SDS-ELECTROPHORESIS OF VIRUS PROTEIN

The molecular weight of virus protein was estimated in 10% (w/v) polyacrylamide gels through SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) following the method described by Maizel (1971).

Virus was dissociated by heating for 2 min in a boiling water bath in the presence of 0.0625M Tris-HCl buffer pH 6.7 containing 0.5% (w/v) SDS and 1% (V/V) 2-mercaptoethanol. Samples of 10-20 microlitres containing virus protein were layered on cylindrical polyacrylamide gels (120x6 mm). The solubilised virus samples were co-electrophoresed for 4 h with molecular weight markers in electrode buffer \(\sim 0.6\%\) (w/v) tris and 2.88% (w/v) glycine in water\(\sim\) at 5mA per tube. After electrophoresis, the
gels were stained overnight with 0.1% (w/v) Coomassie brilliant blue dissolved in equal volume of methanol and water containing 7% (V/V) acetic acid. The gels were destained in water containing 10% (V/V) methanol and 7.5% (V/V) acetic acid. The markers used to estimate the molecular weight of the protein subunit of virus were:

Trypsin inhibitor (MW 20,100 daltons), trypsinogen (MW 24,000 daltons), carbonic anhydrase (MW 29,000 daltons), glyceraldehyde-3-phosphate dehydrogenase (MW 36,000 daltons), egg albumin (MW 45,000 daltons) and bovine serum albumin (MW 66,200 daltons). All these markers were procured from Sigma Chemical Co. Ltd., U.S.A.

CHARACTERISTIC OF VIRAL NUCLEIC ACID

Isolation of viral nucleic acid

The nucleic acid was isolated by phenol-chloroform method. Phenol and chloroform were prepared as detailed elsewhere (Maniatis et al., 1982). Phenol contained 8-hydroxyquinoline to a final concentration of 0.1% (w/v) while chloroform solution contained 4% (V/V) iso-amyl alcohol.

Purified solution containing virus was extracted with a mixture of phenol-chloroform (1:1) followed by another extraction with chloroform only.
2 ml virus preparation + 2 ml mixture of phenol-chloroform (1:1)

Mix the contents on a Vortex mixer till an emulsion forms

Centrifuge for 3 min at 1600 g

Phenol phase discard

Aqueous phase

Centrifuge for 3 min at 1600 g

Chloroform phase discard

Aqueous phase

Add 2.5 volumes of chilled ethanol

Keep overnight at -20°C

Centrifuge for 30 min at 15000 g

Supernatant discard

Pellet suspended in distilled water (viral nucleic acid)
The infectivity of viral nucleic acid prepared by the above method was tested on local lesion host, C. amaranticolor.

Type of nucleic acid

The type of nucleic acid contained in the virion was determined by orcinol (Shatkin, 1969) and diphenylamine (Burtan, 1956) tests for RNA and DNA, respectively.

Orcinol test

Orcinol reagent was prepared by adding 100 mg of orcinol and 100 mg of FeCl₃·6H₂O in 100 ml of concentrated HCl. 1 ml of freshly prepared reagent was added to 1 ml of nucleic acid preparation. The mixture was placed in boiling water bath for 45 min and observed for the development of colour.

Diphenylamine test

Diphenylamine reagent was prepared by mixing 1 gm of diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of H₂SO₄. 1 ml of suitably diluted nucleic acid preparation was mixed with 2 ml of diphenylamine reagent and heated at 100°C for 10 min to observe the development of colour.
ELECTRON MICROSCOPY

Morphology of the virus particle

A small drop of the purified virus preparation was placed by a freshly broken capillary on a formvar coated copper grid with a carbon backing. After two minutes, the excess of sample was soaked out by touching a piece of pointed filter paper at the grid edge. The dried grid was then stained with 2 per cent (w/v) uranyl acetate (pH 4.2) for 90 sec. The samples were alternatively stained with 2 per cent (w/v) phosphotungstic acid (pH 7.0) in identical manner as detailed above. Grids thus prepared were examined under Philip EM 420 Electron Microscope.

ULTRASTRUCTURAL STUDIES

*N. glutinosa* leaves with typical virus symptoms were collected after 13 days of inoculation for the purpose. The leaf areas exhibiting the symptoms were cut and immersed in 3% glutaraldehyde solution in 0.1M phosphate buffer pH 7.5. Leaf segments of 1 mm x 2-3 mm were then cut and transferred to fresh fixative solution in small injection vials. After 6 h, the fixative was drained out and the leaf segments were washed twice with phosphate buffer at an interval of half an hour to remove glutaraldehyde. One per cent osmium tetroxide solution in 0.1M phosphate buffer was then poured over the leaf segments in vials. Vials were covered with aluminium foil and allowed to stand for 2 h. The
osmium tetroxide solution was carefully taken out and the leaf segments were washed with distilled water. They were then dehydrated in graded series of acetone as follows:

1. 30% Acetone - 10 min
2. 50% Acetone - 10 min
3. 70% Acetone - 10 min
4. 95% Acetone - 10 min
5. 100% Acetone - 30 min (two changes each of half an h)

The leaf segments, after dehydration, were cleared in propylene oxide by giving two changes of half an hour each. Then propylene oxide was substituted with 1:1 mixture of epoxy resin and propylene oxide and the leaf segments were left in that for 2 h. Mixture of Spurr's epoxy resin was prepared as follows:

Resin, i.e. vinylcyclohexene dioxide (VCD) - 10 ml
Hardner, i.e. nonenyl succinic anhydride (NSA) - 26 ml
Flexibilizer, i.e. diglycidylether of polypropylene glycol (DER 736) - 06 ml
Accelerator, i.e. dimethylaminoethanol (DMAE) - 0.4 ml

Resin, hardner and flexibilizer were first thoroughly mixed and then accelerator was added and mixed properly.

The aluminium caps were then taken off the vials and propylene oxide allowed to get evaporated. After 8 h, the resin mixture in the vials was replaced with fresh resin mixture and vials left for another 4 to 10 h. The properly infiltrated leaf
segments were then embedded in fresh resin mixture in gelatin capsules orienting them suitably for transverse sectioning. The capsules in proper holders were transferred to an oven set at 70°C for curing of the resin mixture. The properly cured blocks were ready after 24 h. They were then trimmed and ultra-thin sections of silver interference colour (i.e., Ca 70 to 80 nm) obtained with the help of LKB Ultratome Nova. The section ribbons were lifted on copper grids. The sections on grids were then stained as follows:

(a) **Saturated alcoholic uranyl acetate**

This solution was prepared by dissolving excess of uranyl acetate in 50% ethanol followed by centrifugation at 6,000 g. The supernatant was then filtered through Whatman No. 1 filter paper. Drops of this stain were placed on parafilm on slides and sections on grids were immersed in them. They were left in the drops for half an hour at room temperature followed by thorough rinsing in 50% ethanol and two lots of distilled water. The grids were then drained and further stained with lead citrate as given below.

(b) **Lead citrate solution**

This solution was prepared according to the method described by Reynolds (1963) which is as follows. The following three stock solutions needed for the purpose were prepared as detailed below.
1. **Stock solution A**

37.7 g of trisodium citrate monohydrate was dissolved in distilled water and the volume made to 100 ml with distilled water.

2. **Stock solution B**

33.1 g of lead nitrate was dissolved in glass distilled water and the volume made to 100 ml with distilled water.

3. **Stock solution C**

1N sodium hydroxide.

Lead citrate solution was prepared fresh only a few hours before use by mixing the above solutions in the following order.

Sixteen ml distilled water + 3 ml of solution A followed by stirring. Then 2 ml of solution B was added and contents stirred. A thick white precipitate which appeared was then dissolved by adding 4 ml of solution C. The solution obtained was centrifuged at 6,000 g for 10 min to sediment any left over precipitate and the supernatant was filtered through Whatman No.1 filter paper. Drops of the solution after diluting it to 1:4 with 0.01N NaOH were placed on parafilm on slides in Petri dish containing pellets of sodium hydroxide to provide a carbon dioxide (CO₂) free
atmosphere. The sections on grids previously stained with uranyl acetate were floated on these drops for 10 min. They were then washed thoroughly in 0.01N NaOH followed by distilled water, drained, dried and screened under electron microscope, JEOL JEM 108S, at 100 kv.

SEROLOGY

Raising of antisera

Partially purified virus preparation obtained after differential centrifugation was used as antigen for immunization of rabbits. Healthy male albino-rabbits, approximately 2 Kg in weight and six month old were used. One intravenous injection in ear and two intramuscular injections into thigh were given 10 days apart to a single albino rabbit using 1 ml partially purified virus. For intravenous injection, virus was injected into the external marginal vein of the ear formerly shaved and rubbed with xylene to avoid contamination and appearance of clear vein. For intramuscular injection, the virus preparation (1 ml) was emulsified with an equal volume of Freund's incomplete adjuvant (Difco Lab., U.S.A.). The emulsion was divided into two parts and injected in each thigh of the rabbit.

Blood was collected from the ear that was not used for injection by giving sharp incision after 20, 30 and 60 days of final injection. About 15-20 ml of the blood was collected at a
time and allowed to clot at room temperature (20 ± 5°C) for 2 h and kept in a refrigerator for 4 h. Then, serum was separated and centrifuged at 5,000 rpm for 5 min to remove fibrin, blood cells, etc. The straw yellow coloured supernatant was collected and stored in sterile small ampules by adding few crystals of sodium azide (NaN₃).

Serological tests

Dip serology (ISEM)

Trapping of virus particles

0.1 g of N. glutinosa leaves showing typical symptoms after 13-15 days of inoculation were macerated in 1 ml of 0.1M phosphate buffer pH 7.5. Copper grids coated with a colloidian film were floated on PVX (potato virus X) antiserum of 1:5,000 titre diluted with phosphate buffer, for 20 min at room temperature in a humid chamber. Grids were then washed with 30 drops of distilled water and drained by touching their edge with filter paper. They were then immediately floated, film side down, on 0.01 ml drops of the crude virus extract and incubated in a humid chamber at 37°C for 1 h. They were then again washed with 30 drops of distilled water, drained by touching their edge with filter paper and then immediately floated on 2% aqueous uranyl acetate solution for 1 min. Now they were finally drained and allowed to air dry. The grids so prepared were screened under JEOL JEM 108S electron microscope at 100 kv.
Decoration of virus particles

After trapping the virus particles on antiserum coated grids as described above, the grids were again floated on the same antiserum with the same dilution for 1 h at 37°C in a humid chamber. They were then washed with 30 drops of distilled water, drained and immediately floated on 2% aqueous uranyl acetate solution for 1 min. Then, they were finally drained, air dried and screened under the electron microscope.

Tube precipitin test

Serial two fold dilutions of both antiserum and antigen were prepared using 0.85% saline as diluent. Equal proportions of antisera and antigen of different dilutions were mixed together in serological tubes (6x1 cm) and incubated at 37°C in a water bath. The formation of precipitate and intensity was observed using a magnifying glass.

Chloroplast agglutination test

Chloroplast agglutination test was performed as described by van Slogteren (1955). In this test, 5 drops of diluted antiserum were mixed with 2 drops of crude infected plant sap on a microscope slide. Observation for clumping reaction was made under microscope.
**Ouchterlony gel double diffusion test**

Ouchterlony agar gel double diffusion test (Ouchterlony, 1948, 1958, 1962) was used for the antigen antibody reactions. Crude sap and purified virus preparation were tested. The tests were carried out using 90 mm sterilized disposable plastic Petri dishes (Steriware Ltd., India). For the optimum conditions for the formation of immunoprecipitin band using different combinations of gel media and antigen prepared in different ways were studied. The agar gel plates were then incubated in a humid chamber at room temperature and observed after 24 and 48 h.

**Preparation of agar gel media**

Agar gel media for immunodiffusion tests were prepared by the following methods:

1. 0.5 g agarose (Difco Lab., U.S.A.) was melted in 100 ml phosphate buffer (0.1M, pH 7.5) containing 0.85% (w/v) sodium chloride (PBS).

2. 1 g agarose was melted in 100 ml phosphate buffer (0.1M, pH 7.5) containing 0.85% (w/v) sodium chloride (PBS).

3. 1 g agarose was melted in 100 ml phosphate buffer (0.1M, pH 7.5) containing 0.02% (w/v) sodium azide.

4. 1 g agarose was melted in 100 ml phosphate buffer containing 0.85% (w/v) NaCl plus 1% (w/v) SDS.
Preparation of viral antigens

Antigens used in immunodiffusion tests were prepared in the following ways:

1. Infected *N. glutinosa* leaf tissues (10-15 days after inoculation) were triturated in phosphate buffer (0.1M, pH 7.5) with 0.2% (w/v) sodium sulphite (1:1, w/v).

2. Infected *N. glutinosa* leaf tissues (10-15 days after inoculation) were macerated in phosphate buffer (0.1M, pH 7.5) plus 0.85% (w/v) NaCl (1:1, w/v).

Antisera to various flexuous viruses as detailed below were obtained from different sources to establish the serological relationship, if any, with known viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato virus S (PVS)</td>
<td>Dr. I.D. Garg, CPRI, SIMLA (India).</td>
</tr>
<tr>
<td>Potato virus X (PVX)</td>
<td></td>
</tr>
<tr>
<td>Potato virus Y (PVY)</td>
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</tr>
<tr>
<td>Cymbidium mosaic virus (CybMV)</td>
<td>Dr. G. Lebeurier, IBMC, Strasbourg, France.</td>
</tr>
<tr>
<td>Narcissus mosaic virus (NaMV)</td>
<td>Dr. D.Z. Maat, Research Institute for Plant</td>
</tr>
<tr>
<td>(PoAMV)</td>
<td></td>
</tr>
<tr>
<td>Papaya mosaic virus (PaMV)</td>
<td></td>
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<tr>
<td>White clover mosaic virus</td>
<td></td>
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<tr>
<td>(WC1MV)</td>
<td></td>
</tr>
</tbody>
</table>
POSITION OF UTTAR PRADESH IN INDIA

DISTRICT ALIGARH

300 0 300 KILOMETRES
4.1 NATURAL SYMPTOMS

Naturally infected plants of turnip, *Brassica rapa* L. showed mosaic like symptoms on leaves. At advanced stage, the infected plants showed reduction in leaf size, twisting of leaves, floral abnormalities, reduction of root and over-all stunting of plants (Fig.1.0a,b). Only few distorted roots of small size were produced.

4.2 DISEASE INCIDENCE

Survey for recording the incidence of disease during the winter of 1983-84 revealed that the disease incidence after seedling stage to flowering stage ranged between 40-80 per cent. A perusal of Table-1.0 and Fig. 1.1 revealed a gradual increase in incidence and indicated that the plants at all stages of growth were susceptible to infection.

4.3 TRANSMISSION

4.3.1 Sap

The virus causing mosaic disease of turnip and designated as turnip mosaic virus (TuMV) was readily transmitted by sap prepared in 0.1M phosphate buffer pH 7.0 from turnip to turnip, *Nicotiana glutinosa* and various other hosts. The transmission of
disease was 90-100 per cent by sap inoculation using carborundum predusted 2-3 basal leaves of healthy plants.

Table-1.0: Incidence of TuMV at different stages of plant growth during the season 1983-84.

<table>
<thead>
<tr>
<th>Date of observation</th>
<th>Number of plants observed</th>
<th>Number of diseased plants*</th>
<th>Percentage of diseased plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 Oct. 1983</td>
<td>520</td>
<td>52</td>
<td>10</td>
</tr>
<tr>
<td>01 Nov. 1983</td>
<td>640</td>
<td>115</td>
<td>18</td>
</tr>
<tr>
<td>15 Nov. 1983</td>
<td>700</td>
<td>210</td>
<td>30</td>
</tr>
<tr>
<td>01 Dec. 1983</td>
<td>610</td>
<td>244</td>
<td>40</td>
</tr>
<tr>
<td>15 Dec. 1983</td>
<td>485</td>
<td>209</td>
<td>43</td>
</tr>
<tr>
<td>01 Jan. 1984</td>
<td>498</td>
<td>324</td>
<td>65</td>
</tr>
<tr>
<td>15 Jan. 1984</td>
<td>500</td>
<td>400</td>
<td>80</td>
</tr>
<tr>
<td>01 Feb. 1984</td>
<td>526</td>
<td>379</td>
<td>72</td>
</tr>
<tr>
<td>15 Feb. 1984</td>
<td>505</td>
<td>283</td>
<td>56</td>
</tr>
<tr>
<td>01 Mar. 1984</td>
<td>370</td>
<td>189</td>
<td>51</td>
</tr>
<tr>
<td>15 Mar. 1984</td>
<td>430</td>
<td>194</td>
<td>45</td>
</tr>
</tbody>
</table>

*Observation based on apparent symptoms on leaves.

4.3.2 Aphids

Four species of aphids, *Aphis gossypii*, *A. fabae*, *Brevicoryne brassicae* and *Myzus persicae* were used by allowing
them to acquire and transmit the virus in a persistent and non-
persistent manner but they did not evoke any symptoms on healthy
plants. The transmission tests were carried out using *Nicotiana
glutinosa* as a donor and recipient host in all combinations and
ten aphids per plant were invariably used in the study.

4.3.3 Soil

Twenty seedlings of each *Brassica rapa, Nicotiana
glutenosa, N. sylvestris, Datura metel,*
*D. stramonium* and *Trichosanthes anguina* were transplanted in soil
collected from and around the TuMV infected plants. These plants
did not show any apparent symptoms even after 2 months. Back
inoculation tests carried out on local lesion host, *Chenopodium
amaranticolor,* using the sap prepared from randomly selected
5 plants of each group indicated the absence of virus in these
plants. Thus, it appeared that the virus was not transmitted
through soil.

4.3.4 Seed

*Brassica rapa, Nicotiana glutinosa, N. sylvestris,*
*Datura metel, D. stramonium* and *Trichosanthes anguina* plants
raised using seeds collected from infected plants did not show
any apparent symptoms when grown under insect proof condition in
sterilized soil. Back inoculation tests, to ascertain presence/
absence of virus, carried out on local lesion host using the sap prepared from randomly selected 5 plants of each group indicated negative result. Thus, it appeared that the virus was not transmitted through seeds which were collected from infected plants.

4.3.5 Dodder

Only one species of dodder, *Cuscuta reflexa* was used in an attempt to transmit TuMV from diseased *Nicotiana glutinosa* to healthy *N. glutinosa* plants. None of the plants showed any symptoms and no virus could be recovered in back inoculation to *Chenopodium amaranticolor* from dodder inoculated plants. As only one species of dodder was used, no generalization could be made regarding the transmission of the virus by dodder.

4.4 HOST RANGE AND SYMPTOMATOLOGY

Host range of the turnip mosaic virus (TuMV) was investigated by mechanical inoculation of sap from infected plants to a range of test plants. Back inoculations from all inoculated plants were made on *Chenopodium amaranticolor* to ascertain the presence of virus. Following plants developed symptoms as described below and virus was recovered on back inoculation to test plant except where stated otherwise.

*Brassica rapa* L.: The following cvs. were recorded as susceptible to the virus infection.
Brassica rapa L. cv. Sutton Early Snow Ball
  cv. Purple Top White
  cv. Green Globe

Inoculated plants showed systemic symptoms in the form of downward curling of leaves, light and dark green patches followed by various stages of reduction in lamina after 12 days of inoculation. Inoculated plants remained dwarf, bore very few flowers and showed suppression of root system (Fig. 1.2).

Brassica oleracea L. var. botrytis: The following cvs. responded to the virus infection.
  cv. Snow Ball-16
  cv. Katki Faizabadi Kalmi
  cv. Katki
  cv. Dania
  cv. Patna Mid Season

Systemic symptoms appeared on newly emerging leaves in the form of curling, mottling of leaves within 5-6 days followed by dark and light shaded areas and finally the leaves yellowed after 12-14 days of inoculation (Fig. 1.3a), except cv. Katki. Leaves also exhibited various degrees of reduction in lamina.

In cv. Katki the systemic symptoms appeared on newly emerging leaves in the form of mottling, within 5-6 days followed by dark black patches on the light green background, after 12-14
days of inoculation (Fig. 1.3b). Inoculated plants remained
dwarf and produced deformed and reduced sized heads.

**Brassica oleracea** L. var. capitata: The following cvs. were
susceptible to the virus infection.

- cv. Pride of India
- cv. Sutton Express
- cv. Long Blood Red
- cv. Golden Acre
- cv. American Special Ball Head

Systemic symptoms appeared after 10 days of inoculation
on newly emerging leaves in the form of light and dark green areas
(Fig. 1.4). Infected plants remained dwarf.

**Brassica campestris** L.: Inoculated plants showed systemic symp­
toms after 10 days of inoculation in the form of downward curling
of leaves, reduction in lamina, very few flowers and over-all
retardation in the growth of plants (Fig. 1.5).

**Raphanus sativus** L.: The following cvs. were recorded as suscep­
tible to the virus infection.

- cv. Pusa Rashmi
- cv. Pusa Chetaki
- cv. Chinese White
Systemic symptoms appeared on newly emerging leaves in the form of deformation, downward curling, reduction in lamina and very few flowers. The inoculated plants remained dwarf (Fig. 1.6a). But cv. Pusa Rashmi also showed light and dark green areas on newly emerging leaves (Fig. 1.6b).

**Spinacea oleracea** L.: The following cvs. responded to virus infection in different manners.

**cv. Pusa Jyoti:** Inoculated plants showed systemic symptoms in the form of mosaic on newly emerging leaves after 11 days of inoculation and infected leaves were smaller in comparison to healthy ones (Fig. 1.7a).

**cv. Palang Sag** showed systemic symptoms on newly emerging leaves in the form of upward rolling after 12 days of inoculation (Fig. 1.7b).

In cv. Banarjee's Giant Palang, systemic symptoms appeared on newly emerging leaves in the form of upward rolling followed by twisting like a rope (Fig. 1.7c) after 10 days of inoculation.

**Vigna sinensis** Savi: Systemic symptoms appeared one week after inoculation in the form of vein clearing, yellowing, deformation and twisting of leaves (Fig. 1.8).

**Nicotiana benthamiana** Domin.: Mosaic, stunting, mottling and yellowing were observed in newly emerging leaves after 13 days.
of inoculation. The inoculated leaves showed wilting after 15 days of inoculation (Fig. 1c,9). Most of the inoculated plants died before blooming and those surviving produced only a few distorted flowers.

\textit{N. glutinosa} L.: Systemic symptoms appeared after 8 days of inoculation on the newly emerging leaves in the form of vein clearing followed by mosaic after 12 days (Fig. 2.0). Flowers on the inoculated plants were shed.

\textit{N. tabacum} L. cv. NC-95: Inoculated plants showed prominent light and dark green patches on the newly emerging leaves and subsequently reduction in leaf size (Fig. 2.1).

\textit{N. tabacum} L. cv. GT-4: Inoculated plants developed systemic symptoms consisting of light and dark green patches on the newly emerging leaves after 11 days of inoculation. The infected leaves were smaller in size, misshapen and deformed in comparison to healthy ones (Fig. 2.2).

\textit{N. sylvestris} Speg. & Comes: Systemic symptoms appeared after 9 days of inoculation on the newly emerging leaves in the form of vein clearing followed by light and dark green areas. Leaves exhibited various stages of reduction in lamina and were misshapen (Fig. 2.3).

\textit{N. tabacum} L. cv. Lime Green: Systemic symptoms developed after 8 days of inoculation on the newly emerging leaves in the form of vein clearing followed by mosaic.
**N. tabacum** L. cv. Sensation Mixed: Inoculated plants developed systemic symptoms after 8 days of inoculation on the newly emerging leaves in the form of twisting and deformation.

**Petunia hybrida** Vilm.: Systemic symptoms appeared after 9 days of inoculation on the newly emerging leaves in the form of mosaic, mottling, reduction and deformation in lamina and light and dark green patches. The infected leaves were smaller in size as compared to healthy ones (Fig. 2.4).

**N. tabacum** L. cv. White Burley: Necrotic light black rings were formed on the inoculated leaves 5–6 days after inoculation. After 10 days of inoculation the necrotic rings increased a little more, coalesced and the leaves were shed (Fig. 2.5a,b). No systemic symptoms were observed on newly emerging leaves till 2 months.

**N. tabacum** L. cv. Xanthi: Necrotic lesions encircled by light black rings appeared on inoculated leaves after 6 days of inoculation at the 4–5 leaf stage (Fig. 2.6). The inoculated leaves were shed after 8–10 days of inoculation and most of the plants died within 15–20 days of inoculation. No systemic symptoms were observed.

**N. tabacum** L. cv. Harrison's Special: Inoculated leaves of plants developed necrotic lesions after 5 days of inoculation, and the leaves were shed after 12 days of inoculation. No systemic symptoms were observed till 2 months.
N. *tabacum* L. cv. Samsun: Necrotic lesions appeared on the inoculated leaves after 6-7 days of inoculation (Fig. 2.7). The inoculated leaves were shed within 12-15 days after inoculation. Most of the inoculated plants at 4-5 leaf stage died after 20 days of inoculation while mature plants survived. No systemic symptoms were observed till 2 months.

N. *tabacum* L. cv. Anand-3: Necrotic lesions appeared on inoculated leaves after 5 days of inoculation. The inoculated leaves were shed after 15 days of inoculation. Systemic symptoms were not observed till 2 months.

N. *megalosiphon* Heurek & Mueller: Necrotic lesions developed on inoculated leaves 5-6 days after inoculation (Fig. 2.8a). The inoculated leaves were shed within 10-12 days of inoculation while the newly emerging leaves showed mild mosaic, deformation, downward curling and reduction in lamina (Fig. 2.8b).

N. *tabacum* L. cv. CTRI Special: Necrotic lesions encircled with rings appeared on the inoculated leaves after 5-6 days of inoculation (Fig. 2.9). The inoculated leaves were shed after 15 days of inoculation and no systemic symptoms followed.

N. *tabacum* L. cv. Bhopali Pakra: Necrotic lesions encircled with black rings appeared on the inoculated leaves after 5 days of inoculation (Fig. 3.0). The rings increased in size, coalesced and leaves were shed after 10 days. Systemic symptoms were not observed till 8 weeks after inoculation.
**N. tabacum** L. cv. DR-1: The inoculated leaves showed light yellow and necrotic lesions encircled with black rings after 5 days of inoculation (Fig. 3.1). The inoculated leaves were shed within 12-15 days of inoculation. No systemic symptoms appeared till 2 months.

**Trigonella foenum-graecum** L.: Black spherical spots appeared on the inoculated leaves after 6 days of inoculation (Fig. 3.2). The inoculated leaves became yellow and were shed after 15 days of inoculation. Systemic symptoms were not observed till 8 weeks after inoculation.

**Gomphrena globosa** L.: Necrotic lesions circled with red haloes appeared on inoculated leaves after 5 days of inoculation (Fig. 3.3). No systemic symptoms appeared till 2 months.

**Trianthema portulacastrum** L.: Light black necrotic lesions appeared on the inoculated leaves after 5 days of inoculation (Fig. 3.4). The lesions increased in size, coalesced and the inoculated leaves were shed but the newly emerging leaves never showed systemic symptoms.

**Phaseolus lunatus** L. cvs. Lobia Improved Black Seeded and Lobia Pusa Barsati: Red coloured local lesions appeared on inoculated leaves after 7 days of inoculation (Fig. 3.5) and no systemic symptoms developed till 2 months of inoculation.
Vicia faba L. cv. The Sutton: Red coloured local lesions appeared on inoculated leaves after 7 days of inoculation. No systemic symptoms appeared.

Chenopodium amaranticolor Coste & Reyn.: Chlorotic local lesions appeared on inoculated leaves after 4-5 days of inoculation in summer and after 5-7 days in winter (Fig. 3.6). The lesions increased in size, coalesced and leaves were shed in summer within 7 days of inoculation.

C. murale L.: Necrotic local lesions were produced on inoculated leaves after 4-5 days in summer and 5-7 days in winter (Fig. 3.7). The lesions increased in size, coalesced and leaves were shed after 10 days of inoculation.

C. album L.: Chlorotic local lesions appeared on inoculated leaves after 5-7 days of inoculation in winter. The lesions increased in size, coalesced and leaves were shed in summer.

C. quinoa L.: Chlorotic local lesions appeared on inoculated leaves after 5-7 days of inoculation (Fig. 3.8). The lesions increased in size, coalesced and leaves were shed after 11 days of inoculation.

Solanum nigrum L.: Systemic symptom appeared after 10 days of inoculation on newly emerging leaves in the form of light and dark green areas, yellow patches and vein bandings. The leaves exhibited various stages of reduction in lamina and infected leaves were smaller in size as compared to healthy ones (Fig. 3.9).
*Datura metel* L.: Systemic symptoms appeared 9-11 days after inoculation on newly emerging leaves in the form of vein clearing followed by a green mosaic. Inoculated leaves showed yellowing. Leaves also exhibited reduction in lamina (Fig. 4.0).

*D. stramonium* L.: Systemic symptoms appeared after 9-11 days of inoculation on newly emerging leaves in the form of vein clearing starting from the base to tip followed by a green mosaic (Fig. 4.1).

*Cucumis sativus* L. cv. Khira: Systemic symptoms appeared one week after inoculation on the newly emerging leaves in the form of light and dark green areas. The roots were poorly developed and stem cracked longitudinally above the soil. The inoculated plants remained dwarf (Fig. 4.2).

*Trichosanthes anguina* L.: Systemic symptoms appeared after 6-8 days of inoculation on the newly emerging leaves in the form of vein clearing followed by green mosaic and yellow patches (Fig. 4.3).

*Luffa aegyptiaca* Mill.: Systemic symptoms appeared on newly emerging leaves in the form of vein clearing and green mosaic after 11 days of inoculation.

*Momordica charantia* L.: Inoculated plants developed systemic symptoms consisting of yellowing, light and dark green areas on leaves after 9 days of inoculation. Infected leaves exhibited various degrees of deformation.
Hibiscus esculentus L.: The following cvs. were recorded as susceptible to virus infection:

- cv. Sutton Makhmali
- cv. Seven Dhari
- cv. Long Green

Systemic symptoms appeared on the newly emerging leaves in the form of yellowing, light and dark green areas after 12 days of inoculation. The infected plants remained dwarf and leaves were also smaller in comparison to healthy ones. No recovery of virus was found on back inoculation.

Ipomoea purpurea Roth.: The following cvs. were observed as susceptible to virus infection.

- cv. Candy Pink
- cv. Super Giant Mixed
- cv. Azure Blue
- cv. Super Giant Red with White Throat

Systemic symptoms appeared on newly emerging leaves in the form of light and dark green areas and yellowing followed by light black patches after 12 days of inoculation (Fig. 4.4).

Ruellia tuberosa L.: Systemic symptoms appeared after 11 days of inoculation on the newly emerging leaves in the form of vein banding and interveinal yellowing (Fig. 4.5). The plants remained stunted.
**Amaranthus tricolor** L.: Systemic symptoms appeared after 12 days of inoculation on the newly emerging leaves in the form of deformation, mosaic and black patches followed by marginal necrosis (Fig. 4.6). The growth of the plants was severely affected.

**Apium graveolens** L.: Plant developed systemic symptoms consisting of thickening of leaves, downward curling, mosaic (Fig. 4.7) and stunting of the plants.

**Ammi majus** L.: Systemic symptoms appeared one week after inoculation on the newly emerging leaves in the form of curling, mosaic and stunting of plants.

**Phlox drummondii** Hook.: Plants developed systemic symptoms consisting of mosaic, shortening of leaves followed by curling in a peculiar manner (Fig. 4.8).

**Brachycome iberidifolia** Benth.: Plants developed systemic symptoms consisting of malformation, shortening of stem and curling of leaves followed by their complete twisting (Fig. 4.9).

**Ageratum mexicanum** Sims. cv. Blue Mink: Systemic symptoms appeared in the form of green mosaic, curling of leaves and stunting of plants.

**Arctotis stoechadifolia** Berg. cv. Sutton Special Hybrid: Systemic symptoms appeared on the newly emerging leaves in the form of vein clearing followed by light and dark green areas and curling of leaves. Inoculated plants remained dwarf in comparison to healthy ones.
**Calendula officinalis** L. cv. Orange Coronet and Art Shades:
Systemic symptoms appeared in the form of light and dark green areas, mosaic, curling, deformation and mottling of newly emerging leaves after 10 days of inoculation (Fig. 5.0).

**Centaurea imperialis** L. cv. Sutton Giant Mauve: Systemic symptoms appeared on the newly emerging leaves in the form of curling, twisting, green mosaic and deformation of lamina after 9-11 days of inoculation. The inoculated plants remained dwarf in comparison to healthy ones (Fig. 5.1a,b).

**Dimorphotheca aurantiaca** DC.: Plants developed systemic symptoms consisting of mosaic and leaf deformation after 10 days of inoculation. Inoculated plants remained stunted.

**Tagetes erecta** L.: Systemic symptom appeared on the newly emerging leaves after 8 days of inoculation in the form of curling, shortening, deformation and twisting of leaves. Leaves exhibited various degrees of reduction in lamina (Fig. 5.2).

**Tetragonia expansa** Murr.: The inoculated leaves showed light green mosaic followed by yellowing of leaves and finally the leaves were shed after 12-15 days of inoculation.

**Verbena hybrida** Voss.: Systemic symptoms consisted of elongation of internodes, downward curling, twisting and reduction in the size of lamina (Fig. 5.3).
**Carthamus roseus** L. cv. Dwarf Rose Pink: Systemic symptoms appeared 9-11 days after inoculation on the newly emerging leaves in the form of curling, green mosaic and deformation. The inoculated plants remained dwarf in comparison to healthy ones.

**Impatiens balsamina** L.: The following cvs. were found susceptible to virus infection.

- cv. Rose Flowered Crimson
- cv. Rose Flowered White
- cv. Rose Flowered Rose
- cv. Rose Flowered Mixed
- cv. Rose Flowered Violet

The systemic symptoms appeared on the newly emerging leaves in the form of light and dark green patches, mosaic, deformation and various degrees of reduction in lamina (Fig. 5.4). Infected plants remained stunted.

**Convolvulus major** Gilib cv. Picta: Systemic symptoms appeared 8-10 days after inoculation on the newly emerging leaves in the form of light green mosaic and reduction in size of leaves.

**Coccinia indica** Wight & Aran.: Systemic symptoms appeared on the newly emerging leaves after 12 days of inoculation in the form of mosaic and various degrees of reduction in lamina (Fig. 5.5).

**Alyssum maritimum** Lam. cv. Snow Carpet: Plants showed systemic symptoms consisting of vein clearing, mottling and yellow patches on the newly emerging leaves. The older leaves were shed.
Cyamopsis tetragonoloba L.: Systemic symptoms appeared after 9 days of inoculation on the newly emerging leaves in the form of mosaic, curling, deformation, twisting, mottling and various degrees of reduction in lamina (Fig. 5.6).

Iberis amara L.: Inoculated leaves showed yellowing, necrotic spots and were shed after 4 days of inoculation. No systemic symptoms were observed.

Vernonia cineria Less.: Systemic symptoms consisting of mosaic, downward curling of leaves, deformation and reduction in number and size of leaves (Fig. 5.7) were observed after 12 days of inoculation.

Matthiola incana R.Br.: Systemic symptoms appeared on the newly emerging leaves in the form of vein clearing and mosaic mottling after 9 days of inoculation.

Laggeria aurita Sch. Bhip ex C.B. Clarks: Systemic symptoms appeared on the newly emerging leaves in the form of mosaic, rosetting, mottling and reduction in lamina after 13 days of inoculation (Fig. 5.8).

Salvia officinalis L.: Systemic symptoms appeared on the newly emerging leaves in the form of mosaic and reduction in lamina after 9 days of inoculation.

Physalis floridana hydb.: Systemic symptoms appeared on the newly emerging leaves in the form of mosaic and reduction in lamina after 8 days of inoculation (Fig. 5.9).
**Bryophyllum calycinum** Salisb.: Systemic symptoms appeared on the newly emerging leaves in the form of light green mosaic after 15 days of inoculation (Fig. 6.0).

**Eschscholzia californica** Cham. cv. Ivory White: Systemic symptoms appeared on the newly emerging leaves in the form of mosaic after 7 days of inoculation.

**Sida cardifolia** L.: Systemic symptoms appeared on the newly emerging leaves in the form of vein clearing followed by light green mosaic and deformation (Fig. 6.1).

**Commelina benghalensis** L.: Systemic symptoms appeared on the newly emerging leaves in the form of light and dark green patches, downward curling and deformation after 12 days of inoculation. Infected leaves also exhibited various degrees of reduction in lamina (Fig. 6.2).

**Peristrophe bicalyculata** Nees.: Systemic symptoms appeared on the newly emerging leaves in the form of light and dark green patches. The infected leaves were smaller in comparison to healthy ones (Fig. 6.3).

**Non-hosts**

Following plants showed no symptoms (systemic/local) till two months after inoculation and no virus could be recovered when back inoculations from these plants were made on *Chenopodium amaranticolor*.

**Family: Amaranthaceae**

**Amaranthus caudatus** L. cv. Yellow Splendour
Gomphrena globosa L. cv. Globe Amaranth Purple
          cv. Globe Amaranth Mixed

Family: ASTERACEAE

Acrodinum roseum Benth. cv. Sutton Special Mixture
Bellis perennis L. cv. Sutton Dwarf White
Coreopsis tinctoria Nutt. cv. Picta
Cosmos bipinnatus Cav. cv. Double Mixed
Lactuca sativa L.
Sonchus asper Hill.
Zinnia elegans Jacq. cv. Sutton Giant Double Yellow
          cv. Sutton Giant Double Mixed

Family: BORAGINACEAE

Cordia obliqua Willd.

Family: CHENOPODIACEAE

Kochia scoparia Schrad. cv. Trichipylla

Family: CRUCIFERAE

Brassica oleracea L. var. botrytis cv. Sutton Pusi
          cv. Pusa Snow Ball
          cv. Massuria Ball
          cv. Special Indian Snow Ball
          cv. Super Maghi
          cv. Special Agahni (Late)
cv. Special Parijat Pusa

cv. Agahni Jaldbaj

cv. Silver King

cv. American White

cv. Early of India

cv. Kuwari Early

cv. Selected Special Maghi

cv. 235-S

cv. 74-6C

cv. 114-S

cv. 236-S

cv. Snow Ball Elite

Brassica oleracea L. var. capitata cv. Early Express Dewali

cv. Special Pride

cv. Glory

cv. Red Drum Head

cv. Pride of Asia

cv. Express Pointed

cv. Double Express

cv. Sutton Eclipse Drumhead

cv. Sutton Pride of India

cv. September

cv. Glory of Enkhuizen
Brassica rapa L. cv. Pusa Swarnima
   cv. Sutton Golden Ball
Raphanus sativus L. cv. Pusa Himani
   cv. Punjab Sel-5
   cv. Crimson French Breakfast

Family: CUCURBITACEAE

Citrullus vulgaris Schard. var. Fistulosus
Cucumis sativus L. cv. Point Sett
   cv. Poona White Wonder
   cv. Improved Long Green
Cucumis melo L. cv. Foot Kakri
   cv. Kakri
Cucurbita moschata Duchesne ex Poir
Lagenaria vulgaris Ser.
Cucurbita pepo L.

Family: EUPHORBIACEAE

Euphorbia hirta L.
Phyllanthus niruri L.

Family: GRAMINAE

Zea mays L.
Triticum aestivum L.
Family: MALVACEAE

Hibiscus esculentus L. cv. Pusa Swani Packet
  cv. Bhindi N-5
  cv. KS-312
  cv. KS-302 (Red)

Family: NYCTAGINACEAE

Mirabilis jalapa L.

Family: PAPILIONACEAE

Cajanus cajan L.
Medicago falcata L.
Pisum sativum L. cv. Bonneville
  cv. Sutton Show Perfection
  cv. Early Wonder
Vigna radiata (L.) Wilczek.

Family: POLYGONACEAE

Antigonon leptopus Hook. & Arn. cv. Leptopus Pink
  cv. Deep Carmine

Family: PORTULACACEAE

Portulaca oleracea L. cv. Sutton Improved Double Mixed

Family: SOLANACEAE

Capsicum annuum L. cv. Pusa Jawala
  cv. Suryamukhi Black
cv. Suryamukhi Green
cv. Jwala
cv. Bull Nose
cv. Hungarian Wax
cv. Chinese Giant
cv. California Wonder
cv. Elephant Struck
cv. Chilli G-4
cv. Chilli NP64-A
cv. Chilli G-3
cv. Chilli PC-1

Capsicum pendulum Wild.

Lycopersicum esculentum Mill. cv. Mixed Ornamental
  cv. S-12
  cv. Sutton Roma
  cv. Money Maker
  cv. Punjab Chhoara
  cv. Pusa Early Dwarf
  cv. Pusa Ruby
  cv. Pusa 120
  cv. Pant-T₁
  cv. Pant-T₂
  cv. Pant-T₃
  cv. Best of All
  cv. Marglobe Supreme
  cv. Bonny Best
cv. Mikado
cv. VFN-Bush
cv. VFN-8
cv. EC173898
cv. EC173897
cv. EC173896
cv. EC173902

**Nicotiana affinis** Moore

**N. fragrans** Hook. cv. Evening Fragrance

**N. rustica** Schrank

**N. occidentalis** Wheeler

**Solanum melongena** L. cv. Special Banarsi Jhumkeya

  cv. Pusa Kranti
  cv. Pusa Purple Long
  cv. Pant Samrat
  cv. Manjri Gota
  cv. Long White
  cv. Round White
  cv. Baromashi
  cv. Black Beauty
  cv. Pusa Purple Cluster
  cv. Long White Cluster
  cv. Nurki
  cv. Improved Muktakeshi
  cv. Banaras Giant White
Family: UMBELLIFERAE

Daucus carota L. cv. Early Nautes
   cv. Pusa Kesar
   cv. Zino

Family: VIOLACEAE

Viola tricolor L. cv. Supremo Early
   cv. Sutton Pixie Mixed

3 BIO-PHYSICAL PROPERTIES

It is desirable to study properties of a virus in crude sap before any work on its physico-chemical nature is undertaken. These studies provide an idea about the stability and concentration of the virus in the sap. Although these studies have restricted value (Ross, 1964), they are of utmost importance in determining the procedure for the purification and characterization of virus.

Studies on bio-physical properties were carried out using N. glutinosa as donor host of the virus and tests were made on local lesion host, C. amaranticolor. The results were recorded from the crude sap obtained by macerating the infected N. glutinosa leaves with the help of mortar and pestle.
4.5.1 Dilution end point (DEP)

Several dilutions using phosphate buffer (0.1M, pH 7.5) were prepared from crude sap. Each dilution was tested on local lesion host, C. amaranticolor. The virus in crude sap was found to be infectious up to a dilution of $10^{-4}$ but no local lesions were found when the sap was diluted to $10^{-5}$ (Table-1.1).

Table-1.1: Effect of dilution of sap on the infectivity of inoculum of TuMV.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Number of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>67</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>49</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>20</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>11</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>03</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>00</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>00</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 18 leaves of C. amaranticolor.

4.5.2 Thermal inactivation point (TIP)

Ten aliquots of crude sap of 5 ml each were exposed separately in thin walled test tubes at specific temperature
for 10 minutes. The samples, after treatment were cooled immediately and tested on local lesion host, *C. amaranticolor* to evaluate infectivity. The TuMV was inactivated after being heated for 10 minutes at 65°C. Therefore, the TIP was found in the range of 60 to 65°C (Table-1.2).

Table-1.2: Effect of temperature on the stability of TuMV.

<table>
<thead>
<tr>
<th>Temperature in °C</th>
<th>Number of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature (20±5°C)</td>
<td>98</td>
</tr>
<tr>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>55</td>
<td>06</td>
</tr>
<tr>
<td>60</td>
<td>02</td>
</tr>
<tr>
<td>65</td>
<td>00</td>
</tr>
<tr>
<td>70</td>
<td>00</td>
</tr>
<tr>
<td>75</td>
<td>00</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 18 leaves of *C. amaranticolor*.

4.5.3 Longevity *in vitro* (LIV)

Two samples of 25 ml each of crude sap obtained from infected *N. glutinosa* leaves were kept in two sterilized glass vials. One was kept at room temperature (20±5°C) and the other
in a refrigerator (4°C). Each sample was assayed on *Chenopodium amaranticolor* after a specific period of storage (Table-1.3).

Table-1.3: Effect of storage on infectious sap at 20±5°C and 4°C.

<table>
<thead>
<tr>
<th>Storage in h</th>
<th>Average number of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Room temp. (20±5°C)</td>
</tr>
<tr>
<td>00</td>
<td>180</td>
</tr>
<tr>
<td>06</td>
<td>152</td>
</tr>
<tr>
<td>12</td>
<td>138</td>
</tr>
<tr>
<td>18</td>
<td>108</td>
</tr>
<tr>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td>48</td>
<td>34</td>
</tr>
<tr>
<td>72</td>
<td>02</td>
</tr>
<tr>
<td>96</td>
<td>00</td>
</tr>
<tr>
<td>120</td>
<td>00</td>
</tr>
<tr>
<td>144</td>
<td>00</td>
</tr>
<tr>
<td>168</td>
<td>00</td>
</tr>
<tr>
<td>192</td>
<td>00</td>
</tr>
</tbody>
</table>

*Average number of local lesions per leaf based on 18 leaves of *C. amaranticolor*.

The data included in table-1.3 showed that virus in sap remained infectious till 72 h at room temperature (20±5°C) and 144 h at 4°C. No infectivity was observed beyond these periods.
4.6 **PURIFICATION**

4.6.1 **Selection of propagation host**

Four hosts of the virus isolate infecting turnip viz., *Nicotiana glutinosa*, *N. megalosiphon*, *N. sylvestris* and *Cucumis sativus* were compared with regard to virus concentration at different times after inoculation. The plants were mechanically inoculated and assayed for virus concentration at different intervals after inoculation using *C. amaranticolor* as a local lesion host (Table-1.4 and Fig. 6.4).

Table-1.4: Concentration of TuMV in different hosts at different days after inoculation.

<table>
<thead>
<tr>
<th>Host</th>
<th>Average number of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Days after inoculation)</td>
</tr>
<tr>
<td></td>
<td>2  4  6  8  10  12  14  16  18  20  22</td>
</tr>
<tr>
<td><em>N. sylvestris</em></td>
<td>0  0  9  40  94  138  120  70  48  32  28</td>
</tr>
<tr>
<td><em>N. megalosiphon</em></td>
<td>0  0  28  98  160  142  111  30  24  20  20</td>
</tr>
<tr>
<td><em>N. glutinosa</em></td>
<td>0  2  0  8  38  68  126  190  170  30  28  26</td>
</tr>
<tr>
<td><em>Cucumis sativus</em></td>
<td>0  0  24  66  90  82  71  53  42  36  32</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 25 leaves of *C. amaranticolor*.

It is apparent from the Table-1.4 and Fig. 6.4 that the virus attained maximum concentration in about 10 days following
Fig. 6.4

Concentration of turnip mosaic virus in different hosts at different periods after mechanical inoculation.
incubation in *N. megalosiphon* and *C. sativus*. However, in *N. sylvestris* and *N. glutinosa* the maximum concentration was reached after 12 and 14 days of inoculation, respectively. The concentration of the virus in *N. sylvestris* was found to be less than *N. glutinosa* (Fig. 6.4). Therefore, *N. glutinosa* was used as a host for propagation of TuMV.

### 4.6.2 Selection of local lesion host

Four local lesion hosts of the virus isolate infecting turnip viz., *Chenopodium quinoa*, *C. murale*, *C. amaranticolor* and *Gomphrena globosa* were compared to select the most suitable one. The inoculum was prepared from infected *N. glutinosa* and local lesions were counted after 5-7 days of inoculation (Table-1.5).

**Table-1.5:** Number of local lesions on different local lesion hosts.

<table>
<thead>
<tr>
<th>Local lesion host</th>
<th>Average number of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chenopodium quinoa</em></td>
<td>106</td>
</tr>
<tr>
<td><em>C. murale</em></td>
<td>96</td>
</tr>
<tr>
<td><em>C. amaranticolor</em></td>
<td>155</td>
</tr>
<tr>
<td><em>Gomphrena globosa</em></td>
<td>18</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 25 leaves of local lesion hosts.*
On the basis of data presented in Table 1.5, Chenopodium amaranticolor was selected as a suitable local lesion host.

4.6.3 Standardization of extraction procedure

Effect of buffers

Effect of acetate, borate, citrate and phosphate buffers at different levels of pH was compared for extraction of TuMV from infected leaves of N. glutinosa. Partially clarified extract obtained after low speed centrifugation (6,000 rpm for 10 min) was assayed on local lesion host, C. amaranticolor.

Results presented in the Table-1.6 and Fig. 6.5 indicate that the maximum infectivity was obtained with phosphate buffer pH 7.5.

Attempts were also made to determine the most suitable molarity (ionic strength) of phosphate buffer pH 7.5 for virus infectivity. Infected leaves of N. glutinosa were macerated in phosphate buffer pH 7.5 of different molarities separately. The samples after a low speed centrifugation were assayed on C. amaranticolor for comparison of virus infectivity (Table-1.7 and Fig. 6.6).
Table-1.6: Effect of various buffers at different pH levels on the infectivity of TuMV.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Average number of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M Acetate</td>
<td>4.0</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>21</td>
</tr>
<tr>
<td>0.1M Borate</td>
<td>7.0</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>37</td>
</tr>
<tr>
<td>0.1M Citrate</td>
<td>4.0</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>03</td>
</tr>
<tr>
<td>0.1M Phosphate</td>
<td>5.0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>20</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 25 leaves of C. amaranticolor.
Fig. 6.5

Effect of various buffers at different pH values on the infectivity of turnip mosaic virus.
Table-1.7: Relative infectivity of TuMV at different molarities of phosphate buffer pH 7.5.

<table>
<thead>
<tr>
<th>Molarity</th>
<th>Average number of local lesions/leaf*</th>
<th>Per cent relative infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01M</td>
<td>90</td>
<td>53</td>
</tr>
<tr>
<td>0.1M</td>
<td>170</td>
<td>100</td>
</tr>
<tr>
<td>0.2M</td>
<td>92</td>
<td>54</td>
</tr>
<tr>
<td>0.5M</td>
<td>70</td>
<td>41</td>
</tr>
<tr>
<td>1.0M</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>2.0M</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>2.5M</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>3.0M</td>
<td>16</td>
<td>09</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 25 leaves of C. amaranticolor.

It is clear from the Table-1.7 and Fig. 6.6 that the extraction of infected leaves in 0.1M phosphate buffer gave the highest infectivity. But high molarity (3.0M) affected the infectivity adversely.

Thus, 0.1M phosphate buffer pH 7.5 was used for extracting the virus infected tissues.

4.6.4 Concentration of the virus in different parts of the host

Equal amount of infected tissues of root, stem and leaf were homogenized separately with phosphate buffer (0.1M, pH 7.5)
Effect of different molarities of phosphate buffer (pH 7.5) on the infectivity of turnip mosaic virus.
and assayed on local lesion host, \textit{C. amaranticolor} after a low speed centrifugation for comparison of virus concentration.

Results presented in Table-1.8 reveal that the maximum concentration of the virus was present in leaf tissue followed by stem and root.

Table-1.8: Concentration of TuMV in different parts of \textit{N. glutinosa} 14 days after mechanical inoculation.

<table>
<thead>
<tr>
<th>Parts of plant</th>
<th>Average number of local lesions/leaf*</th>
<th>Per cent relative infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Stem</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>Leaf</td>
<td>120</td>
<td>100</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 18 leaves of \textit{C. amaranticolor}.

4.6.5 Effect of additives on infectivity of turnip mosaic virus (TuMV)

To evaluate the effect of different additives on the infectivity of TuMV, sodium sulphite, thioglycollic acid, 2-mercaptoethanol and ethylenediamine tetracetic acid (EDTA) were added separately or in different combinations in phosphate buffer (0.1\%, pH 7.5). Such buffer was then used during maceration of
infected *N. glutinosa* leaf tissues (1:1 w/v). Extract prepared in phosphate buffer (0.1M, pH 7.5) without any additives served as control. Assaying of virus was carried out on local lesion host, *C. amaranticolor* (Table-1.9).

Results presented in Table-1.9 indicate that the combination of 0.1% sodium sulphite and EDTA (0.01M) when added to the extraction medium increased the infectivity of TuMV by 33%. Therefore, the combination of sodium sulphite (0.1%) and EDTA (0.01M) was mixed with phosphate buffer (0.1M, pH 7.5) for extraction of infectious sap.

**Table-1.9: Effect of additives on the infectivity of TuMV.**

<table>
<thead>
<tr>
<th>Additives</th>
<th>Average number of local lesions/leaf*</th>
<th>Per cent increase in infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>A</td>
<td>170</td>
<td>210</td>
</tr>
<tr>
<td>B</td>
<td>170</td>
<td>198</td>
</tr>
<tr>
<td>C</td>
<td>170</td>
<td>178</td>
</tr>
<tr>
<td>D</td>
<td>170</td>
<td>182</td>
</tr>
<tr>
<td>A+B</td>
<td>170</td>
<td>226</td>
</tr>
<tr>
<td>A+D</td>
<td>170</td>
<td>208</td>
</tr>
<tr>
<td>A+B+D</td>
<td>170</td>
<td>212</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 18 leaves of *C. amaranticolor*.

A - Sodium sulphite (0.1%)
B - EDTA (0.01M)
C - 2-Mercaptoethanol (0.1%)
D - Thioglycollic acid (0.1%)
4.6.6 Clarification of extract

By organic solvents

Sap extracted from *N. glutinosa* in EB, i.e. phosphate buffer (0.1M, pH 7.5 containing 0.1% sodium sulphite and 0.01M EDTA) was mixed with different organic solvents either alone or in different combinations and incubated for 15 min at 4°C. Samples were centrifuged for 10 min at 6,000 rpm. The aqueous layer was separated and assayed on *C. amaranticolor*. Sap extracted from *N. glutinosa* in EB only was used as control.

Table-2.0: Effect of some organic solvents on infectivity of sap containing TuMV.

<table>
<thead>
<tr>
<th>Organic solvent</th>
<th>Final percentage of organic solvent</th>
<th>Average no. of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Butanol</td>
<td>20</td>
<td>106</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>20</td>
<td>106</td>
</tr>
<tr>
<td>Chloroform</td>
<td>20</td>
<td>106</td>
</tr>
<tr>
<td>Mixture of butanol + chloroform (1:1)</td>
<td>20</td>
<td>106</td>
</tr>
<tr>
<td>Mixture of carbon tetrachloride + chloroform (1:1)</td>
<td>20</td>
<td>106</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 25 leaves of *C. amaranticolor*. 
Effect of some organic solvents on infectivity of sap containing TuMV.
Results presented in the Table-2.0 and Fig. 6.7 reveal that butanol, carbon tetrachloride and a mixture of carbon tetrachloride and chloroform (1:1) showed adverse effect on virus. A mixture of 20% butanol and chloroform (1:1) was found to be efficient and so was routinely used for clarification of N. glutinosa extract containing TuMV. However, chloroform too gave somewhat satisfactory results.

4.6.7 Precipitation of virus with polyethyleneglycol (PEG 6000 MW)

The precipitation of TuMV from clarified preparation obtained through low speed centrifugation (10,000 rpm for 20 min), PEG (6,000 MW) of different concentration (2, 4, 6 and 8%) was attempted with or without NaCl (1%). The precipitate obtained in each case was suspended in EB and centrifuged at 6,000 rpm for 10 min. The supernatant after diluting it with EB to bring it to the original volume was inoculated on local lesion host, C. amaranticolor to find out whether the virus is precipitated by PEG under the experimental conditions. The clarified preparation obtained after low speed centrifugation was also inoculated on C. amaranticolor for comparison of virus infectivity.

The results given in the Table-2.1 and Fig. 6.8 indicate that all the virus was not precipitated by 2, 4, 6 and 8% PEG with 1% NaCl. The maximum recovery of virus was with 6% PEG without using NaCl. NaCl seems to have deleterious effect on virus infectivity and did not facilitate the precipitation of the virus.
Table-2.1: Effect of PEG and NaCl on the precipitation of TuMV.

<table>
<thead>
<tr>
<th>PEG (6,000 MW)</th>
<th>NaCl (% w/v)</th>
<th>Average number of local lesions/leaf*</th>
<th>Loss of infectivity (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>02</td>
<td>1</td>
<td>152</td>
<td>06</td>
</tr>
<tr>
<td>02</td>
<td>-</td>
<td>152</td>
<td>10</td>
</tr>
<tr>
<td>04</td>
<td>1</td>
<td>152</td>
<td>13</td>
</tr>
<tr>
<td>04</td>
<td>-</td>
<td>152</td>
<td>24</td>
</tr>
<tr>
<td>06</td>
<td>1</td>
<td>152</td>
<td>48</td>
</tr>
<tr>
<td>06</td>
<td>-</td>
<td>152</td>
<td>82</td>
</tr>
<tr>
<td>08</td>
<td>1</td>
<td>152</td>
<td>40</td>
</tr>
<tr>
<td>08</td>
<td>-</td>
<td>152</td>
<td>68</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 25 leaves of C. amaranticolor.

4.7 PURIFICATION PROCEDURE

Several procedures were evaluated for the purification of the present virus isolate, but the results of two methods which were most suitable are detailed here.

Relative infectivity of the samples was determined at different steps of purification by assaying on C. amaranticolor. Data are presented in Table-2.2 and Fig. 6.9.
Fig. 6-8

Effect of PEG (6,000 mw) and NaCl on the precipitation of TuAV.
Macerate 100 g frozen infected *N. glutinosa* leaves in 100 ml EB
Squeeze through muslin cloth
Centrifuge at 6,000 rpm for 10 min
Pellet discard
Supernatant*

20% Chilled mixture of butanol + chloroform (1:1)
Stir for 15 min
Centrifuge at 6,000 rpm for 10 min
Pellet discard
Supernatant*

6% PEG (6,000 Mw)
Stir for 2 h
Centrifuge at 10,000 rpm for 20 min
Supernatant discard
Pellet
Suspend in EB
Centrifuge at 6,000 rpm for 10 min
Pellet discard
Supernatant**
Centrifuge at 30,000 rpm for 90 min
Supernatant discard
Pellet
Suspend in 2 ml 0.1M phosphate buffer pH 7.5
Centrifuge at 6,000 rpm for 10 min
Pellet discard
Supernatant** (Partially purified virus suspension)

*Assayed for virus infectivity on C. amaranticolor
### Table-2.2: Relative infectivity of the virus isolate at different steps of purification by PEG.

<table>
<thead>
<tr>
<th>Steps of purification</th>
<th>Average number of local lesions/leaf*</th>
<th>Per cent relative infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant (after 1st low speed)</td>
<td>108</td>
<td>83</td>
</tr>
<tr>
<td>Butanol + chloroform (1:1) treatment</td>
<td>130</td>
<td>100</td>
</tr>
<tr>
<td>PEG precipitation**</td>
<td>55</td>
<td>42</td>
</tr>
<tr>
<td>After high speed**</td>
<td>32</td>
<td>25</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 18 leaves of C. amaranticolor.

**Virus suspension was diluted to original volume for assaying virus infectivity.

Relative infectivity of the virus isolate at different steps of purification was assayed on C. amaranticolor. Data are presented in Table-2.3 and Fig. 7.0 (for 2nd Procedure).

The virus isolate was purified successfully by the above mentioned procedure, but the second procedure involving differential centrifugation showed a very good concentration of virus particles in comparison to first procedure. The number of virus particles and infectivity were found much greater in the samples of virus prepared by the second procedure. Therefore, the second procedure was adopted for the purification of the virus. All further studies on the properties of the virus were carried out using this method for isolating the virus.
Fig. 6.9
Infectivity of TuMV at different steps of purification involving precipitation by PEG.
PURIFICATION PROCEDURE-II

Macerate 100 g frozen infected *N. glutinosa* leaves in 100 ml EB

Squeeze through muslin cloth

Centrifuge at 2,000 rpm for 10 min

Pellet discard

Supernatant

20% Chilled mixture of butanol + chloroform (1:1)

Stir for 15 min

Centrifuge at 6,000 rpm for 10 min

Pellet discard

Supernatant

1% Triton X-100

Stir for 30 min

Centrifuge at 6,000 rpm for 10 min

Pellet discard

Supernatant

Centrifuge at 35,000 rpm for 120 min

Supernatant discard

Pellet

Suspend in EB

Centrifuge at 6,000 rpm for 10 min

Pellet discard

Supernatant

Centrifuge at 40,000 rpm for 120 min

Supernatant discard

Pellet

Suspend in 2 ml phosphate buffer 0.1M pH 7.5

Centrifuge at 6,000 rpm for 10 min

Pellet discard

Supernatant** (Partially purified virus suspension)

*Assayed for virus infectivity on *C. amaranticolor*
Table-2.3: Relative infectivity of virus isolate at different steps of purification.

<table>
<thead>
<tr>
<th>Steps of purification</th>
<th>Average number of local lesions/leaf*</th>
<th>Per cent relative infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant (After 1st low speed)</td>
<td>148</td>
<td>86</td>
</tr>
<tr>
<td>Butanol + chloroform (1:1) treatment</td>
<td>172</td>
<td>100</td>
</tr>
<tr>
<td>1% Triton X-100 treatment</td>
<td>136</td>
<td>79</td>
</tr>
<tr>
<td>Differential centrifugation I**</td>
<td>112</td>
<td>65</td>
</tr>
<tr>
<td>Differential centrifugation II**</td>
<td>78</td>
<td>45</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 18 leaves of C. amaranticolor.

**Virus suspension was diluted to original volume for virus assay.

4.8 DENSITY GRADIENT CENTRIFUGATION

Further purification of TuMV was achieved by rate zonal density gradient centrifugation. The last traces of plant proteins and other remaining material were removed by rate zonal density gradient centrifugation. One ml partially purified preparation of TuMV was loaded on sucrose density gradients and centrifuged for 90 min at 4,000 rpm in a Beckman SW 50L rotor. The tubes when examined in a dark room by projecting a narrow beam of light down the tube from the top showed a light scattering band (Fig. 7.1). This light scattering band was consistently present. Symptoms
Infectivity of turnip mosaic virus at different steps of purification by differential centrifugation.

Fig. 7.0

- Supernatant (after first low speed)
- Butanol + chloroform treatment
- 1% Triton X-100 treatment
- Differential centrifugation I
- Differential centrifugation II

Average no. of local lesions/leaf on C. amaranticolor
typical of TuMV were induced when material removed from the light scattering band was inoculated on C. amaranticolor and N. glutinosa. Light scattering band appeared reproducibly when the material removed from the light scattering zone was mixed with buffer, centrifuged at 40,000 rpm for 2 h in an angle head rotor to pellet the virus particles present and resuspended in 1 ml of phosphate buffer and layered on top of linear sucrose density column, and centrifuged for 90 min at 40,000 rpm in SW 50L rotor.

4.9 BUOYANT DENSITY

Virus particles in purified preparations banded as a single component with buoyant density of 1.32 g/cm³ when centrifuged to equilibrium in cesium chloride (CsCl) solution.

4.10 ANALYTICAL CENTRIFUGATION

Sedimentation studies of the purified virus preparation was carried out with a Beckman Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. Boundary position was measured by Nickon projection profile device. Sedimentation coefficient was calculated to be 118S. The virus sedimented as a single peak suggesting that the preparation was homogenous and contained a single type of virus particles (Fig. 7.2).
4.11 **UV-ABSORPTION SPECTRUM**

The purified virus preparation was examined in an UV-spectrophotometer. The virus band obtained after density gradient centrifugation was removed, mixed with EB and the virus pelletted by high speed centrifugation. Pellet was resuspended in EB and centrifuged for 10 min at 6,000 rpm. The supernatant was used after proper dilution to determine the UV-absorption spectra of TuMV. Information regarding the UV-absorption spectra of suitably diluted purified virus sample are presented in Table-2.4.

**Table-2.4: Information collected from UV-spectrum curve.**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A maximum</td>
<td>258 nm</td>
</tr>
<tr>
<td>A minimum</td>
<td>242 nm</td>
</tr>
<tr>
<td>A at 260 nm</td>
<td>0.39</td>
</tr>
<tr>
<td>A at 280 nm</td>
<td>0.31</td>
</tr>
<tr>
<td>A 260/280</td>
<td>1.25</td>
</tr>
<tr>
<td>A 280/260</td>
<td>0.79</td>
</tr>
<tr>
<td>Nucleic acid per cent</td>
<td></td>
</tr>
<tr>
<td>(Gibbs and Harrison, 1976)</td>
<td>ca. 5-6%</td>
</tr>
</tbody>
</table>

Purified preparation gave a UV spectrum (220 nm to 300 nm) typical of nucleoprotein (Fig. 7.3). Maximum and minimum absorbance was recorded at 258 and 242 nm, respectively. Amount of nucleic acid in the virus was calculated to be about 5-6% by
Fig. 7.3
UV-absorption spectrum of purified preparation of turnip mosaic virus.
interpolation of the observed data on A 260/280 ratio in the graph (Gibbs and Harrison, 1976).

4.12 MOLECULAR WEIGHT OF COAT PROTEIN SUBUNIT

SDS-electrophoresis of purified virus degraded by SDS and 2-mercaptoethanol showed one major band indicating a single type of protein subunit in the virus particles. The molecular weight of the protein subunit was estimated by interpolating Rf-value of the viral protein into a graph prepared by simultaneous run of the known molecular weight markers (Fig. 7.4). The molecular weight of protein subunit was calculated as ca. 25,000±500 daltons. The data were based on average of three separate determinations.

4.13 CHARACTERISTIC OF VIRAL NUCLEIC ACID

4.13.1 Isolation of viral nucleic acid

Nucleic acid from intact virus particles was isolated by procedure detailed in materials and methods. Positive results were obtained when nucleic acid was tested on C. amaranticolor. When the extracted viral nucleic acid was treated with pancreatic ribonuclease, no infection occurred whereas the controls were infective, suggesting that the infectious preparation was in fact that of RNA. Infectivity of isolated nucleic acid was compared with SI (standard inoculum) from young infected leaves of
FIG. 7.4

Estimation of molecular weight of coat protein subunit of turnip mosaic virus by SDS-PAGE.
*N. glutinosa*, on local lesion host, *C. amaranticolor*, on the basis of dilutions (Table-2.5).

Table-2.5: Infectivity of isolated nucleic acid in comparison to SI on the basis of dilutions.

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>Average number of local lesions/leaf*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SI (TuMV)</td>
</tr>
<tr>
<td>Undiluted</td>
<td>90</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>71</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>48</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>32</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>04</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>00</td>
</tr>
</tbody>
</table>

*Average number of local lesions/leaf based on 8 leaves of *C. amaranticolor*.

The results presented in the Table-2.5 reveal that the isolated nucleic acid was about 10% infectious as compared to the infectivity of SI (parent virus). The results are somewhat imprecise as the amount of actual virus and nucleic acid has not been taken into consideration.
4.13.2 Type of nucleic acid

The orcinol test gave a positive result as evident by the development of intense green colour in a mixture containing orcinol reagent and viral nucleic acid. Diphenylamine test which indicates the presence of DNA in sample was negative.

The positive orcinol test indicated the presence of RNA in the virion.

4.14 ELECTRON MICROSCOPY

Morphology of the virus particles

Samples removed from the visible infectious zone in density gradient tube were diluted with buffer and sucrose was removed by pelleting the virus by a high speed centrifugation. The pellet was resuspended in phosphate buffer pH 7.5 and the suspension clarified by centrifuging at 6,000 rpm for 10 min. The suspension was examined in a Phillips EM-420 electron microscope. Preparation negatively stained with 2% (w/v) uranyl acetate revealed the presence of flexuous rod shaped particles (Fig. 7.5), ca. 580 nm in length and 13 nm in width.

4.15 ULTRASTRUCTURAL STUDIES

Mesophyll cells of infected N. glutinosa leaves showed the following peculiar ultrastructural features (inclusion bodies):
1. Cytoplasmic inclusions containing bundles of virus particles and other cytoplasmic contents, 
2. Cytoplasmic laminar inclusion, and 
3. Cytoplasmic banded inclusions consisting of compact or loose aggregate of virus particles.

First type of inclusions were found near the nucleus and diameter of virus particles was 10.5 nm. The other cell contents in these inclusions were cytoplasmic ribosomes measuring 20-21 nm in diameter and vacuoles containing electron dense materials (Fig. 7.6).

Second type of inclusions were quite characteristic as they contained laminar sheeth of about 3-5 nm in thickness and studded on both sides with ribosome like bodies about 14 nm in diameter. They also contained vacuoles filled with fibrillous contents and few virus particles were also present near the laminar sheeth (Fig. 7.7).

The third type of inclusions were banded inclusions formed by orderly arrangement of intact virus particles layered with a periodicity. The periodicity was equal to about 520 nm (Fig. 7.8a,b).
4.16 SEROLOGY

4.16.1 Dip serology

4.16.1.1 Clumping or trapping of virus particles

A large number of flexuous rod shaped virus particles were trapped (Fig. 7.9) on grid coated with PVX-antiserum (potato virus X antiserum). The particles measured 490-580 nm in length and ca. 13 nm in width. The helical arrangement of proteins subunits could also be discerned (Fig. 7.9).

4.16.1.2 Decoration of virus particles (ISEM)

Virus particles were trapped on PVX-antiserum coated grids when incubated with PVX-antiserum showed a high degree of binding of antibodies with them (Fig. 8.0).

4.16.2 Tube precipitin test

Tube precipitation tests carried out with different combination of antigen and antiserum produced granular precipitate. With the help of this technique, various combination of two-fold dilution of antigen and antiserum revealed the antigen end point as 1:256 and antiserum end point as 1:512 (Table-2.6).
Table-2.6: Homologous reaction of antigen and antiserum in tube precipitin tests.

<table>
<thead>
<tr>
<th>Dilution of antigen</th>
<th>Intensity of tube precipitin in different dilutions of antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted</td>
</tr>
<tr>
<td>Undiluted</td>
<td>+3</td>
</tr>
<tr>
<td>1:1</td>
<td>+3</td>
</tr>
<tr>
<td>1:2</td>
<td>+3</td>
</tr>
<tr>
<td>1:4</td>
<td>+3</td>
</tr>
<tr>
<td>1:8</td>
<td>+3</td>
</tr>
<tr>
<td>1:16</td>
<td>+3</td>
</tr>
<tr>
<td>1:32</td>
<td>+3</td>
</tr>
<tr>
<td>1:64</td>
<td>+2</td>
</tr>
<tr>
<td>1:128</td>
<td>+</td>
</tr>
<tr>
<td>1:256</td>
<td>+</td>
</tr>
<tr>
<td>1:512</td>
<td>-</td>
</tr>
</tbody>
</table>

+3 Strong
+2 Moderate
+ Weak
- No ppt

Dilution end point of antiserum - 1:512
Dilution end point of antigen - 1:256
4.16.3 Chloroplast agglutination test

Chloroplast agglutination tests were performed on glass slides. Drops of antigen and antiserum were mixed on a glass slide and incubated for 15 min at room temperature (20±5°C). The chloroplast and cell components became readily and completely enmeshed in the net work of precipitate.

4.16.4 Ouchterlony gel double diffusion test

Double diffusion tests in plates were carried out using different combination of gel media to determine a suitable gel media for the formation of precipitin band of the virus isolate under investigation. The suitable gel medium consisted of phosphate buffer 0.1M, pH 7.5 containing 1% agarose, 1% SDS (sodium dodecyl sulphate) with 0.85% NaCl where visible and clear immunoprecipitin band was formed. Viral antigen (clarified sap from infected N. glutinosa leaves) prepared in phosphate buffer (0.1M, pH 7.5) with 0.85% (w/v) NaCl (1:1 v/v) showed best results in Ouchterlony gel double diffusion tests.

Purified virus preparation and crude sap from infected N. glutinosa plants reacted specifically with the antiserum prepared against the present virus in double diffusion tests. Fig. 8.1 shows the results of a test in which purified preparation of TuMV, sap from TuMV infected N. glutinosa plants and sap extracted from healthy N. glutinosa plants was set up in the wells
surrounding a central well containing TuMV antiserum. Precipitin line was formed between the antiserum well and the well containing purified virus preparation and also between the antiserum well and the well containing sap extracted from infected \textit{N. glutinosa} plants. No precipitin line was formed between the antiserum well and the well in which sap from healthy \textit{N. glutinosa} plants was set up.

Fig. 8.2 shows the results of a test in which antigen was tested against the antiserum of potato virus X (PVX). Sap from healthy as well as TuMV infected \textit{N. glutinosa} plants and purified preparation of TuMV were set up separately in wells surrounding a central well containing potato virus X (PVX) antiserum. Well defined precipitin curve was formed between the antiserum well and the well containing sap extracted from infected \textit{N. glutinosa} plants, and also between the antiserum well and the well containing purified virus preparation. No precipitin band was formed between the antiserum well and the well in which sap from healthy \textit{N. glutinosa} plants was set up.

The virus under study (TuMV) showed no serological reaction with antisera against the following flexuous viruses: potato virus Y, potato virus S, potato aucuba mosaic virus, cymbidium mosaic virus, narcissus mosaic virus, papaya mosaic virus and white clover mosaic virus.