MATERIALS AND METHODS
Chapter III

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3.1. Host and the inoculum:

The host plant for the research work is taken to *Vigna radiata* L. Wilczek [Syn. *Phaseolus aureus* Roxb (mung bean)]. The cultivated variety of mung bean is selected to Pant mung-I for experimental work.

The inoculum virus is mung bean mosaic virus (MBMV). All the experiments of the present study were carried out in an insect proof chamber which was regularly fumigated to maintain insect free conditions. Plants were grown in clay pots of the size 30 cm containing mixture of sterilized sand-loam and compost (1:1:2) and were watered uniformly and regularly.

The virus MBMV was isolated from naturally infected mung bean plant (*Vigna radiata* L.) Wilczek (Syn. *Phaseolus aureus* Roxb) growing in the field. Stock culture of mung bean mosaic virus maintained on mung bean cultivated variety Pant mung-I in the insect proof chamber, where no other host susceptible to these virus was grown. The virus culture was transferred to young and healthy mung bean cv. Pant mung-I at frequent inoculation.
3.2. **Preparation of virus inoculum:**

The virus inoculums was prepared by macerating the leaf tissue showing distinct symptoms in a sterilized mortar and pastle with 0.05 M phosphate buffer (Ph 7.0) at the rate of one ml per gram of tissue. The juice was passed through muslin cloth and the filtrate was used as the inoculum in all the experiments.

The inoculation was made by gently rubbing on the upper surface of the primary leaves of the plant with the help of finger dipped in infective sap. During inoculation, care was taken to ensure uniform pressure and spread of inoculum. Leaves was slightly dusted with 600 mesh carborundum powder. Prior to inoculation, the inoculated leaves are washed immediately with a jet of distilled water. The control plants were treated similarly, using the neutral phosphate buffer solution only.

Seeds of different species of legumes was obtained from “Narendra Dev University of Agriculture and Technology, Kumarganj Faizabad”, “Hind seed company, Faizabad” and “Americal certified seed company, Faizabad”. Before sowing, seeds were treated with 0.1 per cent $H_2Cl_2$ for 2 minutes and washed thrice with tap water and dried in shade.

While, studying the host-range of the virus, attempts were made to recover the virus from inoculated plants. Usually, the inoculum was taken from young leaves but in the absence of apparent symptoms older leaves were also used. For verifying the presence of viruses in all suspects and systematically infected plants, mechanical inoculation were made to test plants (mung bean cv. Pant mung-1)
All the glasswares required for determining the properties of virus isolates were sterilized before use. The condition of insectory and the methods of culturing and handling the insects was as same described by Watson (1936, 1938 and 1972)

3.3. EXPERIMENTAL PLAN

During experimental period, cultivated variety of mung bean i.e., Pant mung-1 as host plant and virus isolate mung bean mosaic virus for systemic multiplication was taken. Test plant used for different experiments were grown in 30 cm clay pots having sand, loam, compost mixture (1:1:2). Each experiment was repeated thrice and average are given in the results.

For study the effect of virus infection on different physiological aspects of nodulation, at different periods of infections, seven days old mung bean cv. Pant mung-1 seedlings of the first group (100 seedlings) were inoculated with neutral phosphate buffer to serve as the healthy control. While, those of second group were inoculated with mung bean mosaic virus (MBMV).

The plants of each group was harvested at 15th 30th 45th 60th 75th days of inoculation at the rate of 20 plants per harvest. Out of 20 plants of each harvest, one half (10 plants) were harvested in the morning. (8o0 to 100 1ST). The harvested samples were used for studying nodulation, leg-haemoglobin and nitrate–reductase activity. The remaining half (10 plants) was used for studying the different physiological aspects of the nodules by the following methods ——
3.3.1. **Nodulation:**

The soil and compost mixture in which seedlings was grown was tested for presence of *Rhizobia*. Therefore, seed were not artificially inoculated with *Rhizobia* because the soil mixture was already infected with them. Mung bean plants were uprooted carefully to avoid damaging of root system. Nodules from each plant were collected by removing the soil in running water through 40 mesh screen and collecting the root nodules from the screen. Those nodules adhering with roots are picked up individually with the help of sterilized forceps.

Collected nodules are surface dried with filter papers, sealed in small polythene bag and stored at 4°C. Nodules from each group of plants were counted, weighted and their volume estimated to known the size. For dry weight, nodules was kept in an oven maintained at 80 ± 5°C for 24 hours.

3.3.2. **Nitrate-reductase enzymes (NR):**

The nitrate-reductase enzymes was measured by the method described by Srivastava (1974). 500 mg of fresh weight plant sample (of the same age) from both healthy and infected was taken. The sample was incubated separately in test tubes containing incubation mixture in dark at 30°C. The incubation mixture of each test tube contains 8.0 ml of phosphate buffer (PH = 7.0), One ml of 0.1 M potassium nitrate and 1.0 ml 5 per cent normal propanol.

After 30 minutes, 1.0 ml of incubation mixture was taken and 1.0 ml each of sulphonamide (1 per cent sulphonamide in N-HCl) and
2-Naphthyl ethylene-diamine-dihydrochloride (0.02 per cent) were added and the optical density was read after 10 min. at 540 nm in Beckman spectrophotometer. After words the incubated material was crushed in distilled water and the volume was made to 50 ml before centrifuging it at 10,000 rpm. To the 1.0 ml of cleared supernatant, 1.0 ml of sulphonamide and 1.0 ml of 2-naphthyle ethylene-diamine-dihydrochloride solution was added and OD read as above.

The total NO₂ produced per hour per gram fresh weight of the sample calculated by adding the NO₂ yield of two solutions which determined by matching the two optical density readings with a standard curve prepared with different dilution of sodium nitrate solution.

3.3.3. **Leg-haemoglobin:**

To estimate leg-haemoglobin from the nodules, procedure described by Tu et al. (1970 b), was followed. Nodules washed several times with distilled water to remove the soil and then surface dried with filter papers and then packed in polythene bag, frozen before extractions of leg-haemoglobin. One gram of nodule was homogenised in 5 ml of 0.1 N-KOH and it centrifuged for 10 minutes at 12,000 rpm. To 1.5 ml of supernatant, 1.0 ml water and 0.5 ml 5N-KOH was mixed with 0.1 gram of Na₂S₂O₄ for reduction. Optical density of this solution was taken just after 10 minute at 537, 557 and 577 nm by using Beckman Spectro-photometer. The OD for leg-haemoglobin is calculated by the following formula-

\[
\text{OD leg-haemoglobin} = \frac{\text{OD}_{557} - \frac{1}{2} (\text{OD}_{537} + \text{OD}_{577})}{\text{OD}_{557}}
\]
3.3.4. **Water soluble reducing sugar:**

Anthrone colorimetric method as described by Snell and Snell (1961) was followed to determine the reducing sugar content of the plant sample. 50 mg of dried sample was homogensied with 10 ml of distilled water. After adding activated charcoal for clearing, the homogenate then centrifuged and supernatant was filtered through Whatman filter paper -No 1. To 2 ml of filtrate, 0.5 ml of 2 per cent anthrone reagent (2 g anthrone, recrystallised from benzene in 100 ml of ethyl acetate) added in the test tube. Subsequently, 5 ml of concentrated H₂SO₄ (AR) was carefully layered and tube was swirled gently.

The colour intensity of the product noted after 10 minutes in absorptiometer at 620 nm. The amount of sugar was determined from standard curved prepared with glucose (AR) and expressed as mg / 100 mg dry weight of the sample.

3.3.5. **Water soluble non-reducing sugars:**

The non-reducing sugars was measured by inverting them to reducing sugars by the method of Somogyi (1952). In 10 ml of the filtrate (as obtained in the case of reducing sugars) 5 ml of 0.5N – HCl was added. The content was digested in a boiling waterbath to half an hours. The flask then cooled and the content neutralized with 0.5 NaOH using a drop of methyl red as indicator. The solution was made slightly acidic by adding a drop of acetic acid and the volume made to 25 ml with distilled water. Sugar content of this solution was estimated as described for reducing sugars and the amount of inverted sugars calculated by subtraction. This was multiplied by
the factor 0.93 to obtained the amount of non-reducing sugar present in the sample.

3.3.6. **Total water soluble sugars:**

The sum of amount of reducing and non-reducing sugars gave the value for total water soluble sugars.

3.3.7. **Starch content:**

The starch content was determined according to the method described by Snell and Snell (1961). The dried sample (50 mg) was homogenized with 10 ml of 40 per cent perchloric acid. After mixing charcoal for clearing, the homogenate, centrifuged and filtered through Whatman filter paper No-1.

To 2 ml of the filtrate, 10 ml of anthrone reagent (1 g of anthrone dissolved in 700 ml of concentrated H$_2$SO$_4$ and diluted to 1 litre) was added. This was treated at 100°C for 12 min. and cooled rapidly to room temperature. The colour intensity of the solutions was measured with a photoelectric colorimeter at 630 nm and compared with that of glucose standard. For obtaining quantitative value of starch, a conversion factor (0.9) was used, based on the fact that 0.9gm of starch yield approximately 1gm glucose on hydrolysis.

3.3.8. **Total nitrogen:**

The amount of total nitrogen was determined from dried plant material digested by micro-method of Doneen (1932). To 100 ml of plant sample in a test tube (2.5x20cm), one ml of nitrogen free concentrated H$_2$SO$_4$ containing 10 g salicylic acid per 20 ml was added. After through
mixing, it then allowed to stand for 10 minutes; To this mixture, 0.3 g. of sodium thio-sulphate added and heated gently until fumes appeared at the mouth of test tube. It then cooled and 1.0 ml of 60 per cent perchloric acid, containing 0.5 g CuSO₄ per 500 ml was added.

The mixture was digested by heating very gently until foaming ceased. The cleared mixture was further heated for 2 minutes. The digest was then transferred to a 100 ml volumetric flask by washing with double distilled water for several times and the final volume was made to 100 ml. To 1.0 ml of this solution in a test tube (1x10 cm), 9.0 ml of double distilled water and 1.0 ml of Nessler’s reagent, as modified by Jackson (1949) was added. The colour intensity measured in Hilger pattern Biochem. Absorptiometer at 430 nm, a standard solution of ammonium sulphate (AR) was taken, and treated similarly and its colour intensity measured at the same wave length. Total nitrogen calculated by the following formula.

\[
\text{Reading of Unknown } \times \text{ Factor} = \frac{\text{Conc. of Standard}}{\text{Reading of Standard}}
\]

3.3.9. **Total protein:**

The oven dried plant sample (100 mg) homogenized with 10 per cent trichloro acetic acid in a mortar. The homogenate was centrifuged at 1500 rpm and precipitate placed in an oven at 70 °C for drying. The total nitrogen present in the dried precipitate was determined by the aforesaid micro method of Doneen (1932). The protein content of the material calculated by multiplying this value of the total nitrogen by factor 6.25.
3.3.10. **Nitrate – nitrogen:**

The nitrate–nitrogen of the plant tissues estimated by the method Humphries (1956). Nine ml of 0.35 per cent silver sulphate solution was added to 100 mg of finely powdered plant material in a 50 ml beaker. The beaker was swirled around quickly and immediately after word 1 ml of 13.8 per cent sodium phosphate solution (pH–6.5) then added into it. In order to precipitate the excess silver, the mixture was allowed to stand for 2 hours to extract nitrate and subsequently filtered through whatman filter paper No-1. Two ml of the filtrate was taken in a 15 ml centrifuge tube to which 2 ml of 0.5 per cent copper sulphate solution, 2 ml of water and 0.59 g of calcium hydroxide and magnesium carbonate mixture (1:2) were added. This centrifuge after keeping for an hour. Two ml of the supernatant was taken in a 25 ml beaker and evaporated to dryness in a water bath.

After cooling, 1 ml of phenol-disulphonic acid was added into a beaker quickly, making certain that all the residue comes into immediate contact with the acid. After keeping for 10 min. approximately 10 ml water and an excess of 50 per cent ammonium hydroxide solution was added. The final volume made upto 25 ml and the colour compared with that of standards in a photoelectric colorimeter at 430 nm.

The standard made by preparing 0, 1, 2 and 3 ml of standard potassium nitrate solution (1 ml = 0.07 mg N as nitrate) into separate centrifuge tube, making each to 4.0 ml. CuSO₄ solution (0.5 per cent) and 0.59g Ca(OH)₂ – MgCO₃ mixture (1:2) added to the tube and the mixture was treated as aforesaid.
3.3.11. **Nitrite-nitrogen:**

Nitrite-nitrogen was estimated by Humphrie’s (1956) method. According to this method 100 mg powdered plant material extracted with 10 ml distilled water in a water bath at 40°C for 40 min. and filtered. Two ml of filtrate taken in a 50 ml of volumetric flask and after adding 40 ml water and 2 ml of (1:1) sulphonamide acid solution (3:3g sulphanilic acid dissolved in a mixture of 750 ml water and 250 ml glacial acetic acid) and Nephthylamine solution (0.5g naphthylamine in 100 ml boiled water; diluted to 1 litre containing 250 ml glacial acetic acid), the final volume was made upto 50 ml. It allowed to stand for 30 min. before comparing the colour with standard in photoelectric colorimeter at 520 nm. The standard solution was made with sodium nitrate (AR) and treated as aforesaid for colour development.

3.3.12. **Ammonical nitrogen:**

20 ml of dried and powdered plant material extracted with 10 ml of 10 per cent H₂SO₄ in a water bath at 100°C for 15 minutes. Known aliquots of the solution was taken to estimate ammonical nitrogen by the method of Stroganov (1964). Before distillation of ammonia from the sample, the apparatus was free from NH₃. For this purpose, a small amount of magnesium oxide was added to 100 ml of tap water which distilled till approximately 1/3 of water remained in the flask. Now 25 ml of sample poured into the flask and distilled. When the volume of distillate reached 25 ml marked in the receiver; it was removed and stoppered. Nitrogen content of the distillate was estimated by Nesslerization as described earlier for total nitrogen.
3.3.13. **Total free amino acids:**

It was determined by colorimetric method of Wiggins and Williams (1955). 50 mg dried sample was homogenized with 10 ml of 80 per cent ethanol. After mixing activated charcoal for clearing, the homogenate centrifuged and filtered through whatman filter paper No-1. The filtrate (1.0 ml), after adding 2.5 ml of 1 per cent ninhydrin solution (In isopropanol) and 2.5 ml of acetate buffer (pH-5.4), was heated in a boiling water bath for 30 minutes, then cooled to room temperature. After cooling 5 ml aqueous isopropanol (1:1) was added and mixed thoroughly. Absorbance of the inoculum measured in a Hilger pattern Biochem, Absorptiometer at 580 nm. The amount of free amino acids was determined from standard curve prepared with leucine and expressed as mg / 100 mg dry weight.

3.3.14. **Total phosphorus:**

The method as described by Humphries (1956) was followed to estimate the total phosphorus in plant samples in the term of phosphate.

Dry powdered plant material (50 mg) taken in a silica basin and 5 ml of 0.1 N magnesium nitrate solution added to it. It was thoroughly mixed by a glass rod which finally washed with a few drop of water. The mixture evaporated to dryness on a sand bath and ignited at 500°C in a muffle furnace for 20 min. to a light gray or white ash. After cooling, 10 N sulphuric acid (10ml) was slowly added washing down the ash to the centre of the basin. This was digested gently on a water bath for 15 min. breaking the ash with a glass rod. After cooling, it was diluted with 20 ml water and filtered through whatman filter paper No. 1. The filter paper was washed with hot water and the volume of the filtrate made to 100 ml, 15 ml of this
solution taken in a 50 ml volumetric flask and 1 ml of 10 N sulphuric acid was added to it. 1.0 ml of ammonium molybdate solution (8.3 per cent) and distilled water added to make the volume approximately 45 ml. This was shaken well and 1.0 ml of 1:200 dilution with water of 40 per cent stanus chloride solution in concentrated HCl was added. Again, after shaking, water added quickly to make the final volume to 50 ml. The solution allowed to stand for 20 min. before measuring colour intensity. The standard phosphate solution was also treated similarly and the colours of the standard and unknown solution compared in a photoelectric colorimeter at 600 nm.

3.3.15. Inorganic phosphorus:

It was estimated in term of phosphate by the method described by Snell and Snell (1954). 50 mg of dried sample homogenized with 10 ml of distilled water and after clearing with activated charcoal, it was filtered. To 5.0 ml of filtrate, 1 ml of 1:2:6 H₂SO₄, then added. In addition, 1.0 ml of 4 per cent Na₂SO₄ solution and 1.0 ml 7 per cent sodium molybdate solutions were also added. After mixing by inversion, 1 ml of 1:200 dilution with water of 40 per cent stanous chloride solution in concentrated HCl was added. This solution then diluted to 10 ml with water and mixed. After 20 minutes, absorbance read at 540 nm against a reagent blank in a colorimeter. The amount of inorganic phosphorus obtained with the help of phosphate standard.

3.3.16. Organic phosphorus:

The amount of organic phosphorus was calculated by deducting the amount of inorganic phosphorus from that of the total phosphorus.
3.3.17. **Bacterial population:**

Nodules and soil bacteria (*Rhizobium*), cultured on mannitol-agar medium. The composition of the medium was as follows:

- **Mannitol** 15.0 g
- **K$_2$HPO$_4$** 0.4 g
- **Mg SO$_4$** 0.2 g
- **NaCl** 0.2 g
- **CaCO$_3$** 3.0 g
- **Agar** 15.0 g
- **Yeast extract with water** 100 ml
- **Distilled water** 1000 ml

The pH of medium maintained at 7.0. The medium and distilled water used were sterilized under 15 lb pressure and 121$^\circ$C temperature for 15 min. The glassware sterilized for 2 hours at 170$^\circ$C to 180$^\circ$C in a hot air oven.

Fresh nodule samples which sterilized as above, was washed thoroughly with distilled water. 100 mg of nodule sample homogenized with 100 ml distilled water in a mortar. This was further diluted with 100 times and 1.0 ml of diluted suspension transferred in each of 5 petridishes (10 cm) before pouring the medium into them. Culture was incubated at 25$^\circ$C for 24 hours. Bacterial colonies was then counted and their population calculated as the number of bacterial colonies per gram of nodule.

The bacterial population of the pot soil was estimated just after each harvest. Care was taken to remove all the nodules from the pot soil. The soil was taken from pots, mixed thoroughly and only 100 mg was processed
for bacterial population. The bacterial population of the soil also cultured by
dilution method as described above.

3.3.18. Soil nitrogen:

The soil nitrogen was estimated by procedure described by Mishra (1968). 10 g of dried soil sample obtained from pots and passed through 100
mesh sieve, taken into a 300 ml Kjeldahl flask and moistened with 25 ml
distilled water. The flask was allowed to stand for 30 min. and then 20 g of
sodium sulphate-plus-catalyst mixture (i.e, 20g of CuSO₄, 3 g MgO and 1 g
of Se powder mixed with 20 parts of anhydrous sodium sulphate and mixture
is labelled as sodium sulphate catalyst) mixed with 35 ml of concentrated
H₂SO₄. To mixed the content, the flask was gently swirled and digestion at
low heat was started. After 10 to 30 min., when frothing stopped, the heat
was raised till the acid boiled and condensed approximately one third way up
the neck of flask. The digestion was continued at the same heat and the flask
was rotated at intervals until the organic matter was destroyed and the digest
become light yellow. Heating stopped after completion of digestion and the
flask then cooled. To cooled digest, 100 ml of double distilled was added,
mixed well and allowed the flask to stand for 3 to 4 min. The supernatant
liquid to flask was transferred to a 80 ml flask capacity.

Taking care leave behind as much as possible. The residue of flask
washed 4-5 or more times with 50 to 60 ml double distilled water each time
and the flask filled till the mark. The supernatant was filtered through a
Buchner funnel fitted with whatman No-42 filter paper. The filtrate
collected in a beaker and the nitrogen content of this solution was estimated
by Nesslerization as described earlier for total nitrogen.