Chapter- 3

MATERIALS AND METHODS
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The different materials used and the methods employed during the course of proposed experimental programme are generalized as follows:

Selection of test plant:

*Cicer arietinum* Linn. var. Rachna (Chickpea), *Vigna radiata* Linn. Var. Soona Mung (Green gram) and *Pisum sativum* Linn. var. Harbhajan (Pea) were used as the test plants. The seeds of the above mentioned plants were obtained from National Seed corporation (NSC), New Delhi, India.

Selection of Test Pathogen:

The root-knot nematode, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 was selected as test pathogen.

Preparation and Sterilization of Soil Mixture:

A mixture of sandy-loam soil and compost was prepared in the ratio of 3:1 (soil: manure). This soil mixture was filled in 15 cm earthen pots at the rate of 1kg per pots. The soils in each pot were moistened with sufficient water before sterilization. Later pots with soil were sterilized in an autoclave at 15 lbs pressure for 20 minutes.
Raising and Maintenance of Chickpea, Green gram and Pea seedlings:

Seeds were surface sterilized with mercuric chloride (0.1%) and washed thrice with sterile distilled water. Surface sterilize seeds of chickpea, green gram and pea were sown in pots having sterilized soils @ three seeds per pot each. At three-leaf stage, extra plants were removed and one plant per pot was kept as test plant. Watering was done when required.

Raising and Maintenance of Nematode Inoculum:

Single egg mass obtained from eggplant (aubergine) roots infected with *Meloidogyne incognita* was surface sterilized with chlorox (calcium hypochloride) for 5 minutes and then washed thrice in sterilized distilled water. The egg mass was then allowed to hatch in sterilized distilled water at 27°C. The second stage juvenile (J2) thus obtained were first identified as *Meloidogyne incognita* and then used for further inoculation of eggplants to maintain pure culture and regular supply for experimental work. For counting 5 ml suspension of larvae were placed in counting dish and counted under stereoscopic microscope (Southy, 1970).

Preparation of Plant Extracts:

The ten plants namely *Abutilon indicum* Linn., *Alternanthera philoxeroides* (Mart.) Griseb., *Eclipta alba* Linn., *Euphorbia hirta* Linn., *Launaea nudicaulis* Hool., *Lindenber gia indica* Linn., *Pluchea lanceolata* Oliv., *Ranunculus sceleratus* Linn., *Stellaria media* (Linn.) Vill., *Tridax procumbens* Linn., *Tagetes erecta* L. and *Azadirachta indica* A. Juss. were collected from around the University campus of AMU., Aligarh. Leaves of different plants @ 20
g and 30 g each separately, were washed with distilled water, chopped and macerated in grinder to have a paste. Then paste was mixed with enough distilled water to make it 100 ml, filtered through four-ply muslin cloth. The prepared solution served as stock solution (S) for treatment of three host plants.

To test the mortality and hatching of the root-knot nematode the paste of 100g leaves of different plants mixed with distilled water to make it 100ml. The prepared solution served as stock solution. Further dilutions i.e. S/2, S/10, S/100 and S/1000 were prepared from stock solution (S).

Preparation of different concentrations:

From the above stock solution (S) of 20g and 30g the following concentrations were prepared.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Extract</th>
<th>Distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>1ml (S)</td>
<td>--</td>
</tr>
<tr>
<td>S/2</td>
<td>1ml (S)</td>
<td>1ml</td>
</tr>
<tr>
<td>S/10</td>
<td>1ml (S)</td>
<td>9ml</td>
</tr>
<tr>
<td>S/100</td>
<td>1ml (S/10)</td>
<td>9ml</td>
</tr>
<tr>
<td>S/1000</td>
<td>1ml (S/100)</td>
<td>9ml</td>
</tr>
</tbody>
</table>

Mortality:

Egg masses of root-knot nematode, *Meloidogyne incognita* were kept for hatching in coarse sieves (1mm) having tissue paper and placed in petri dish containing sterilized distilled water just touching its lower side. Freshly hatched larvae were collected in a beaker daily for three days.
Five ml of aqueous suspension containing about 1000 freshly hatched larvae of *Meloidogyne incognita* were poured over 1cm diameter sieves of 350-meshes/ linear inch (Alam et al, 1973). The sieves were inverted over petri dish of 40mm diameter and the nematodes washed down with the help of 5 ml plant extract of different plants separately.

Petri dishes containing sterilized distilled water alone served as control. There were three replicates for each treatment. The petri dishes were sealed with petroleum jelly and kept at 28°C temperature. Numbers of mobile and immobile larvae were counted after 12, 24, 48 and 72 hours of exposure. The death of larvae was ascertained by transferring the immobilized larvae into water for 1 hour and percent mortality was calculated.

**Hatching:**

Egg masses were taken from thoroughly washed roots of eggplant infected with the root-knot nematode, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949. Five egg masses of an average size were kept in 40mm petri dishes containing 5 ml solution of different concentration of leaf extracts separately. Petri dishes containing sterilized distilled water only serve as control. There were three replicates for each treatment. Petri dishes were sealed with white petroleum jelly and were left for 48 hours at 28°C temperature. The total numbers of hatched larvae were counted with the help of counting dish under stereoscopic microscope.
Preparation of dry leaves:

The leaves of *Ranunculus sceleratus*, *Tagetes erecta* and *Azadirachta indica* were collected from the university campus of A.M.U., Aligarh. The fresh leaves were washed thoroughly with distilled water and shade dried for the period of three weeks and grind in a mixer grinder into a powdered form. The powder samples were stored at room temperature for further analysis.

Preparation of cold and hot aqueous extract of dry leaves:

20g and 30g powder sample of *Ranunculus sceleratus*, *Tagetes erecta* and *Azadirachta indica* were dissolved in distilled water at the rate of 20g/100ml of distilled water and 30g/100ml of distilled water for one night in room temperature to obtained cold aqueous extracts. Whereas for hot aqueous extracts the conical flasks were kept on the water bath for $\frac{1}{2}$ an hours and cool it down. The extracts so prepared were squeezed through muslin cloth and Whatman’s filter paper No. 1. The extracts so obtained were put in conical flask and stored in a refrigerator, used as stock solution (S). From stock solution (S) further dilutions were prepared.

Preparation of ethyl acetate, chloroform and butanol extract of dry leaves:

20g and 30g dry leaves powder was dissolved in 100ml of ethyl acetate, chloroform and butanol separately in a conical flask. Then all these solution were transferred into the separating funnel separately and shaked the separating funnel vigorously for 30min.

Later the ethyl acetate, chloroform and butanol fractions were taken out. This process was repeated twice to have pure ethyl acetate, chloroform and butanol
fractions. These fractions were dried under vacuum dryer and obtained residues. These residues were dissolved separately in 100ml of distilled water and served as stock solution (S) for further test.

**Preparation of Carbofuran treatment:**

To test the mortality and hatching of root-knot nematode, *Meloidogyne incognita*, 1g of carbofuran was mixed with 100ml of distilled water. The prepared solution serves as stock solution (S). Further, dilutions i.e. S/2, S/10, S/100 and S/1000 were prepared from the above solution.

**Inoculation Technique of Carbofuran:**

Different doses of carbofuran @ 1g a.i./kg of soil and 1.5 g a.i./kg of soil were applied after a week of nematode inoculation.

**Inoculation Techniques of aqueous leaf extracts of different plants:**

The three weeks old seedling (three leaf stage) of chickpea, green gram and pea were inoculated with 1000 (one thousand) freshly hatched second stage juveniles (J2) of *Meloidogyne incognita*. Nematode inoculum was poured uniformly all around the roots by exposing them. After inoculation the exposed roots were covered by leveling the soil properly.

*erecta* L. and *Azadirachta indica* A. Juss. were poured around the roots of chickpea, green gram and pea seedling @ 20g and 30g per plant.

**Determination of Plant Growth:**

The plants were harvested 60 days after inoculation. Root system was gently washed with tap water taking utmost care to avoid loss or injury to the root system during the complete operation. The excess water of plant was removed by putting them between the folds of blotting sheets.

The plants were cut with a sharp knife just above the base of the root emergence zone. Fresh weight of the root and shoot of all the replicates was taken in gram. Root and shoot length of plants was measured with the help of meter scale. After taking fresh weight of roots and the shoots, these were kept in bamboo envelopes and placed in an incubator for 48 hours at 80°C to obtain their dry weights.

**Root nodule estimation:**

Nodules were estimated by counting the number of nodules per root system and percentage of nodulation reduction was calculated.
**Determination of Nematode population:**

The final population of the root-knot nematode in the soil and root tissues was determined (Christii, 1946).

Root-knot index was recorded as under

1 = No galls no egg masses.
2 = 1-10 galls / egg masses
3 = 11-30 galls / egg masses
4 = 31-100 galls / egg masses
5 = 101 and above galls / egg masses.

**Nematode Population Estimation:**

For extraction of nematodes from the soil 250 g sub samples of well mixed soil from each treatment was processed through Cobb’s sieving and decanting method followed by modified Bearman funnel technique (Southey, 1986). The nematode suspension was collected after 24 hours and number of nematodes was counted in counting dish by taking 2ml suspension from each sample under the stereoscopic microscop. Mean of the three such samples was obtained and the population of nematodes per kg soil was calculated.

For estimation of nematode population in roots, 1.0gm of root from each replicated treatment was macerated for 45 seconds in an electrically operating wearing blender in enough water. Counting was done from the suspension thus obtained as described above. The total final population was obtained by adding the soil as well as root population and the reproduction factor (R/f) was calculated by the formula of Oostenbrink (1966) as follows:
\[ R_f = \frac{P_f}{P_i} \]

Where \( P_f \) represent the final population and \( P_i \) represent the initial population of the nematodes.

**Estimation of Chlorophyll:**

For estimation of chlorophyll contents, 1g fresh leaf pieces were crushed in mortar and pestle containing 50 ml of 80% acetone. The crushed material was filtered through Whatman’s (No.2) filter paper. The filtrate was transferred to 100ml volumetric flask and the volume was made up to the mark with 80% acetone. The transmittance was read at 645 and 663nm on ‘Spectronic 20’ colorimeter. The amount of chlorophyll a, b, and total chlorophyll was determined as mg/g fresh leaf according to the formula given by Mac Lachlan and Zalik (1963).

**Estimation of Protein:**

Protein content in the leaves was estimated by the method of Lowry et al (1951). Preparation of Reagent: Six reagents were needed for estimation of protein i.e. A, B, C, D, E and F.

**Reagent A:** Equal quantity of (1:1) 2% \( \text{Na}_2\text{CO}_3 \) and 0.1N NaOH prepared in double distilled water.

**Reagent B:** 0.5% CuSO\(_4\) + 1% sodium tartarate in 1:1 ratio prepared in double distilled water.

**Reagent C:** 50 ml of reagent A + 1ml of reagent B.

**Reagent D:** Reagent B + \( \text{Na}_2\text{CO}_3 \) (1:1 ratio)
Reagent E: Folin Phenol Reagent.

Reagent F: 40g of NaOH dissolved in one litre of double distilled water to mark 1N-NaOH solution.

For extraction of soluble and insoluble protein, 50mg of dried sample was taken. Then it was crushed in 10-15ml of phosphate buffer and filtered through muslin cloth. It was transferred to a centrifuge tube with repeated washing and volume was made up to 5ml with double distilled water. The extract was then centrifuged at 4,000 rpm for 5 minutes and the supernatant was collected for soluble protein.

Estimation of soluble Protein:

For the estimation of soluble protein 1ml of supernatant was transferred to a 10ml test tube and 5ml of reagent C was added. The solution was mixed well and allowed to stand for 10 minutes at room temperature. 0.5ml of reagent E was added rapidly with immediate mixing. After 30 minutes, the blue coloured solution appeared. This solution was transferred to a colorimetric tube and its intensity was measured by reading its optical density (O.D) at 660nm in ‘Spectronic 20’ colorimeter. A blank was run simultaneously.

Estimation of Insoluble Protein:

To the residue of 5 ml of 5% trichloroacetic acid was added. The solution was allowed to stand at room temperature for 30 minutes after thorough shaking. It was then centrifuged at 4,000 rpm for 10 minutes and the supernatant was discarded. 5ml of 1N NaOH was added to the residue and mixed well by
shaking. The residue was allowed to stand in water bath at 80°C for 30 minutes. Then it was cooled and centrifuged at 4000 rpm. The supernatant together with three washing with 1N NaOH, was collected in a 25ml volumetric flask with 1N NaOH. One ml of NaOH extract was transferred to a 10 ml test tube and 5 ml of reagent E was added rapidly with immediate mixing. After 30 minutes the intensity of the blue solution was measured at 660nm using a ‘Spectronic 20’ colorimeter.

**Standard for Protein:**

40mg of egg albumin was taken in a 100ml of volumetric flask, to which 1-2ml of 0.1N NaOH was added. The contents of flask were stirred carefully and placed on a water bath for a short period for heating. After the albumin became solubilized the volume of the flask was made up to the mark by double distilled water. From this solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 ml was pipetted to ten different test tubes, the solution in each test tube diluted to 1ml by adding 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.0ml of double distilled water respectively. In each test tube 5ml of reagent C was mixed and allowed to stand for 10 minutes at room temperature. 0.5ml of reagent E was then added rapidly with immediate mixing. The optical density of the solution was read at 660nm using a ‘Spectronic 20’ colorimeter. A blank was also run simultaneously.

**Statistical analysis:**

The data obtained were analysed statistically and significance of variance was calculated at P<0.05 and P<0.01 levels.