Chapter-3

Materials
And
Methods
MATERIALS AND METHODS

The root-knot nematode *Meloidogyne incognita* and the root-rot fungus, *Rhizoctonia solani* were selected as test pathogens and purple false eranthemum, *Pseuderanthemum atropurpureum* was used as a test plant during the course of investigation. The different materials used and the methods employed for the proposed experimental programmes are generalized as follows:

3.1. PREPARATION AND STERILIZATION OF SOIL MIXTURE:

Sandy loam soil (sand 71%, silt 21%, clay 8% and pH 7.3) which is commonly found in Aligarh was collected from a fallow field of Aligarh Muslim University Farm. The soil was then passed through a 16 mesh sieve to remove stone particles and debris etc. Soil and farmyard manure (FYM) in the ratio of 3:1 were thoroughly mixed and clay pots (25 cm top diameter) were filled with this soil @ 4 kg / pot. A little amount of water was poured in each pot to just wet the soil before proceeding for sterilization in autoclave at 20 lb pressure for 20 minutes. Sterilized pots were allowed to cool down at room temperature before they were used for experiments.

3.2. RAISING AND MAINTENANCE OF TEST PLANT:

The test plant, purple false eranthemum (*P. atropurpureum*) vegetatively propagated by stem cuttings having 3-4 pairs of leaves were planted in the month of March in large sized earthen pots, containing sterilized soil mixture to develop roots and raise the cuttings. When the cuttings were about 6 week old and have produced sufficient roots, they were ready for transplantation. Thereafter, these well established and healthy rooted cuttings were transplanted singly in clay pots (25 cm top diameter) containing 4 kg sterilized soil mixture. These cuttings were then used for experimental purposes throughout the course of investigation.

3.3. RAISING AND MAINTENANCE OF PURE CULTURE OF ROOT-KNOT NEMATODE *MELOIDOGYNE INCognITA RACE-3*:

The pure culture of *Meloidogyne incognita* Race-3 was raised on tomato (*Lycopersicon esculentum* cv. Pusa Ruby) using a single egg mass collected from
infected plant of *P. atropurpureum*. The egg mass was surface sterilized by treating it with 0.1% aqueous solution of calcium hypochlorite for about two minutes. Treated egg mass was washed five times in sterilized distilled water. The eggs, in the egg mass were allowed to hatch out at 28 ± 2 °C on a coarse sieve (16 mesh), layered with tissue paper and kept in a Petri dish (10 cm diameter) containing sufficient amount of sterilized distilled water to obtain second stage juveniles (J2) of root-knot nematode.

Three week old seedling of tomato cv. Pusa Ruby transplanted in clay pots, containing sterilized soil were inoculated with the second stage juveniles (J2) of *M. incognita* Race-3.

After six weeks, some amount of soil from near the root zone and roots of inoculated plant were examined separately to confirm the establishment and multiplication of root-knot nematode. After three months, the plant was cut at the ground level and the root-knot nematode (J2) was extracted from the pot soil according to Cobb’s decanting and sieving method followed by Baermann’s funnel technique (Southey, 1986). The second stage juveniles of root-knot nematode were also obtained from the egg masses collected from the galled roots of tomato. Nematodes so obtained were used for inoculation of fresh tomato seedlings grown in several clay pots containing sterilized soil. The juveniles of root-knot nematode infected the root and multiplied there on tomato plants. Similarly, tomato seedlings were further inoculated from time to time in order to maintain a regular supply of nematode inoculum. Culture of *M. incognita* Race-3 multiplied and maintained in this way was, thereafter, used for obtaining required inoculum.

### 3.4. PREPARATION OF INOCULUM OF ROOT-KNOT NEMATODE:

After 2-3 months, the egg masses from heavily infected roots of tomato on which pure culture of *M. incognita* Race-3 multiplied were handpicked with the help of sterilized forcep. These egg masses, after being washed in distilled water, were placed on sieve layered with tissue paper. The sieve was placed over Petri dish (10 cm in diameter) containing water. The water level was kept in such a way that it just touched the lower portion of the sieve having egg masses. A series of such assemblies were kept to obtain large number of second stage juveniles required for inoculations. After every 24 hours, the hatched out juveniles (J2) were collected along with water from the Petri dish in a beaker and fresh water was added to the Petri dishes.
The water suspension of root-knot nematode was thoroughly stirred for making homogenous distribution of nematodes before taking 2 ml of suspension in the counting dish for counting the number of second stage juveniles of root-knot nematode under the stereoscopic microscope (Southey, 1986). An average of five counts was taken to determine the density of nematode in the suspension. Volume of water in the nematode suspension was so adjusted that each one ml contained about 100 nematodes. It was done by either adding more water or decanting the excess amount of water so that 10 ml of this suspension contained 1000 second stage juveniles of the root-knot nematode, *M. incognita* Race-3.

### 3.5. ISOLATION OF FUNGI FROM RHIZOPLANE AND ROTTED ROOTS OF *PSEUDERANTHEMUM ATROPURPUREUM*:

Single *Pseuderanthemum atropurpureum* plant showing distinct root galls and root-rot symptoms were randomly selected from each locality viz., A.M.U. Campus, Akrabad, Atrauli, Bijuli, Chandaus, Chharra, Gangiri, Gonda, Hasangarh, Harduaganj, Jalali, Kasimpur, Khair, Raya and Tapal of Aligarh district. These were kept in separate polythene bags and stored in refrigerator at 5-8 °C until processed for isolation of fungi.

The excess amount of soil adhering to the roots was removed by shaking the root system. The soil firmly attached to root was scrapped with the help of camlin hair brush and collected in a Petri dish. The soil thus obtained was thoroughly mixed and 20 mg soil dispersed across the bottom of 100 mm diameter Petri dish to which 15-20 ml of sterilized, melted and cooled potato dextrose- agar (PDA) [Peeled potato=200g, Dextrose=20g, Agar=20g and Distilled water=1000ml] was poured. The soil particles were distributed throughout the medium by rotating the Petri dish. Five plates were poured for each sample and all operations were performed in the inoculation chamber of Laminar-air-flow unit. The inoculated Petri dishes were incubated in BOD (Biological Oxygen Demand) incubator at a temperature 28± 2 °C. Fungi that grew after 5-6 days of incubation were identified and sequentially purified.

The specific identification of fungal species was done by comparing the morphological and cultural characters as described by Samson (1974) for *Paecilomyces lilacinus* (Thom) Sam., Gilman (1967) for *Aspergillus niger* (Van Tiegh.), *Cladosporium epiphyllum* Pers., *Cunninghamella verticillata* Paine., *Mucor*
Weh., *Verticillium glaucum* Bon. and Bissett (1991) for *Trichoderma* spp. The identity of the fungal species was further reconfirmed by Prof. P. N. Chowdhry (Retd.), Division of Mycology and plant Pathology, IARI, New Delhi, India.

The serial washing technique was employed to isolate fungus from infected roots as suggested by Harley and Waid (1955). Small portion of rotted roots of each plant were transferred to a sterilized dish containing sterilized distilled water and gently made free of soil particles. The roots were then transferred to another dish and the process was repeated till the time all the soil particles were removed. The roots of each plant were cut into small pieces approximately of 5mm size and transferred to Petri dishes containing 0.1% mercuric chloride solution. After about a minute, the root pieces were washed at least three times in distilled water and soaked on filter paper. Five of these root pieces were then placed in each Petri dish containing sterilized potato-dextrose agar medium (PDA) with the help of sterilized forcep under aseptic conditions in inoculation chamber of Laminar- air-flow unit. These inoculated Petri dishes were incubated at 28± 2 °C for 10 days. The fungus which developed on root segments was examined and identified on confirmation of it’s identity as *Rhizoctonia solani*, it’s pure culture was prepared.

### 3.6. RAISING AND MAINTENANCE OF FUNGUS CULTURE:

The isolated and identified fungi were further raised on Richard’s liquid medium having the following composition:

- Potassium nitrate - 10.00g
- Potassium dihydrogen phosphate - 5.00g
- Magnesium sulphate - 2.50g
- Ferric chloride - 0.02g
- Sucrose - 50.0g
- Distilled Water - 1000ml

The medium was prepared, filtered through muslin cloth and sterilized in an autoclave at 15 lb pressure for 15 minutes in 250 ml Erlenmeyer flasks, each containing about 80 ml of Richard’s medium. Small bits of the fungal mycelium of *R. solani* and other tested fungi viz., *A. niger*, *C. epiphyllum*, *C. verticillata*, *M. hiemalis*, *P. lilacinus*, *T. atroviride*, *T. asperellum*, and *Verticillium glaucum* were separately transferred to these conical flasks. Inoculated flasks were incubated in BOD incubator
at 28±2°C for about 15 days to allow growth of the fungus to be used for further studies. The pure culture of these fungi was continuously maintained on PDA contained in the test tubes by re-inoculation of the fungus after every one month.

3.7. PREPARATION OF FUNGAL INOCULUM:

After incubating the conical flasks for about 15 days, the liquid medium was filtered through Whatman filter paper No. 1. The mycelial mat was gently washed in distilled water to remove the traces of medium and pressed between the folds of blotting paper to remove the excess amount of water. Inoculum was prepared by mixing 10 g mycelial mat in 100 ml of sterilized distilled water and blending it for 30 seconds in a ware blender. Thus each 10 ml of this suspension contained 1.0 g of the fungus.

3.8. INOCULATION TECHNIQUES:

Seven days after transplantation, rooted cuttings of *P. atropurpureum* were inoculated with freshly hatched second stage juveniles (J2) of *M. incognita* Race-3 and/or mycelial mat of *R. solani*. Just before inoculation, the feeder roots of *P. atropurpureum* were exposed by carefully removing the top layer of soil. Thereafter, the required quantity of root-knot nematode suspension and/or fungus suspension was poured uniformly all around the exposed roots using sterilized pipette. After inoculation, the exposed roots were immediately covered by levelling the soil properly.

Untreated and un-inoculated plants served as control. Throughout the course of study, all the treatments were replicated three times and pots were arranged in a randomized complete block design in an open field, exposed to natural sunlight. The plants were irrigated with tap water on alternate days.

3.9. EXPERIMENTS: The experiments conducted during the study, consisted of following aspects:

(I) SURVEY OF PLANT PARASITIC NEMATODES ASSOCIATED WITH *PSEUDERANTHEMUM ATROPURPUREUM* GROWING IN ALIGARH, DISTRICT OF WESTERN UTTAR PRADESH:

The survey of 15 different localities viz., A.M.U. Campus, Akrabad, Atrauli, Bijuli, Chandaus, Chharra, Gangiri, Gonda, Hasangarh, Harduaganj, Jalali, Kasimpur,
Khair, Raya and Tapal of Aligarh district of Western Uttar Pradesh was carried out in the month of March to May 2009 to collect the soil samples from the rhizosphere of *P. atropurpureum*. The total 345 soil samples along with roots were taken at the depth of about 6 to 10 inch with the help of soil auger and each soil sample measured about 500 cm³. The soil and root samples were kept in separate polyethylene bags, sealed tightly and labelled with details of host, locality and date of collection. Samples were stored in refrigerator at 5-8 °C until processed for nematode extraction. The soil from each polythene bag was thoroughly mixed and a sub sample of 200 cm³ soil was used to extract the nematodes by using Cobb’s decanting and sieving method followed by Baermann funnel technique (Southey, 1986). After the extraction of nematodes, the nematode genera were identified and population of each genus was estimated. Thereafter the average population of the nematode present in each locality was calculated. The absolute frequency, relative frequency, absolute density relative density and prominence value of each nematode genus were determined as suggested by Norton (1978).

(II) IDENTIFICATION OF ROOT KNOT NEMATODE SPECIES:

Perineal pattern of the females was used as basis for identifying the species of root-knot nematode. Five heavily infected root-knot nematode plants of *P. atropurpureum* were randomly selected from each locality viz., A.M.U. Campus, Akrabad, Atrauli, Bijuli, Chandaus, Chharra, Gangiri, Gonda, Hasangarh, Harduaganj, Jalali, Kasimpur, Khair, Raya and Tapal of Aligarh district to identify the species of *Meloidogyne*. The galled roots from each infected *P. atropurpureum* plants were first washed gently with tap water to free it from soil particles and immersed into a beaker containing boiling 0.1% lactophenol, acid fuchsin and continued boiling for about 3 minutes. Thereafter, mature female nematodes were randomly teased out from the galls and transferred onto a clean glass slide in a drop of lactophenol. The posterior portion of the female was cut carefully with a sharp razor blade and body contents were cleaned. Further, trimming of perineal was done and finally mounted for observation. At least ten slides of perineal pattern were prepared from each plant. In addition to this, ten slides were also prepared containing at least five second stage juveniles of root-knot nematode isolated from soil of each plant, for studying its morphological characters such as head shape and stylet morphology including the length of second stage juvenile. The identification and conformation of the species
was made by comparing the characteristic features of second stage juvenile and perineal pattern with that of description given by Eisenback et al. (1981). Based on the above characteristics, the said root-knot nematode species was identified as *M. incognita* and *M. arenaria*.

**(III) IDENTIFICATION OF DIFFERENT RACES OF *MELOIDOGYNE* SPP.:**

Seventy isolates of root-knot nematode (5 isolates from each locality) were randomly collected from the *P. atropurpureum* plants growing in different localities of Aligarh district of Uttar Pradesh. Out of these isolates, 55 isolates of *M. incognita* were collected from A.M.U. Campus, Akrabad, Atrauli, Bijuli, Chandaus, Chharra, Gangiri, Gonda, Hasangarh, Harduaganj, Jalali, Kasimpur, Khair, Raya and Tapal. Whereas, 15 isolates of *M. arenaria* were collected from Akrabad, Atrauli, Bijuli, Chandaus, Gangiri, Harduaganj and Kasimpur. Pure culture of these isolates were raised and maintained for the identification of races of *Meloidogyne* spp. The methods of raising and maintenance for each isolate of *Meloidogyne* spp. collected from different localities were same as described in section 3.3.

The identification of races of *M. incognita* and *M. arenaria* infecting *P. atropurpureum* grown in different localities of Aligarh district was made on the basis of their response to the differential host plants *viz.*, Cotton, Deltapine 61; tobacco, NC 95; pepper, Early California Wonder; peanut, Florunner and tomato, Rutgers as suggested by Hartman and Sasser (1985). Watermelon, Charleston Gray was not used in the race identification test, since it was a differential host of *M. hapla*. Twenty one days old cuttings of test plants grown in 6 inch clay pots containing 1 kg of sterilized soil/pot were inoculated with 2000 J2 of either *M. incognita* or *M. arenaria* isolate. After 60 days of inoculation, the number of egg masses and galls per root system were counted and index prepared on the basis of following formula,

\[
\begin{align*}
0 &= \text{No galls or eggmasses} \\
1 &= 1-2 \text{ galls or eggmasses} \\
2 &= 3-10 \text{ galls or eggmasses} \\
3 &= 11-30 \text{ galls or eggmasses} \\
4 &= 31-100 \text{ galls or eggmasses} \\
5 &= \text{More than 100 galls or eggmasses}
\end{align*}
\]
The host plants having an average gall and eggmass index of 2 or less were considered resistant (-), whereas, the plants with an average gall and eggmass index of more than 2 were considered susceptible (+). The data was compared with the following Table-A as proposed by Taylor and Sasser (1978).

**Table A: Response of Races of *M. incognita* and *M. arenaria* to the North Carolina Differential Host Test plants.**

<table>
<thead>
<tr>
<th>Meloidogyne species and Physiological Races.</th>
<th>Differential Host Plants</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td><em>M. incognita</em></td>
<td></td>
</tr>
<tr>
<td>Race-1</td>
<td>-</td>
</tr>
<tr>
<td>Race-2</td>
<td>-</td>
</tr>
<tr>
<td>Race-3</td>
<td>+</td>
</tr>
<tr>
<td>Race-4</td>
<td>+</td>
</tr>
<tr>
<td><em>M. arenaria</em></td>
<td></td>
</tr>
<tr>
<td>Race-1</td>
<td>-</td>
</tr>
<tr>
<td>Race-2</td>
<td>-</td>
</tr>
</tbody>
</table>

* (-) indicates a resistant host; (+) indicates a susceptible host.

**(IV) STUDIES ON THE PATHOGENICITY OF *MELOIDOGYNE INCOGNITA* RACE-3 AND *RHIZOCTONIA SOLANI* ON *PSEUDERANTHEMUM ATROPURPUREUM*:**

In order to determine the inoculum threshold level of root-knot nematode, *M. incognita* Race-3, the rooted cuttings of *P. atropurpureum* were inoculated with 250, 500, 1000, 2000, 4000, 8000 and 16000 freshly hatched second stage juveniles (J2) of *M. incognita* per kg soil. Similarly, to determine the inoculum threshold level of *R. solani*, the rooted cuttings of *P. atropurpureum* were inoculated with 0.25, 0.50, 1.0, 2.0, 3.0 and 4.0 g mycelial mat of *R. solani* per kg soil. Un-inoculated plants served as control.
(V) STUDIES ON THE EFFECT OF INDIVIDUAL, CONCOMITANT AND SEQUENTIAL INOCULATION OF MELOIDOGYNE INCognita RACE-3 AND RHIZOCTONIA SOLANI ON PLANT GROWTH AND DISEASE DEVELOPMENT IN PSEUDORANTHEMUM ATROPURPUREUM:

In order to study the effect of early establishment of *M. incognita* Race-3 or *R. solani* on plant growth parameters, root-rot and nematode multiplication, the *P. atropurpureum* cuttings after seven days of transplantation were inoculated with the test pathogens viz., *M. incognita* Race-3 (@ 2000 J2 / kg soil) and *R. solani* (@ 3g mycelial mat / kg soil) either individually or in various combinations of simultaneous, and pre and post inoculations. This experiment was conducted to find out whether the interaction between the two pathogens is additive or synergistic. The experiment was designed according to the following schedule:

1. Un-inoculated (control).
2. Inoculated with *R. solani*.
3. Inoculated with *M. incognita* Race-3.
4. *Rhizoctonia solani* inoculated 15 days prior to *M. incognita* Race-3.
5. *Meloidogyne incognita* Race-3 inoculated 15 days prior to *R. solani*.
6. Concomitantly inoculated with *M. incognita* Race-3 and *R. solani*.

(VI) STUDIES ON THE INTERACTIVE EFFECT OF VARYING INOCULUM LEVELS OF ROOT-KNOT NEMATODE, MELOIDOGYNE INCognita RACE-3 AND ROOT-ROT FUNGUS, RHIZOCTONIA SOLANI ON PSEUDERANTHEMUM ATROPURPUREUM:

To study the interactive effect of varying inoculum levels of *M. incognita* Race-3 and *R. solani* on the plant growth, nematode multiplication and root-rot, the *P. atropurpureum* cuttings after seven days of transplantation were inoculated concomitantly with variable inoculum levels of *M. incognita* Race-3 and *R. solani*. Besides, the cuttings were also individually inoculated with variable inoculum levels of *M. incognita* Race-3 and *R. solani*. The experiment was designed according to the following schedule.

1. Un-inoculated (control).
2. Inoculated with *R. solani* @ 2 g mycelium / kg soil.
3. Inoculated with *R. solani* @ 3 g mycelium / kg soil.
4. Inoculated with *R. solani* @ 4 g mycelium / kg soil.
5. Inoculated with *M. incognita* Race-3 @ 1000 J2 / kg soil.
6. Inoculated with *M. incognita* Race-3 @ 2000 J2 / kg soil.
7. Inoculated with *M. incognita* Race-3 @ 4000 J2 / kg soil.
8. Concomitantly inoculated with *M. incognita* Race-3 (@ 1000 J2 / kg soil) and *R. solani* (@ 2 g mycelia mat / kg soil.)
9. Concomitantly inoculated with *M. incognita* Race-3 (@ 1000 J2 / kg soil) and *R. solani* (@ 3 g mycelia mat / kg soil.)
10. Concomitantly inoculated with *M. incognita* Race-3 (@ 1000 J2 / kg soil) and *R. solani* (@ 4 g mycelia mat / kg soil.)
11. Concomitantly inoculated with *M. incognita* Race-3 (@ 2000 J2 / kg soil) and *R. solani* (@ 2 g mycelia mat / kg soil.)
12. Concomitantly inoculated with *M. incognita* Race-3 (@ 2000 J2 / kg soil) and *R. solani* (@ 3 g mycelia mat / kg soil.)
13. Concomitantly inoculated with *M. incognita* Race-3 (@ 2000 J2 / kg soil) and *R. solani* (@ 4 g mycelia mat / kg soil.)
14. Concomitantly inoculated with *M. incognita* Race-3 (@ 4000 J2 / kg soil) and *R. solani* (@ 2 g mycelia mat / kg soil.)
15. Concomitantly inoculated with *M. incognita* Race-3 (@ 4000 J2 / kg soil) and *R. solani* (@ 3 g mycelia mat / kg soil.)
16. Concomitantly inoculated with *M. incognita* Race-3 (@ 4000 J2 / kg soil) and *R. solani* (@ 4 g mycelia mat / kg soil.)

(VII) **STUDIES ON THE LIFE-CYCLE OF MELOIDOGYNE INCognITA RACE-3 ON PSEUDERANTHEMUM ATROPURPUREUM IN PRESENCE AND ABSENCE OF RHIZOCTONIA SOLANI:**

Penetration and life cycle of *M. incognita* Race-3 was studied on *P. atropurpureum*. The present investigations were carried out in months of August-September and the prevailing temperature during this period was found to range between 35 to 37°C. Six week old rooted cuttings of *P. atropurpureum* were singly transplanted into 6 inch clay pots containing 1 kg sterilized soil mixture. After one week of transplantation, one set of 150 cuttings was simultaneously inoculated with freshly hatched 2000 second stage juveniles of *M. incognita* Race-3 and 3 g fungal
suspension of *R. solani* and the other set of 150 cuttings was individually inoculated with same inoculum level of *M. incognita* Race-3 served as control.

Observations on penetration and developmental stages of the nematode were recorded from three cuttings of each set after every 24 hours (first being after 12 hours) and continued till the completion of life-cycle. The complete root system of the tested plants was carefully removed from the soil, washed gently in tap water and stained in boiling 0.1% acid fuchsin, in lactophenol followed by washing in tap water and keeping in plain lactophenol for further differentiation.

The developmental stages of *M. incognita* were identified and designated as (A, B, C, D and E) as described by Triantaphyllou and Hirschmann (1960).

A = pre parasitic II\(^{nd}\) stage juvenile (filiform shaped)
B = parasitic II\(^{nd}\) stage juvenile (spindle shaped)
C = III\(^{rd}\) and IV\(^{th}\) stage of juvenile (sausage shaped)
D = moulted IV\(^{th}\) stage juvenile (moulted sausage shaped)
E = adult females (sac shaped) and males (filiform shaped).

The number of juveniles (*J*\(_2\*)) penetrated and their different developmental stages found in roots were counted under a stereomicroscope. The percentage of juveniles penetrated was calculated against the initial inoculum level of *M. incognita*. Similarly, the per cent of females and males developed against the total juveniles penetrated was calculated. The number of eggs/egg mass and the population of nematode/kg soil were also estimated (Southey, 1986).

(VIII) EFFICACY OF NEMATICIDE, SOIL FUNGI, CHOPPED LEAVES AND SAWDUST OF SOME PLANTS ON THE GROWTH OF *PSEUDERANTHEMUM ATROPURPUREUM*:

The eight soil fungi viz., *Aspergillus niger*, *Cladosporium epiphyllum*, *Cunninghamella verticillata*, *Mucor hiemalis*, *Paecilomyces lilacinus*, *Trichoderma atroviride*, *Trichoderma asperellum*, and *Verticillium glaucum* @ 1.0 g mycelium + conidia /kg soil; saw dust of some plants viz., babool (*Acacia nilotica* (L.) Willd. ex Delile), cedrus (*Cedrus deodara* (Roxb.) Don), chir pine (*Pinus roxburghii* Sarg.) sal (*Shorea robusta* Roth), sheesham (*Dalbergia sissoo* Roxb.), teak (*Tectona grandis* L.) and neem (*Azadirachta indica* A. Juss.) @ 10 g /kg soil; green chopped leaves of some plants viz., *Argemone mexicana* Linn. (Mexican prickly poppy), *Cannabis*
sativa L. (Sunhemp), Cassia angustifolia Vahl. (Indian Senna), Coccinia cordifolia (L.) Cogn. (Ivy gourd), Coleus forskohlii Briq. (Medicinal Coleus), Duranta erecta L. (Golden dewdrop), Parthenium hysterophorus Linn. (Congress Grass), Phyllanthus niruri L. (Stonebreaker), Tinospora cordifolia (Thunb.) Miers. (Heartleaf Moonseed) and Withania somnifera (L.) Dunal (Indian Ginseng) @ 20 g / kg soil and a nematicide, carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) as a bare root-dip treatment in aqueous solution (100 ppm) were tested individually to see their effect on plant growth parameters of *P. atropurpureum*. The method of application of these additives was similar as described following in sections (IX), (X) and (XI). Six week old well established rooted cuttings of *P. atropurpureum* were transplanted in the treated pots. The pots were kept in an open field exposed to natural light conditions. The plants were irrigated with tap water on alternate days. Untreated plants served as control. Afterwards, these treatments were also used for the purpose of management of *M. incognita* Race-3 - *R. solani* disease complex in *P. atropurpureum*. The management measures are described separately as follows.

(IX) EFFICACY OF NEMATICIDE AS BARE ROOT-DIP TREATMENT AND SOME SOIL FUNGI ON THE MANAGEMENT OF *MELOIDOGYNE INCognita* RACE-3 - *RHIZOCTONIA SOLANI* DISEASE COMPLEX IN *PSEUDERANTHEMUM ATROPURPUREUM*:

The efficacy of soil fungi, green chopped leaves and saw dust of some plants was compared with a generally recommended nematicide, carbofuran used as a bare root-dip treatment for the management of disease complex caused by *M. incognita* Race-3 and *R. solani* in *P. atropurpureum*. The six week old rooted cuttings of *P. atropurpureum* were uprooted and washed gently three to four times with tap water to remove the adhering soil particles. The roots of these cuttings were dipped in aqueous solution of carbofuran (Furadan 3G) at a concentration of 100 ppm a.i for three hours. These cuttings were immediately transplanted singly into clay pots (25 cm top diameter) containing 4 kg sterilized soil and after 7 days of transplantation, the cuttings were simultaneously inoculated with *M. incognita* Race-3 (2000 J₂ /kg soil) and *R. solani* (3.0 g mycelial mat / kg soil). Thereafter, pots were kept in an open field exposed to natural light conditions. The plants were irrigated with tap water on alternate days. Untreated-uninoculated plants served as control-I and untreated-inoculated (*M. incognita* Race-3 and *R. solani*) served as control-II.
The eight fungi viz., *Aspergillus niger*, *Cladosporium epiphyllum*, *Cunninghamella verticillata*, *Mucor hiemalis*, *Paecilomyces lilacinus*, *Trichoderma atroviride*, *Trichoderma asperellum*, and *Verticillium glaucum* were used separately for the management of *M. incognita* Race-3 - *R. solani* disease complex in *P. atropurpureum*. The culture of these fungi was raised on Richard’s liquid medium in order to obtain mycelial mat. Thereafter, the mycelial mat of each fungal culture was blended with water to obtain fungal suspension for the purpose of inoculation as described earlier in the section 3.7 and 3.8. The six week old rooted cuttings of *P. atropurpureum* were transplanted singly into the clay pots containing 4 kg sterilized soil. The cuttings after 7 days of transplantation were concomitantly inoculated with *M. incognita* Race-3 (2000 J2 /kg soil) and *R. solani* (3.0 g mycelial mat / kg soil). At the same time soil fungi @ 1.0 g mycelium + conidia /kg soil were also inoculated in the root system of *P. atropurpureum* according to the schedule given below. These pots were kept in an open field exposed to natural light conditions. The plants were irrigated with tap water on alternate days. Untreated-uninoculated plants served as control-I and untreated-inoculated with *M. incognita* Race-3 and *R. solani* served as control-II.

**Schedule of various treatments:**

1. Untreated-uninoculated [control-I]
2. Untreated-inoculated with *M. incognita* Race-3 (Mi) + *R. solani* (Rs) [control-II]
3. *Aspergillus niger* + Mi + Rs
4. *Cladosporium epiphyllum* + Mi + Rs
5. *Cunninghamella verticillata* + Mi + Rs
6. *Mucor hiemalis* + Mi + Rs
7. *Paecilomyces lilacinus* + Mi + Rs
8. *Trichoderma atroviride* + Mi + Rs
9. *Trichoderma asperellum* + Mi + Rs
10. *Verticillium glaucum* + Mi + Rs
11. Carbofuran + Mi + Rs
EFFICACY OF GREEN CHOPPED LEAVES OF SOME PLANTS ON THE MANAGEMENT OF *MELOIDOGYNE INCognITA* RACE-3 - *RHIZOCTONIA SOLANI* DISEASE COMPLEX IN *PSEUDERANTHEMUM ATROPURPUREUM*:

Fresh green leaves of ten plants viz., *Argemone mexicana*, *Cannabis sativa*, *Cassia angustifolia*, *Coccinia cordifolia*, *Coleus forskohlii*, *Duranta erecta*, *Parthenium hysterophorus*, *Phyllanthus niruri*, *Tinospora cordifolia* and *Withania somnifera* were collected from different places of Aligarh and used for the management of *M. incognita* Race-3 – *R. solani* disease complex in *P. atropurpureum*. The leaves were washed thoroughly with tap water, surface sterilized with 0.1% calcium hypochlorite and repeatedly washed in sterilized distilled water. The leaves of each plant were then cut into small pieces and amended separately into the sterilized soil @ 20 g /kg soil and mixed thoroughly. Thereafter, chopped leaves amended soil was transferred into clay pots (25 cm top diameter) @ 4 kg soil /pot. These pots were kept in an open field exposed to natural light conditions. The plants were irrigated with tap water on alternate days to keep the soil moist and facilitate the complete decomposition of organic material. After two weeks of waiting period six week old rooted cuttings of *P. atropurpureum* were transplanted in these pots. The cuttings, after 7 days of transplantation were concomitantly inoculated with *M. incognita* Race-3 (2000 J₂ /kg soil) and *R. solani* (3.0 g mycelial mat /kg soil) and the experiment was designed according to the schedule given below. Untreated-uninoculated plants served as control-I and untreated-inoculated with *M. incognita* Race-3 and *R. solani* served as control-II.

**Schedule of various treatments:**

1. Untreated-uninoculated [control-I]
2. Untreated-inoculated with *M. incognita* (Mi) + *R. solani* (Rs) [control-II]
3. *Argemone mexicana* + Mi + Rs
4. *Cannabis sativa* + Mi + Rs
5. *Cassia angustifolia* + Mi + Rs
6. *Coccinia cordifolia* + Mi + Rs
7. *Coleus forskohlii* + Mi + Rs
8. *Duranta erecta* + Mi + Rs
9. *Parthenium hysterophorus* + Mi + Rs
10. *Phyllanthus niruri* + Mi + Rs
11. *Tinospora cordifolia* + Mi + Rs
12. *Withania somnifera* + Mi + Rs

(XI) EFFICACY OF SAWDUST OF SOME PLANTS ON THE MANAGEMENT OF MELOIDOGYNE INCognita RACE-3 - RHiZOCtONIA SOLANI DISEASE COMPLEX IN PSEUDERANTHEMUM ATROPURPUREUM:

Sawdust of babool (*Acacia nilotica*), cedrus (*Cedrus deodara*), chir pine (*Pinus roxburghii*), sal (*Shorea robusta*), sheesham (*Dalbergia sissoo*), teak (*Tectona grandis*) and neem (*Azadirachta indica*) were used in the management of *M. incognita* – *R. solani* disease complex in *P. atropurpureum*. The sawdust of these plants was separately mixed thoroughly with soil @ 20g/kg soil. The sawdust amended soil was filled into clay pots @ 4 kg soil/pot. These pots were kept in an open field exposed to natural sunlight. The plants were irrigated with tap water on alternate days to keep the soil moist and facilitate the complete decomposition of sawdust. After two week of waiting period, six week old rooted cuttings of *P. atropurpureum* were transplanted in these pots. The cuttings, after 7 days of transplantation were concomitantly inoculated with *M. incognita* Race-3 (2000 J2/kg soil) and *R. solani* (3.0 g mycelial mat/kg soil) and the experiment was designed according to the schedule given below. Untreated-uninoculated plants served as control-I and untreated-inoculated with *M. incognita* Race-3 and *R. solani* served as control-II.

**Schedule of various treatments:**

1. Untreated-uninoculated [control-I]
2. Untreated-inoculated with *M. incognita* (Mi) + *R. solani* (Rs) [control-II]
3. Babool sawdust + Mi + Rs
4. Cedrus sawdust + Mi + Rs
5. Chir Pine sawdust + Mi + Rs
6. Sal sawdust + Mi + Rs
7. Sheesham sawdust + Mi + Rs
8. Teak sawdust + Mi + Rs
9. Neem sawdust + Mi + Rs
(XII) INTEGRATED MANAGEMENT OF MELOIDOGYNE INCognita
race-3 – RHIZOCTONIA SOLANI DISEASE COMPLEX IN
PSEUDERANTHEMUM ATROPURPUREUM BY USING
CARBOFURAN AS BARE ROOT DIP TREATMENT, FUNGAL
BIOCONTROL AGENTS, CHOPPED LEAVES AND SAWDUST OF
SOME PLANTS:

After the testing of nematicide- carbofuran, soil fungi, chopped leaves and
sawdust of different plants for the management of M. incognita Race-3 - R. solani
disease complex in P. atropurpureum, the effective fungal biocontrol agents viz., P.
lilacinus and T. atroviride; chopped leaves of A. mexicana, P. niruri, P.
hysterophorus and T. cordifolia; sawdust of neem, teak and sheesham and nematicide,
carbofuran (Furadan 3G) were used for the integrated management. The selection of
the effective agents for the integrated management was done by using those agents
which played a significant positive role in the protection of P. atropurpureum from
disease complex caused by M. incognita Race-3 – R. solani. Six week old rooted stem
cuttings of P. atropurpureum already treated with carbofuran as root-dip were
transplanted singly into 25cm top-diameter clay pots, each containing 4 kg sterilized
soil treated with fungal biocontrol agent and either chopped leaves or sawdust in
different combinations. The doses and method of application of these additives
including root-dip treatment with carbofuran were similar as described earlier in
section (IX), (X) and (XI). Seven days after transplantation, plants were inoculated
simultaneously with M. incognita (2000 J2 / kg soil) and R. solani (3.0 g mycelial mat
/ kg soil) and the experiment was designed according to the schedule given below.

Schedule of various treatments:

1. Untreated – un-inoculated [control-1].
2. Untreated - inoculated with M. incognita (Mi) + R. solani (Rs) [control-II].
3. Carbofuran (100ppm) + P. lilacinus (1.0g mycelium + conidia / kg soil) + A.
mexicana (20.0g chopped leaves / kg soil) + Mi + Rs
4. Carbofuran (100ppm) + P. lilacinus (1.0g mycelium + conidia / kg soil) + T.
cordifolia (20.0g chopped leaves / kg soil) + Mi + Rs
5. Carbofuran (100ppm) + P. lilacinus (1.0g mycelium + conidia / kg soil) + P.
niruri (20.0g chopped leaves / kg soil) + Mi + Rs
6. Carbofuran (100ppm) + *P. lilacinus* (1.0g mycelium + conidia / kg soil) + teak sawdust (20.0g / kg soil) + Mi + Rs

7. Carbofuran (100ppm) + *P. lilacinus* (1.0g mycelium + conidia / kg soil) + Sheesham sawdust (20.0g / kg soil) + Mi + Rs

8. Carbofuran (100ppm) + *P. lilacinus* (1.0g mycelium + conidia / kg soil) + *P. hysterophorus* (20.0g chopped leaves / kg soil) + Mi + Rs

9. Carbofuran (100ppm) + *P. lilacinus* (1.0g mycelium + conidia / kg soil) + Neem sawdust (20.0g / kg soil) + Mi + Rs

10. Carbofuran (100ppm) + *T. atroviride* (1.0g mycelium + conidia / kg soil) + *A. mexicana* (20.0g chopped leaves / kg soil) + Mi + Rs

11. Carbofuran (100ppm) + *T. atroviride* (1.0g mycelium + conidia / kg soil) + Neem sawdust (20.0g / kg soil) + Mi + Rs

12. Carbofuran (100ppm) + *T. atroviride* (1.0g mycelium + conidia / kg soil) + *P. hysterophorus* (20.0g chopped leaves / kg soil) + Mi + Rs

13. Carbofuran (100ppm) + *T. atroviride* (1.0g mycelium + conidia / kg soil) + *P. niruri* (20.0g chopped leaves / kg soil) + Mi + Rs

14. Carbofuran (100ppm) + *T. atroviride* (1.0g mycelium + conidia / kg soil) + Sheesham sawdust (20.0g / kg soil) + Mi + Rs

15. Carbofuran (100ppm) + *T. atroviride* (1.0g mycelium + conidia / kg soil) + *T. cordifolia* (20.0g chopped leaves / kg soil) + Mi + Rs

16. Carbofuran (100ppm) + *T. atroviride* (1.0g mycelium + conidia / kg soil) + teak sawdust (20.0g / kg soil) + Mi + Rs

3.10. RECORDING OF OBSERVATIONS:

(a) **DETERMINATION OF ABSOLUTE FREQUENCY, RELATIVE FREQUENCY, ABSOLUTE DENSITY, RELATIVE DENSITY AND PROMINENCE VALUE OF PLANT PARASITIC NEMATODES:**

Absolute frequency, relative frequency, absolute density relative density and prominence value of each nematode genus were determined according to Norton, 1978.

\[
\text{Absolute frequency} = \frac{\text{Number of samples containing a species}}{\text{Total number of samples collected}} \times 100
\]
Relative frequency = \( \frac{\text{Frequency of species}}{\text{Sum of frequencies of all species}} \times 100 \)

Absolute density = \( \frac{\text{Number of individuals of a species in a sample}}{\text{Volume of sample}} \times 100 \)

Relative density = \( \frac{\text{Number of individuals of a species in a sample}}{\text{Total of all individuals in a samples}} \times 100 \)

Prominence value = Absolute density \( \sqrt{\text{Absolute frequency}} \)

(b) PLANT GROWTH DETERMINATION:

The plants were uprooted after 150 days of inoculation and their roots were gently washed in slow running tap water to avoid losses and injury to roots during the entire operation. For measuring length and dry weight, the plants were cut with a sharp knife just above the base of root emergence. The length of the shoots and roots was recorded in centimetres from the cut end to the top of first leaf and the longest root respectively. The weight of plant was recorded in grams. For measuring dry weight, the root and shoot were kept in paper made envelopes for drying in an oven running at 60°C for 2-3 days. The percentage reduction/ stimulation in plant growth parameters (Plant length, fresh weight, dry weight, shoot length, root length, number of leaves and leaf area) of different treatments in relation to control was calculated.

(c) ESTIMATION OF CHLOROPHYLL AND CAROTENOID CONTENT:

The chlorophyll content in the fresh leaf was estimated as suggested by Mackinney (1941). One gram of finely cut fresh leaves of \textit{P. atropurpureum} was ground to a fine pulp using a mortar and pestle after pouring 20 ml of 80% acetone. The mixture was centrifuged at 5000 rpm, for 5 minutes. The supernatant was collected in 100 ml volumetric flask. The residue was washed three times, using 80% acetone. Each washing was collected in the same volumetric flask and volume was made up to 100 ml mark, using 80% acetone. The absorbance was read at 645, 663 and 440 nm against the solvent (80% acetone) blank on spectrophotometer. The chlorophyll content present in the extract (microgram per milliliter) was calculated using the following equations:
\[ C_a (\text{mg/L}) = 12.7 (A_{663}) - 2.69 (A_{645}) \]
\[ C_b (\text{mg/L}) = 22.9(A_{645}) - 4.68(A_{663}) \]
\[ C_{a+b} (\text{mg/L}) = 20.2 (A_{645}) + 3.02 (A_{663}) \]
\[ C_k (\text{mg/L}) = 4.70 (A_{440}) - 0.27 (\text{Chl}_{a+b}) \]

Where \( C_a \) is the concentration of chlorophyll a, \( C_b \) is the concentration of chlorophyll b, \( C_{a+b} \) is the concentration of total chlorophyll and \( C_k \) is the concentration of carotenoids (\( \mu \text{g/ml} \)). \( A_{663}, A_{645}, A_{440} \) are the absorption values of leaves where wavelengths were 663nm, 645nm and 440 nm respectively.

From the above calculated values of \( C_a, C_b, C_{a+b} \) and \( C_k \) (\( \mu \text{g/ml} \)), the concentration of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids (mg/g) can be calculated as follows.

\[ X_a = C_a \times V/W \]
\[ X_b = C_b \times V/W \]
\[ X_{a+b} = C_{a+b} \times V/W \]
\[ X_k = C_k \times V/W \]

Where \( X_a \) is the concentration of chlorophyll a, \( X_b \) is the concentration of chlorophyll b, \( X_{a+b} \) is the concentration of total chlorophyll and \( X_k \) is the concentration of carotenoids (mg/g leaf). \( W \) (g) is the weight of sample and \( V \) (ml) is the volume of mixed extracting solution (80% acetone).

**(d) ESTIMATION OF LEAF AREA:**

The leaf area was calculated by using a graph sheet having small squares of area 1 cm\(^2\) each. The leaf was placed over the graph sheet and an outline was traced. The number of squares inside the traced outline were counted. The partially squares which were at least half covered by the leaf were also counted and those which were less than half covered were left. The total number of squares counted gives the leaf area in cm\(^2\).

**(e) ESTIMATION OF NUMBER OF LEAVES:**

The number of leaves were estimated by counting the number of leaves per plant.
(f) NEMATODE POPULATION ESTIMATION:

For extraction of nematode (J2 and male) from soil, the soil from the pots of each treatment was mixed thoroughly and a sub-sample of 200 g soil was processed through sieves according to Cobb’s decanting and sieving method followed by Baermann funnel technique (Southey, 1986).

The nematode suspension of each sample was collected in a beaker and volume was made upto 100ml. For proper distribution of nematode, the suspension was bubbled with the help of pipette and 2ml suspension from each sample was drawn and transferred to a counting dish. The number of nematode was counted in five replicates for each sample. Mean of the five such countings was calculated and the final population of *M. incognita*/kg soil was determined.

To estimate the root population of nematode (J2, J3, J4, mature female and male), 1.0 g root randomly collected from each replicate and cut into small pieces of about 1-2 cm and then root was macerated with enough water in an electrically operated waring blender for about 30 to 40 seconds. The macerate was collected in a beaker and volume was made up to 100 ml. The number of nematode was counted as described above and total number of nematode for the whole root system of each replicate was then calculated. Reproduction factor (Rf) of root-knot nematode was calculated by the formula R=Pf/Pi, where, Pf represents the final and Pi represents initial population of nematode.

(g) ESTIMATION OF NUMBER OF GALLS:

The galls produced by root-knot nematode (*M. incognita*) were estimated by counting the number of galls per root system.

(h) ESTIMATION OF ROOT-ROT PERCENTAGE:

In order to determine the percentage of root-rot caused by *R. solani* in *P. atropurpureum*, the roots were washed gently several times to remove the adhering soil particles. About 15 pieces (5 cm in length) of lateral roots were randomly selected and cut from different portions of the root system of *P. atropurpureum*. Thereafter, from 15 pieces of each root sample, 5 pieces were randomly selected for calculation of root-rot. Each piece of root was observed and measured for the rotted portion in the form of dark brown to black coloured areas on the root surface. The percentage of root-rot was calculated by taking into account the length of rotted portion in relation
to total length of root pieces. The following formula was used to calculate the percentage of root-rot.

\[ \text{Root-rot (\%)} = \frac{\text{Length of rotted area on root pieces}}{\text{Total length of root pieces}} \times 100 \]

(i) ASSESSMENT OF COLLAR-ROT, CROWN-ROT SYMPTOMS AND MORTALITY OF PSEUDERANTHEMUM ATROPURPUREUM PLANTS:

*Pseudoranthemum atropurpureum* plants inoculated with *M. incognita* and/or *R. solani* were observed during the growth period for appearance of symptoms. The assessment of mortality, collar-rot and collar-rot symptoms in the plant were determined according to Sharma (2012). The symptoms of collar-rot were recorded on the particular day of appearance of rotting upto 1-2 cm all around the base of the stem (collar region). The assessment of crown-rot symptoms in the present study was based on visual observation, aerial symptoms showing yellowing, drying and shedding of leaves followed by rotting, present in the length of 1-2 cm around the circumference of any one of the branches of *P. atropurpureum* plant after inoculation. The mortality of plants was determined on the day when plants collapsed and toppled down due to severe rotting at the base of stem. The plant growth parameters, number of galls/root system, nematode population, collar-rot, crown-rot and root-rot symptoms were not recorded in the treatments which resulted in mortality of plants.

STATISTICAL ANALYSIS:

The data obtained were analyzed statistically using SPSS for windows (SPSS Inc., Chicago, IL, USA ver. 12.0). Analysis of variance (ANOVA) was performed according to the design of the experiment and F-value was calculated at P= 0.05 and P= 0.01. For significant data, critical difference (C.D.) was calculated. Treatment means were compared using C.D. values at P= 0.05 and discussed accordingly.