# MATERIALS AND METHODS

The general procedure followed in different experiments is summarised below:

## 3.1. SOIL STERILIZATION:

Sandy loam soil (coarse sand 1.3 percent, fine sand 63.00 per cent, silt 15.0 per cent and clay 20.0 per cent) collected from the nearby fields was mixed with farm yard manure in 5 : 1 proportion. The mixture was steam sterilized at the pressure of 1.4 Kg per $cm^2$ for three hours in autoclave for three consecutive days. Such steam sterilized soil mixture was used in different experiments.

## 3.2. DISINFESTATION OF POTS:

The earthen pots of 15 cm - diameter were washed with water and disinfested with 4.0 per cent formaldehyde (formalin 40 EC). The formalin was allowed to get evaporated before using such pots for research work.

## 3.3. RAISING TOMATO SEEDLINGS:

Earthen pots of 15 - cm diameter were filled with steam sterilized soil mixture. The seeds of tomato cv. Pusa Ruby, previously sterilized with 1 : 1000 mercuric chloride (Hussey and Barker, 1973) were seeded at the rate of 2 kg/hectare.

Recommended agronomical practices in vogue were followed for raising healthy seedlings. Two weeks old seedlings were used for transplanting in various experiments unless stated otherwise. 3.4.

## 3.4 STUDIES OF SOIL MYCOFLORA :

### 3.4.1. ISOLATION OF RHIZOSPHERE MYCOFLORA :

For rhizosphere studies , the seeds after having disinfection were sown in the autoclaved soil and fungi were isolated from the rhizosphere of tomato at intervals of 15 days, from seedling to the maturation of the plant. For analysis, five plants were removed carefully and brought to laboratory in sterile containers. The plants were shaken to remove superfluous soil from the root system. Under aspectic conditions each plant was taken from the container and placed on the sterile glass plate. The root system was spread out and the soil particles adhering to the root surface were removed. With the help of micro spatula the soil was transferred to petridishes containing 10 ml of melted and cooled peptone dextrose agar medium (Saburaud,1910) in following constituents :

| | |
|---|---|
| Peptone | ---- 10 gm |
| Dextrose | ---- 40 gm |
| Agar | ---- 20 gm |
| Distillled Water | ---- 1 l |

The plates were rotated before solidification of agar medium

in order to disperse the soil particles evenly. After pouring and inoculating, the petridishes were inoculated at 28 C, and the fungus which developed after one week, were examined and indentified. The frequency of fungi was calculated by the following formula :

$$\text{Frequency} = \frac{\text{Number of plates containing a particular fungus}}{\text{Total number of plates poured}} \times 100$$

In order to determine the population, the soil held on flattered tip of the needle was transferred to each plate. Later the average weight of each transfer was determined. Subsequently the population was determined by containing the number of colonies developed in all plates and then transferring the figures to the number of colonies developed in one gram of soil.

For determining the relative abundance (RA) of the fungi the formula suggested by the Mc lean and Cook (1957) was employed which is as follows:

$$RA = \frac{\text{Total number of colonies of a fungus}}{\text{Total number of colonies of all the fungi}}$$

For control the fungi were isolated from the indentical soil without a plant. It was termed as non-rhizosphere. The technique used was the same as described above.

### 3.4.2.ISOLATION OF RHIZOPLANE MYCOFLORA:

For studying rhizoplane mycoflora, the serial root washing technique of Harley and Waid (1955), was employed. The roots were cut into small pieces. These root pieces were subjected to 30 washings in sterile distilled water. The root pieces were then transferred to sterilized petridishes containing 10 ml of melted and cooled peptone dextrose agar medium. The petridishes were incubated at 28 + 2 C for one week. The fungi which developed after one week were examined and indentified. Frequency and relative abundance was calculated as done in case of rhizosphere study.

## 3.5. MAINTENANCE OF PURE CULTURE OF FUNGI:

The pure culture of fungi encountered during isolation from the plate was maintained on peptone dextrose agar (PDA) medium and subcultured fornightly by transfer of fungal growth on fresh slants of the same medium.

## 3.6. MAINTENANCE OF PURE CULTURE OF NEMATODES

### 3.6.1. *M. incognita*:

Single egg-mass obtained from infected brinjal root, grown in farmers field at Aligarh - India was surface sterilized in 1 : 500 solution of calcium hypochloride for 5 minutes and washed it

thrice in sterilized distilled water and allowed to hatch in distilled water at 28 + 2 C. The seedlings of susceptible cultivar of tomato Pusa Ruby were inoculated with the larvae hatched from a single egg-mass. Subsequent inoculation of plants were from such infections to develop culture.

3.6.2 R. reniformis :

For raising the population of R. reniformis eggs from single gravid females treated with 1 : 500 hypochloride , were transferred in sterilized distilled water for hatching. Pre-adult females so obtained were placed near the roots of tomato cv. Pusa Ruby grown in sterilized soil.

After 60 days, the plants were inoculated from the culture thus raised in oroder to have regular supply of the inoculum.

3.7. RAISING AND MAINTENANCE OF DIFFERENT FILTRATE OF FUNGI:

The fungi were grown in Richard's medium contained in 250 ml Erlenmeyer flasks and incubated at 25 C. After 15 days, the contents of the flask were filtered through Whatman filter paper and the mycelial mat was used for inoculation while the filtrate for other studies.

## 3.8. PREPARATION OF FUNGAL INOCULUM AND INOCULATION:

The mycelial mat for inoculation purpose was obtained by growing the fungus on 50 ml Richard's medium (Riker & Riker, 1936) contained in 250 ml Erenmeyer flasks. The flasks were inoculated with disc of 5 mm diameter of freshly cut peptone dextrose agar medium with the mycelial mat on the top from actively growing fungus.After incubating the inoculated flasks for ten days at room temperature, the mycelial mat along with medium was wahshed with distilled sterile lukewarm water to remove trace of medium and gently pressed between the fold of a sterile filter paper to remove excess water. The fungal mycelial mat was weighted and blended with sterilized water in proper quantity at medium speed in blender for ten seconds. Freshly macerated mycelial suspension was used for inoculating. The soil was inoculated with, 0.25, 0.50, 1.00 and 2.00 gm of the fungus per kg soil.

## 3.9. EFFECT OF SAPROPHYTIC FUNGI ON THE GROWTH OF PATHOGENIC FUNGI:

Aspergillus niger, A. flavus, A. nidulans, Rhizopus nigricans, Penicillium notatum, P. digitatum were tested against the F. oxysporum f.sp.lycopersici, were selected as saprophytic fungi; and Rhizoctonia solani, Alternaria solani and Pythium digitatum as pathogenic fungi.

### 3.9.1. THE EFFECT WAS DETERMINED BY USING AGAR-DISC METHOD AND CULTURE FILTRATE METHOD

The studies were made on peptone dextrose agar in petri-dishes. The centre of the flask containing PDA was inoculated with the test pathogenic fungus. On either side of the inoculated portion, towards the edge of the plate, a small bit of mycelium of saprophytic fungi was placed. For control the plates inoculated with the fungus alone. The difference in growth of the fungus indicated the inhibition.

### 3.9.2 CULTURE FILTRATE METHOD:

The culture filtrate of saprophytic test fungi were obtained as given in para- 3.7. The culture filtlrate of the fungi (5 ml) were poured to each 250 ml erlenmeyer flasks containing Richard's medium. The flasks were inoculated with pathogenic fungus by transferring a small bit of the mycelium. The flasks were incubated at 25 C. After a week of incubation, the mycelial mat was separated out. The mat was dried at 40 C for 72 hrs., and subsequently, cooled in dessicator having anhydrous calcium chloride. The mycelium mat was weighed. The dry weight of the actual mat was calculated in gm by substrating the weight of the filter paper. There were three replicates with 10 flasks of each treatment. The growth of the fungi in Richard's medium alone was taken as control. The difference in the dry weight of the mycelium was taken as the inhibition in growth.

## 3.10. STUDIES ON THE EFFECT OF DIFFERENT INOCULUM LEVEL OF *R. solani*, *A. solani*, *F. oxysporum* f.sp. *lycopersici* AND *P. aphanidermatum* ON GROWTH OF PLANTS.

The determine the effect of initial inoculum levels of the fungi on growth of plants, four levels viz, 0.25, 0.50, 1.00 and 2.00 gm mat/kg of soil were tried alongwith control (no fungus). Pots of 15-c-d were filled in with sterilized soil mixture as per section 3.1 and 3.2. The seedlings raised in the manner described in 3.3 were inoculated with different inoculum levels as mentioned above. Growth was determined after 60 days. The disease index was calculated as per guidance on formula given by Subudhi and Raut (1994);

$$DI = \frac{oa + 1b + 2c + 3d + 4c}{a + b + c + d + e}$$

where --

0 = no disease
1 = 1-10% damage of plants
2 = 11-20% damage of plants
3 = 21-49% damage of plants
4 = 50% or more damage of plants.

a, b, c, d and e indicate number of infected plants in the respective grades.

## 3.11. EFFECT OF INOCULATING TOMATO cv. Pusa Ruby WITH DIFFERENT INOCULUM LEVELS OF M. incognita AND R. reniformis SEPARATELY ON GROWTH OF PLANTS AND MULTIPLICATION OF NEMATODES:

A pot culture experiment having 10 treatments was designed. The pots were filled with steam sterilized sandy loam soil raised in the maner and described in para. 3.3, and inoculated with root-knot/reniform nematode separately. Each treatment was replicated five times. The growth parameters, nematode population and RKI were determinded after 60 days were recorded as per details given in 3.17.

## 3.12. EFFECT OF INOCULATING SEEDS WITH FUNGI ON GERMINATION OF SEED OF TOMATO cv. Pusa Ruby.

### 3.12.1. SEED-DRESSING WITH FUNGAL CULTURE:

The seeds after having surface sterilized with mercuric chloride were coated with the spores of all the fungi isolated from rhizosphere separately by rolling them over 4 to 6 days old sporulating culture. The seeds so treated (100) were seeded into sterilized soil. The emergence of seedlings was determined after 5,8 and 12 days. The seedling emergence in uninfected soil served as control. The damping-off was also observed. There were five replicates of each treatment.

### 3.13.2 SEED TREATED WITH CULTURE FILTRATE:

The seeds after having disinfected were soaked in culture filtrate of different concentration, S, S/2, S/10, S/100 and S/1000 for 24 hrs. Same number of seeds were soaked in distilled water as control. The seeds after soaking were removed and spread out on moist filter paper enlarge petridishes. Each plate contained 50 seeds. There were ten petriplates in each treatment. At the end of 12 days of incubation at 30 C the number of germinating seeds was determined. The incidence of damping-off was also determined.

### 3.14. EFFECT OF VARIOUS COMBINATION OF BOTH NEMATODES AND PATHOGENIC FUNGI ON SEEDLING EMERGENCE:

Previously sterilized soil mixture 30 cc/tray as in para section 2.1 was placed into plastic trays of 20 X 20 cm tray and nematised separately and concomitantly with fungi as per following scheme (Table - A).

| Treatments | |
|---|---|
| Rs | Mi |
| As | As + Mi |
| Fo | Fo + Mi |
| Pa | Pa + Mi |
| Rs + As | Rs + Mi |
| Rs + Fo | Rs + As + Mi |
| Rs + Pa | Rs + Fo + Mi |
| As + Pa | Rs + Pa + Mi |
| As + Fo | As + Pa + Mi |

| | |
|---|---|
| Fo + Pa | As + Fo + Mi |
| Rs + As + Fo | Fo + Pa + Mi |
| Rs + As + Pa | Rs + As + Fo + Mi |
| As + Fo + Pa | Rs + As + Pa + Mi |
| Fo + Pa + Rs | As + Fo + Pa + Mi |
| Rs + As + Fo +Pa | Fo + Pa + Rs + Mi |
| | Rs + As + Fo + Pa + Mi |
| Rr | Rr |
| As + Rr | Mi |
| Fo + Rr | Mi + Rr |
| Pa + Rr | Rs + Mi + Rr |
| Rs + Rr | As + Mi + Rr |
| Rs + As + Rr | Fo + Mi + Rr |
| Rs + Fo + Rr | Pa + Mi + Rr |
| Pa + Rs + Rr | Rs + As + Mi + Rr |
| As + Fo + Rr | Rs + Fo + Mi + Rr |
| Fo + Pa + Rr | Pa + Rs + Mi + Rr |
| Rs + As + Fo + Rr | As + Pa + Mi + Rr |
| Rs + As + Pa + Rr | As + Fo + Mi + Rr |
| As + Fo + Pa + Rr | Fo + Pa + Mi + Rr |
| Fo + Pa + Rs + Rr | Rs + As + Fo + Mi + Rr |
| Rs + As + Fo + Pa + Rr | Rs + As + Pa + Mi + Rr |
| | As + Fo + Pa + Mi+ Rr |
| | Fo + Pa + Rs + Mi + Rr |
| | Rs + As + Fo + Pa + Mi + Rr |

Mi = nematode, M. incognita (1000 J2/kg soil)
Rr = nematode, R. reniformis (1000 g.f./kg soil)
Rs = Fungus, Rhizoctonia solani
As = Fungus, Alternaria solani
Pa = Fungus, Pythium aphanidermatum
Fo = Fungus, Fusarium oxysporum f.sp. lycopersici
0.50 = indicates weight of muycelium mat (g)/kg soil.

In each tray surface sterilized seeds of tomato cv. Pusa Ruby were sown @ 2.00 seeds/tray. Trays were arranged in compltely randomised design with three replications. Seedling emergence was recorded at 5, 8 and 12 days after seedling. Other parameters recorded were viz; average galls, gall index were rated as given para section - 3.17.

## 3.15. EFFECT OF DIFFERENT DILUTIONS OF CULTURE FILTRATE OF FUNGI ON HATCHING AND MORTALITY OF *M. incognita* AND *R. reniformis*:

The fungi wer grown in the manner given in 3.7. The culture filtrate was obtained and was termed as standard (S). It was diluted to S/2, S/10, S/100 and S/1000 by adding the required amount of sterilized distilled water. Eggmass (5) of root-knot nematode and reniform nematode were transferred in cavity blocks containing 5 ml of culture filtrate of different concentrations of various fungi. After 12/24, 48 and 72 hrs the number of larvae hatched of root-knot nematode and pre adults of reniform nematode was determined number of larvae or pre adult hatched in distilled water served as control.

Similarly, for determining the mortality of larvae, the hatched larvae/ pre-adults (100) were transferred into cavity block containing 5 ml of culture filtrates of different concentrations of various fungi. The larvae were examined after 12, 24, 48 and 72 hrs. The number of immobilised larvae was counted. In order to ascertain whether they are dead, the immobilised larvae were placed in setrile water. If they do not regain mobility after 6 hrs were considered as dead. Five replicates were maintained of each treatment.

## 3.16. EFFECT OF INTERACTION BETWEEN PATHOGENIC FUNGI AND NEMATODES ON TOMATO cv. Pusa Ruby:

To study the interaction between fungi viz. *F. oxysporum* f.sp. *lycopersici*, *Rhizoctonia solani*, *Alternaria solani*, *Pythium aphanidermatum* and *Aspergillus niger* separately and nematodes (*M. incognita* and *R. reniformis*) the seedlings of tomato plant cv. Pusa Ruby were inoculated as follows:

| Treatments | |
|---|---|
| | Mi |
| | Rr |
| | F0.50 |
| | F1.00 |
| | F2.00 |
| Simultaneous | Inoculation |
| | An0.50 + F0.50 |
| | Mi + Rr |
| | Mi + F0.50 |
| | Mi + F1.00 |
| | Mi + F2.00 |
| | Mi + An0.50 |
| | Mi + An0.50 + F0.50 |
| | Mi + Rr + F0.50 |
| | Mi + Rr + F1.00 |

Mi + Rr + F2.00

Rr + F0.50

Rr + F1.00

Rr + F2.00

Rr + An0.50

Rr + An0.50 + F0.50

Sequential inoculation

| NaF | NbF |
|---|---|
| Mi +> F1.00 | Mi <+ F1.00 |
| Mi +> F2.00 | Mi <+ F2.00 |
| Mi +> An0.50 | Mi <+ An0.50 |
| Mi +> An0.50 + F0.50 | Mi <+ An0.50 + F0.50 |
| Mi + Rr +> F0.50 | Mi + Rr <+ F0.50 |
| Mi + Rr +> F1.00 | Mi + Rr <+ F1.00 |
| Mi + Rr +> F2.00 | Mi + Rr <+ F2.00 |
| Rr +> F0.50 | Rr <+ F0.50 |
| Rr +> F1.00 | Rr <+ F1.00 |
| Rr +> F2.00 | Rr <+ F2.00 |
| Rr +> An0.50 | Rr <+ An0.50 |
| Rr +> An0.50 + F0.50 | Rr <+ An0.50 + F0.50 |

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Mi = M. incognita (1000 J2/Kg soil).
Rr = R. reniformis (1000 gravid female/kg soil).
An = A. niger.
F = Fungus.
0.50, 1.00, 2.00 = Mycelium weight (g)
NaF (+>) = Nematode inoculated after one week of fungus inoculation.
NbF (<+) = Nematode inculated before one week of fungus inoculation.

The pots (15 cm-d) were filled with sterilized soil mixture as per section 3.1 and 3.2. The seedlings (15 days old) were inoculated with 0.50, 1.00, 2.00 gm mycelium mat /pot and 1000 J2/gravid nematodes /pot. The inoculum was contained in 100 sterilized water /pot was added. Proper agronomical practices were followed whenever required. Each treatment was replicated three times in completely randomised design. The observations were recorded as per section 3.17.

3.17. **OBSERVATIONS:**

Observations were recorded in all the inoculation studies after 60 days unless stated otherwise. The plants were uprooted, washed in running water and paper dried. The length and fresh weight of root and shoots were determined. The plants were dried at 60 C for 2 days. After cooling dry, weight was also determined.

Nematode population in soil/root was also determined. The nematodes were dissected/isolated from roots (Byrd et al.1983) and soil by Sieving + decantation method of Cobb and Baermann funnel technique (Southey, 1986).

The root-knot index was determined as follows the 0-5 scale (Taylor and Sasser, 1978):

0 = No galls no eggmasses

1 = 1 to 10 galls

2 = 11 to 30 galls

3 = 31 to 100 galls

4 = 101 to 200 galls

5 = 201 and above galls

The number of egg-masses was determined by dissecting the roots under the stereoscopic microscope. The Rf (reproduction factor) was calculated by dividing the final population (Pf) with the inoculated levels (Pi).

$$Rf = \frac{Pf}{Pi}$$

## 3.18. EFFECT OF LEAF EXTRACTS OF PLANTS ON NEMATODE AND RHIZOSPHERIC FUNGI:

Fourteen plant species viz;Ricinus communis, Argemone mexicana, Azadirachta indica, Cannabis sativa, Caltropis procera, Chenopodium album, Datura stramonium, Eucalyptus globulus, Lantana indica, Madhuca indica. Portulaca oleracea, Cassia fistula. Tagetes erecta and Thuja orientalis belonging to families, Euphorbiaceae, Papaveracae, Meliaceae, Cannabinaceae, Asclepiadaceae, Chenopodiaceae, Solanaceae, Myrtaceae, Verbenaceae, Sapotaceae, Portulaceae, Leguminosae, compositae and Cupressaceae respectively were selected for this purpose.

### 3.18.1 PREPARATION OF THE PLANT LEAF EXTRACT:

Freshly collected leaves of plants (50 g) were washed with water for 2-3 times and were ground in pestle and mortar with 100 ml distilled water. It was passed through muslin cloth. This extract was centrifuged @ 4000 r.p.m for 5 minutes and supernatant was filtered and sterilized by vaccum filtration through a 0.45 um size filter. It was termed as standard (S). It was diluted with sterile distilled water to obtain S/2, S/10, S/100 and S/1000.

### 3.18.2 EFFECT OF LEAF EXTRACT ON THE HATCHING AND MORTALITY OF *M. incognita* AND *R. reniforms*.

The details of technique employed for determining the effect of extract on hatching + mortality of larvae of both the nematodes were the same as detailed in para section 3.15 with culture filtrate of fungi except that culture filtrate was replaced with leaf extract.

### 3.18.3 EFFECT OF LEAF EXTRACT OF PLANTS ON GROWTH OF RHIZOSPHERIC FUNGI.

Richard's medium of double strength was used for determining the effect of leaf extracts on growth of fungi. Measured quantity of the 'S' standard extract was added to the medium so as to get S/2, S/10, S/100 and S/1000 as the final concentration. For 'S' concentration the medium was itself made in the concentration of leaf extract.

The flasks containing the medium with different concentration of extracts of various plants were inoculated with fungi. There were three replicates with 10 flasks in each treatment/ replicate. After 7 days of incubation at 28 C, the dry weight of the mycelium was determined as detailed in para section -3.9.2. The growth of the fungi in unamended medium served as control.

### 3.18.4 EFFECT OF SOIL AMENDMENT WITH CHOPPED LEAVES OF CERTAIN PLANTS ON INTERACTION BETWEEN FUNGUS (*F. oxysporum* f.sp. *lycopersici*; *A. niger*) AND NEMATODES viz; *M. incognita* and *R. reniformis*.

Chopped and finely ground leaves of the plants @ 50, 100 and 150 gm/kg soil were mixed with sterilized soil contained in 15 cm pots as para section 3.1 and 3.2. Each treatment was replicated thrice. The pots were watered regularly for ensuring proper decomposition of the amendment. After a week - long waiting period, 2 weeks old seedlings of tomato cv. Pusa Ruby raised in the manner given in para section - 3.3 were transplanted singly to each pot. These seedlings were inoculated as follows (Table - C).

| S.No. | Treatment |
|---|---|
| | Mi |
| | Rr |
| | Mi +> Cl100 |

Rr +> Cl100

F0.50 +> Cl100

Simultaneous inoculation

Mi + F0.50

Rr + F0.50

Mi +> F0.50 +> Cl100

Rr +> F0.50 +> Cl100

| NaF | NbF |
|---|---|
| Mi + F0.50 | Mi + F0.50 |
| Rr + F0.50 | Rr + F0.50 |
| Mi + F0.50 +> Cl100 | Mi + F0.50 +> Cl100 |
| Rr + F0.50 +> Cl100 | Rr + F0.50 +> Cl100 |

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| | |
|---|---|
| Mi | = M. incognita |
| Rr | = R. reniformis |
| Cl | = Chopped leaves |
| 100 | = leaves weight (g) |
| F | = Fungus (F. oxysporum f.sp. lycopersici) |
| 0.50 | = Mycelial weight in gm. |
| NaF (+>) | = Nematode inoculated after a week of fungus inoculation. |
| NbF (<+) | = Nematode inoculated before a week of the fungus inocualtion. |

Data on plant growth parameters, disease (Rf) of nematodes were calculated in the manner given in section - 3.17. Throughout the studies, appropriate checks were maintained. The data were subjected to statistical analysis.