MATERIALS
AND
METHODS
3. MATERIALS AND METHODS

3.1. Selection of test crops and pathogens:

Two nematode species, viz., the root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood and the reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira and two fungi, viz., the wilt-fungus, *Fusarium oxysporum* f. *ciceri* (Padwick) Subram. and the root-rot fungus, *Macrophomina phaseolina* (Tassi) Goid. were selected for the present study. Experiments with chickpea, *Cicer arrietinum* L. were conducted during winter season and for green gram/mungbean, *Phaseolus aureus* Roxb. during summer season. Respective rhizobia of the test plants were used as and when required. The wilt-fungus, *F. oxysporum* f. *ciceri* and the test nematodes were used in experiments concerning chickpea, whereas, in case of mungbean, the root-rot fungus, *M. phaseolina* along with the nematode species were used.

3.2. Preparation of inoculum of the nematodes:

Separate cultures of the root-knot nematode, *M. incognita* and the reniform nematode, *R. reniformis* were maintained on tomato in concrete microplots.

In case of root-knot nematode, eggmasses were collected from the infected roots of tomato plants with the help of forcep and were placed on small coarse sieve (1 mm pore size) fitted with moist tissue paper and placed in petri dishes (10
cm diameter) containing water. Second stage juveniles, which were hatched out, were collected along with water from petridishes after every 24 hrs. Fresh water was added to the petridishes after withdrawing the nematode suspension everytime. This process was repeated up to 5-7 days. These second-stage juveniles served the inoculum of the root-knot nematode.

For the extraction of the reniform nematode, soil was collected from around the roots of heavily infected tomato plants growing in the microplots. The soil was processed for extraction of the reniform nematode by using Cobb's sieving and decanting method along with modified Bearmann's funnel technique (Southey, 1986). The nematode suspension thus obtained, served the inoculum of the nematode.

Separate suspensions of the test nematodes, were gently stirred for making homogeneous distribution of nematodes and then 5 ml suspensions were transferred to the counting dish (Southey, 1986) and the numbers of 2nd stage juveniles in case of *M. incognita* and immature females in case of *R. reniformis* in each sample were counted under stereoscopic microscope. An average of five counts were made in each case to determine the density of nematodes per unit volume of these suspensions.

3.3. Preparation of inoculum of the fungi:

Pure cultures of the wilt-fungus, *Fusarium oxysporum* f. *ciceri* and the root-rot fungus, *Macrophomina phaseolina*, were
obtained from Indian Agricultural Research Institute (IARI), New Delhi. Pathogenicity of the fungi was tested on their respective hosts before use in different experiments. The fungi were maintained in culture tubes containing potato-dextrose-agar (P.D.A.) which were prepared from the following constituents:

\[
\begin{align*}
\text{Potato} & = 250.00 \, \text{g} \\
\text{Dextrose} & = 17.00 \, \text{g} \\
\text{Agar} & = 20.00 \, \text{g} \\
\text{Distilled water} & = 1000.00 \, \text{ml}
\end{align*}
\]

First the peeled pieces of the potato were boiled in distilled water and the extract so obtained (after removing the potato pieces) was mixed with dextrose and agar and boiled again. The medium in liquid condition (when still hot) was transferred to culture tubes which were plugged with cotton plugs. These tubes were then autoclaved. After cooling of the P.D.A., the test fungi were transferred to these culture tubes in an aseptic chamber by using all precautions prescribed to such an operation.

The inocula of the wilt-fungus, *Fusarium oxysporum* f. *ciceri* and the root-rot fungus, *Macrophomina phaseolina* were raised on Richard's liquid medium (Riker & Riker, 1936) having the following composition:

\[
\begin{align*}
\text{Potassium nitrate} & = 10.00 \, \text{g} \\
\text{Potassium dehydrogen phosphate} & = 5.00 \, \text{g} \\
\text{Magnesium sulphate} & = 2.50 \, \text{g} \\
\text{Ferric chloride} & = 0.02 \, \text{g} \\
\text{Sucrose} & = 50.00 \, \text{g} \\
\text{Distilled water} & = 1000.00 \, \text{ml}
\end{align*}
\]
One hundred ml of the above medium was transferred to 250 ml Erlenmeyer flasks which were plugged with cotton plugs covered with butter paper. Then these flasks were autoclaved. Small bits of the test fungi were transferred to these conical flasks in an aseptic chamber taking all the precautions prescribed for such an operation. These fungi were incubated for 15 days in an incubator running at 28°C temperature.

After the incubation period, the mycelial mats were removed and then gently passed between sterile blotting sheets to remove the excess amount of liquid. The inoculum was prepared by mixing 10 g fungal mycelium in 100 ml of sterilized distilled water in a waring blender for few seconds (Stemerding, 1964). In this way each 10 ml of this homogenate contained 1 g of fungal mycelium.

3.4. Varietal reaction to the nematodes:

Sandy loam soil, which is commonly found in Aligarh was collected from a fertile field in Aligarh Muslim Univeristy Agriculture Farm and were passed through a coarse seive (1 mm pore size) to remove stone particles and debris, etc. Compost manure at the rate of 1 g N/kg soil was added and thoroughly mixed with the soil. Fifteen cm clay pots were filled with 1 kg of the soil-compost mixture and then these pots were autoclaved and used for further studies.
Four uniform sized seeds of different cultivars of chickpea (Annigeri, C-375, Chaffa, E-100, EC-1538, F-61, F-404, JG-62, H-355, K-4, Hima, JGC-1, JG-23, JG-24, JG-221, ICC-391WR, BG-225, ICC-3103WR, ANM-123, BG-209, ICC-202WR, BG-220, Pusa-209 and K-850) and green gram/mungbean (K-851, T-44, PDM-11, PDM-54, PDM-84-139, PDM-146, 4/395 and ML-137) were surface sterilized in 0.1% mercuric chloride for 2 minutes, then thoroughly washed in sterile distilled water and sown in the pots containing sterilized soil. After emergence, the seedlings were thinned and only one seedling was allowed to grow in each pot. When the plants attained the age of three weeks these were separately inoculated with 50,500 and 5000 2nd stage juveniles of *M. incognita* oimmature females of *J. reniformis*. Uninoculated plants served as control. There were 5 replicates for each treatment including uninoculated control.

For the inoculation of plants with the nematodes, appropriate amounts of nematode suspension (according to the inoculum level) were poured around the root-surface which was exposed by removing small amount of soil. After inoculation, the exposed roots were covered by levelling the soil properly. The pots placed on a greenhouse bench in a randomised manner. Necessary weeding and watering were done as and when required.

Another experiment was also established on similar pattern using bacterized seeds. For chickpea, *Bradyrhizobium*
(chickpea type) and for mungbean, *Rhizobium phaseoli*, obtained from IARI, New Delhi, were used for bacterization of seeds. Sucrose solution (5%) was used as sticker and it was mixed with respective rhizobial culture. Seeds were mixed with this mixture in such a manner that a uniform coating formed on their surface. These bacterized seeds were dried at room temperature and then sown. Further steps were same as described above.

Both the experiments (with bacterized/unbacterized seeds) were terminated 100 days after seed germination and plant length (cm) / fresh weight (g), number of pods and other parameters were determined.

Nitrate reductase activity (NRA) in the leaves was estimated by the procedure of Jaworski (1971). The leaves were collected in the early morning, then cut into small pieces. Freshly harvested leaf tissue (200 mg) suspended in 5 ml mixture of 0.1 M phosphate buffer (pH 7.5), 0.02 M KNO₃, 5% propanol, and 2 drops of chloramphenicol (0.5 mg/ml) in sealed tube was incubated at 25°C in the dark for 60 minutes. NRA was measured by NO₂⁻ production which was detected by treating 0.4 ml of the incubation mixture with 0.3 ml each of 1% sulfanilamide in 3 M HCl and 0.02% N-1-nephthyl-ethylenediamine hydrochloride for 20 minutes. After appropriate dilution the absorption peak at 540 nm was measured with the help of Bouch & Lomb Spectronic-20 spectrophotometer.
Chlorophyll content of leaf was estimated by the method of Hiscox & Israelstam (1979). One hundred milligram of leaf pieces were placed in a vial containing 7 ml DMSO (Dimethyl Sulphoxide) and the chlorophyll was extracted into the fluid at 65°C by incubating it for 60 minutes. The extract was transferred to a graduated tube and made up to 10 ml with DMSO and assayed immediately. A sample of 3.0 ml chlorophyll extract was transferred in covette and the OD values at 645 and 663 nm were read in spectronic-1001 spectrophotometer against DMSO blank.

Number of root-galls per plant was determined in case of root-knot nematode-inoculated plants. While in case of reniform nematode, the pot soil from each treatment was separately processed after the termination of the experiment according to Cobb's sieving and decanting method followed by the modified Bearmann's funnel technique (Southey, 1986). Nematode population in roots was determined by macerating the root-pieces in a Waring blender for few seconds and then counting their numbers. Reproduction factor (R) of the nematode was calculated following the method of Oostenbrink (1966) by dividing the final population with the initial population (pf/pi).

In the experiment where bacterized seeds were used, root-nodule index (on 0-5 scale) was determined in case of chickpea on the basis of visual observation (0 = no nodulation
1 = very light nodulation, 2 = light nodulation, 3 = moderate nodulation, 4 = heavy nodulation and 5 = very heavy nodulation). Whereas, in case of mungbean, the root-nodules were counted per plant.

For both the experiments (with bacterized/unbacterized seeds) separate sets of 5 plants for each treatment were also kept for studying the effect of the nematode infection on pollen fertility. Pollen fertility (in percentage) at flowering stage was estimated by the method of Brown (1949) using stainability of pollen grains. The pollen grains which took up stain and had a regular outline were considered fertile while those which were empty without stain and had irregular shape were considered sterile.

3.5. Varietal reaction to the fungi:

Similar experiments, as in 3.4, were established to study the varietal reaction of chickpea to Fusarium oxysporum f. ciceri and green gram/mungbean to Macrophomina phaseolina. Three inocula of the fungi were used: 0.5, 1.0 and 1.5 g mycelium/plant. The inocula were prepared in the same manner as described in 3.3. Inoculations with fungi were made in the same manner as with the nematodes as described in 3.4.

Recording of the data was done in the same manner as described in 3.4. In case of mungbean, root-rot index was determined on 0-5 scale: 0 = no root-rot, 1 = very light root-rot, 2 = light root-rot, 3 = moderate root-rot, 4 = moderately severe root-rot, 5 = very severe root-rot.
3.6. Interrelationship of the pathogens:

Four surface sterilized seeds of chickpea cv. K-850 or mungbean cv. K-851 were sown in pots which were prepared as in 3.4. After germination, only one plant was allowed to grow per pot. Three-week old plants were inoculated with the test pathogens according to the following schedule:

1. No pathogen (control)
2. Meloidogyne incognita (MI) alone (5000 juveniles/plant)
3. Rotylenchulus reniformis (RR) alone (5000 immature females/plant)
4. Fungus (F) alone (1.0 g mycelium/plant)
5. MI + RR*
6. MI - RR**
7. RR - MI**
8. MI + F*
9. MI - F**
10. F - MI**
11. RR + F*
12. RR - F**
13. F - RR**
14. MI + RR + F*

(Note: * Simultaneous inoculations; ** First named pathogens inoculated 15 days prior to the second named pathogens; In case of chickpea the wilt-fungus, F. oxysporum f. ciceri was used whereas in case of mungbean, the root-rot fungus, Macrophomina phaseolina was used.)

The above experiments were done by using bacterized as well as unbacterized seeds separately. There were five replicates for each treatment including uninoculated control. Aftercare such as weeding, watering, etc. was done as and when required. The experiments were terminated 100 days after seed germination. Recording of the final data was done as described in 3.4 and 3.5.
3.7. Effect of the pathogens on growth stages of plants:

This experiment was, in a way, similar to the experiment described in 3.6. However, the recording of the data was done at different growth stages of plant (e.g., vegetative, anthesis, pre-blooming, full-blooming, post-blooming, pod-setting, pod-maturing) as per procedure described in 3.4 and 3.5. The inoculation schedule with different pathogens was as follows:

1. No pathogen (control)
2. Nematode alone (5000 specimens/plant)
3. Fungus alone (1.0 g mycelium/plant)
4. Nematode + Fungus

3.8. Effect of the pathogens inoculated at different age of plants:

This experiment was also conducted on same lines as in 3.6, however, the inoculations with the pathogens were made at different age of plants, e.g., 2,3,4,5,6,7,8 weeks. The inoculation schedule with the pathogens was same as in 3.7. The experiment was terminated 100 days after seed germination. Recording of the data was done as described in 3.4 and 3.5.

3.9. Effect of oil-seed cakes and nematicides on the pathogens and plant growth:

Fifteen cm clay pots were filled with 1 kg farm soil and autoclaved. These were then treated with oil-seed cakes of castor (*Ricinus communis* L.), neem (*Azadirachta indica* Juss.), mustard (*Brassica compestris* L.), duan (*Eruca sativa* L.), and nematicides.
Mill.) @ 1 g N/pot and nematicides, viz., aldicarb [2-methyl-2-(methylthio) propionaldehyde-O-methyl(carbamoyl) oxime], carbofuran [2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl-carbamate], dimethoate [O,O-dimethyl S-(methylcarbamoylmethyl) phosphorodithioate] @ 1 g a.i./pot. The pots receiving the nematicides were also supplemented with inorganic fertilizers in the form of urea @ 1 g N/pot), superphosphate (@ 0.5 g P/pot) and murate of potash (@ 0.5 g K/pot). The pots receiving oil-seed cakes were watered to ensure proper decomposition. After a week long waiting period surface sterilized seeds (bacterised/unbacterized) of chickpea cv. K-850 and mungbean cv. K-851 were sown separately at the rate of four seeds per pot. However, thinning was done after seed germination and only one plant was allow to grow per pot. Each set of plant with different treatments was inoculated with the pathogens according to the scheme given in 3.7.

Both the test nematodes were used with *F. oxysporum* f. *ciceri* in case of chickpea and *M. phaseolina* on mungbean. Each treatment was replicated five times. Aftercare such as weeding, watering, etc. was done when necessary. Recording of the data was done 100 days after seed germination as in 3.4 and 3.5.

3.10. Field experiment:

The experimental field situated at Aligarh Muslim University Agriculture Farm was thoroughly ploughed and
small beds measuring 6m² prepared, leaving 0.5 meter wide buffer zone between them. The beds were treated with oil-seed cakes (castor, neem, mustard, duan) @ 110 kg N/ha and nematicides (aldicarb, carbofuran, dimethoate) @ 1 kg a.i./ha. The nematicides were also supplemented with inorganic fertilizers: urea @ 110 kg N/ha, superphosphate @ 55 kg P/ha and murate of potash @ 55 kg K/ha. Untreated beds and those receiving inorganic fertilizers alone (at above rates) served as control. Each treatment was replicated five times which were arranged in a randomized manner. Immediately after treating the soil, the beds were watered and after one week, seeds of chickpea cv. K-850 or mungbean cv. K-851 were sown. These experiments were conducted under two separate conditions, viz., normal ploughing (20 cm deep) and deep ploughing (40 cm deep). The experiment was terminated 100 days after seed germination. Nematode population in each bed was determined before treating the soil as well as after terminating the experiment by processing of the representative soil sub-samples with Cobb's sieving & decanting and Bearmann's funnel techniques. Frequency of fungi from the rhizosphere soil (on dry weight basis) was also determined by dilution plate method of Dickinson & Pugh (1965). With the help of a sterilized pipette, 1 ml of 1:1000 dilution was transferred to sterilized petridishes and 10 ml of melted cooled peptone dextrose agar medium (Martin, 1950) was poured. Petriplates were rotated gently in order to equally
distribute the solution. The composition of peptone dextrose agar medium (Martin, 1950) was:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>20.0 g</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
<tr>
<td>Rose bengal</td>
<td>1:30000</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>30 μg/ml</td>
</tr>
</tbody>
</table>

For each treatment 20 petriplates were used. Petriplates were incubated at 28°C and the fungi which developed after one week were examined and identified. The frequency of fungi was calculated by the formula of Mclean & Cook (1957):

\[
\frac{\text{Number of plates containing a particular fungus}}{\text{Total plates poured}} \times 100
\]

Recording of the data with respect to the plant growth characters was done as in 3.4 and 3.5.

The effect of different treatments was also investigated in the next growing season. The field was again prepared in the same manner with normal ploughing treatment for all the beds, which were given half-dose of inorganic fertilizers. Chickpea was grown in beds where mungbean was growing in the preceding season whereas mungbean was grown in the beds where chickpea was grown in the preceding season.

Termination of the experiments and recording of the data were done on same line as for the preceding experiments.