Materials & Methods
The most widely occurring and economically important pathogens viz. reniform nematode (*Rotylenchulus reniformis* Race-3) and root-rot fungus (*Fusarium solani*) in the area and an economically important legume host, chickpea (*Cicer arietinum* var. Kranti) were selected for the present study. The experiments embodying the thesis were conducted in the presence and absence of heavy metal pollutants viz. chromium and nickel. These heavy metals were used in the form of their chloride salts.

### 3.1: PREPARATION AND STERILIZATION OF SOIL MIXTURE:

Sandy loam soil (Table-A) collected from a fallow field near to the campus of Aligarh Muslim University was sieved through 16 mesh sieve and mixed with organic manure in the ratio of 4:1, respectively. Throughout the course of studies, unless stated otherwise, 6 inch pots were filled with this soil mixture @ 1 kg/pot. A little amount of water was poured in each pot to just wet the soil before transferring to an autoclave for sterilization at 20 PSI pressure for 20 minutes. Sterilized pots were allowed to cool down at room temperature before use for experiments.

### 3.2: RAISING AND MAINTENANCE OF TEST PLANTS:

Seeds of chickpea var. Kranti were surface sterilized with 0.1% mercuric chloride (HgCl₂) for two minutes, then washed thrice in sterilized distilled water and treated with chickpea strain of *Rhizobium* before sowing. Sucrose solution (5%) was used as a sticker for bacterization. The bacterised seeds, dried at room temperature, were sown @ 5 seeds / pot and after their germination seedlings were thinned to one plant per pot. One week old, well established and healthy seedlings were used for experimental purposes throughout the course of investigation.
Table-A: Physical and chemical properties of soil and tap water used for the experimental purposes.

Sandy loam soil = sand (69%) + slit (23%) + clay (8%)

pH value of soil = 7.0

Cation Exchange Capacity (CEC) of soil = 4.0

Carbon Nitrogen (C/N) ratio = 9.5

Initial concentration of chromium (Cr) in soil = 2 ppm

Initial concentration of nickel (Ni) in soil = 4 ppm

Initial concentration of chromium (Cr) in water = 1 ppm

Initial concentration of nickel (Ni) in water = 1.20 ppm
3.3: RAISING AND MAINTENANCE OF NEMATODE CULTURE:

The pure culture of reniform nematode, *Rotylenchulus reniformis* was raised on castor plants using a single eggmass collected from infected roots of chickpea. The eggmass was surface sterilized by treating it with 1:500 aqueous solution of chlorox (Calcium hypochlorite) for 5 minutes as described by den Ouden (1958). Treated eggmass was washed thrice with distilled water. The eggs, in egg mass were allowed to hatch out at 28±2°C in BOD incubator on a coarse sieve lined with double layered tissue paper and kept in a Petri dish containing sufficient amount of sterilized distilled water. Castor seedling growing in 12 inch earthen pots containing autoclaved soil was inoculated with the reniform nematodes so obtained. Reniform nematodes were extracted from the pot soil after two months through graded sieves of 16, 60 and 400 mesh according to modified Cobb’s sieving and gravity method followed by Baermann funnel technique (Southey, 1986). Reniform nematodes so obtained were used for inoculating fresh castor seedlings grown in 12 inch earthen pots containing sterilized soil. The immature females of reniform nematode infested the roots and multiplied there in respective pots. After 6-8 weeks, a little of soil from the root zone was examined to confirm the establishment and multiplication of the nematode. After 2-3 months, the plants were cut at the ground level and soil was processed for the extraction of nematodes by the technique mentioned earlier. The roots were thoroughly washed under running tap water, cut into small pieces and transferred near the root zone of castor seedlings growing in large earthen pots locally called as “Nand” containing about 20 Kg sterilized soil. Separate soil suspension containing males and females (1:1) of *R. reniformis* was also transferred with the help of sterilized pipette to the root zone of castor seedling. Castor seedlings were inoculated from time to time to maintain the regular supply of nematode inoculum.
Culture of *R. reniformis* multiplied and maintained in this way was, thereafter used for obtaining required inoculum.

### 3.4: PREPARATION OF NEMATODE INOCULUM:

For preparing the inoculum of *R. reniformis* soil was collected from the root zone of heavily infected castor plants in which pure culture of nematode was raised. This soil was processed for extraction of immature females of reniform nematode using the technique mentioned earlier.

The water suspension of nematode was thoroughly stirred for making homogenous distribution of nematodes before taking 10 ml suspension in counting dish for counting the number of immature females of reniform nematode under the stereoscopic microscope. An average of three counts was taken to determine the density of nematodes in suspension.

Volume of water in the nematode suspension was so adjusted that each ml contained about 100 immature females. It was done by adding more water or decanting the excess amount of water so that 10 ml of this suspension contained 1000 immature females of *R. reniformis*. The male and female ratio of reniform nematode in the inoculum was 1:1, respectively.

### 3.5: ISOLATION OF FUNGUS FROM THE INFECTED CHICKPEA ROOTS:

The chickpea plants exhibiting root-rot symptoms were collected in polythene bags from the infected field at Mathura Road, Aligarh, where chickpea was cultivated on a large scale. Serial washing technique was employed to isolate *F. solani* from the infected root tissues (Harley and Waid, 1955). Infected pieces of roots were transferred to sterilized dish containing sterilized water and gently freed of soil particles. The root pieces were then transferred to another dish and the process was repeated till such time that all the adhering soil particles were removed. The root pieces were then cut into approximately 5 mm pieces and transferred to a 100 mm diameter Petri dish containing 0.1 % mercuric chloride solution. After about two minute, the root
pieces were given three successive washings in sterilized distilled water and soaked on filter paper. Five of these root pieces were plated in each of the 10 Petri dishes containing Potato Dextrose Agar (P.D.A.) with the help of sterilized forceps under aseptic conditions. These inoculated Petri dishes were incubated at 28 ±2⁰C for about 10 days. The fungus that developed on the root segments was examined and identified. On confirmation of its identity as *Fusarium solani*, its pure culture was prepared.

3.6: ISOLATION OF FUNGAL BIOCONTROL AGENTS FROM THE RHIZOSPHERE OF CHICKPEA:

About 25 chickpea plants were collected in polythene bags from the same field as mentioned above. Excess amount of soil adhering to the roots was removed by shaking the root system. The soil still left adhering to the roots was scrapped and collected over the butter paper with the help of sterilized needle. The soil thus obtained from each plant was thoroughly mixed and one microspatula of this soil was transferred to a Petri dish to which 15 – 20 ml of sterilized, melted and cooled potato dextrose agar was poured later. Ten plates were poured for each sample performing all operations under aseptic conditions. The inoculated Petri dishes were incubated at 28 ±2⁰C in BOD incubator. The fungi that developed were examined and identified after 10 days of inoculation. On confirmation of their identity as *Trichoderma harzianum* and *Paecilomyces lilacinus*, pure culture of these fungal biocontrol agents were prepared.

3.7: RAISING AND MAINTENANCE OF FUNGAL CULTURES:

The fungal inoculum of *F. solani*, *T. harzianum* and *P. lilacinus* was further raised on modified Richard’s medium as used by Harman *et al.* (1991), having following composition:
MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium nitrate (KNO₃)</td>
<td>10.00 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (KH₂PO₄)</td>
<td>5.00 g</td>
</tr>
<tr>
<td>Magnesium sulphate (MgSO₄·7H₂O)</td>
<td>1.30 g</td>
</tr>
<tr>
<td>Ferric chloride (FeCl₃)</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.00 g</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>150.00 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000.00 ml</td>
</tr>
</tbody>
</table>

The prepared medium was sterilized in an autoclave at 15 PSI pressure for 15 minutes in 250 ml Erlenmeyer flasks each containing about 100 ml of medium. Small bits of the mycelium of *F. solani* were transferred to these conical flasks. Same procedure was repeated for raising and maintenance of *T. harzianum* and *P. lilacinus*. Inoculated flasks were incubated at 28±2°C in BOD incubator for about 20 days to allow the fungal growth to be used for further studies.

Pure culture of *F. solani*, *T. harzianum* and *P. lilacinus* was continuously maintained on P.D.A. contained in test tubes by inoculation of the respective fungus after every 20 days.

**3.8: PREPARATION OF FUNGAL INOCULUM:**

After incubating the flasks with the test fungi for about 20 days, the liquid medium was filtered through Whatman’s filter paper No. 1, the mycelial mat was washed in distilled water to remove the traces of medium and gently pressed between the folds of blotting paper to remove the excess amount of water. Inoculum was prepared by mixing 10 g fungus (mycelial mat + spores) in 100 ml of sterilized distilled water and blending it for 30 seconds in a Waring blender (Stemerding, 1964). Thus each 10 ml of this suspension contained 1.0 g of fungus.
3.9: INOCULATION TECHNIQUES:

One week old chickpea seedlings were inoculated with either 1000 immature females of *R. reniformis* or 3g of *F. solani* throughout the course of investigations. Feeder roots of seedlings just before inoculations were exposed by removing the top layer of soil and the required quantity of nematode suspension / fungal inoculum was poured uniformly all around the exposed roots using sterilized pipette. Exposed roots were immediately covered after the inoculation by levelling the soil properly, followed by light irrigation.

Throughout the course of studies all the treatments were replicated three times. The pots were arranged in a randomized block design in an open field exposed to natural light and irrigated with tap water (Table-1) on alternate days.

3.10: PREPARATION OF STOCK SOLUTIONS OF HEAVY METALS:

Stock solution of chromium (1000 ppm) was prepared by using chromium chloride (CrCl$_3$.6H$_2$O), considering atomic weight of chromium as 51.9961 and molecular weight of the salt as 266.48. Similarly for nickel, the stock solution (1000 ppm) was prepared form Nickel chloride (NiCl$_2$. 6H$_2$O) taking into account atomic weight of Ni as 58.6934 and the molecular weight of the salt as 237.71. Appropriate amounts of stock solutions were added to distilled water so as to get the desired concentration of the heavy metals used in different experiments.

3.11: PREPARATION OF STOCK SOLUTION OF HEAVY METALS FOR EXPERIMENTS CONCERNING NEMATODE HATCHING AND MORTALITY:

Stock solution of chromium and Nickel were prepared in the same manner as described in 3.10. Desired dilutions viz. 25, 50, 100,200 and 400
ppm of heavy metals was obtained by adding appropriate quantities of stock solution to the nematode suspension.

3.12: PREPARATION OF STOCK SOLUTIONS OF HEAVY METALS FOR POT EXPERIMENT:

Stock solution of different concentration viz. 250, 500, 1000, 2000 and 4000 ppm of heavy metals were prepared in the same manner as described in 3.10. One hundred ml solution of each concentration was added to 1 Kg autoclaved soil per pot so as to get 25, 50, 100, 200 and 400ppm (on dry weight basis) of the metals in relation to known quantity of the soil used per pot.

3.13: EXPERIMENTS:

(i) RACE IDENTIFICATION OF RENIFORM NEMATODE, ROTYLENCHULUS RENIFORMIS:

The ten populations of reniform nematode, collected randomly from chickpea growing areas of Mathura Road and Khair Road locations of Aligarh were used for race identification. Among these populations five (MR-1, MR-2, MR-3, MR-4 and MR-5) were collected from Mathura Road and another five populations (KH-1, KH-2, KH-3, KH-4 and KH-5) were collected from Khair Road. These populations were cultured and maintained on castor grown in 15 inch earthen pots as described earlier.

Seeds of castor var. CH-1, cotton var. H-777, cowpea var. Pusa Komal, Bajra (Pennisetum typhoides var. Pusa 23) and mustard var. Pusa Bold were sown in 6 inch earthen pots containing sterilized soil. Soon after germination seedlings were thinned to one seedling per pot and one week well established seedlings were inoculated with 1000 immature females of reniform nematode / pot. All these crops except mustard which is a rabi season crop, were grown in kharif season.
MATERIALS AND METHODS

For observations of eggmasses per root system in each treatment, plants were depotted after 5 weeks of inoculation, roots were washed free of soil and then observed directly under stereoscopic binocular. The roots were then stained with acid fuchsin (Byrd et al., 1983) for recording number of females of *R. reniformis* per root system. Each treatment was replicated thrice.

Categorization of races of reniform nematode was done as suggested by Prasada Rao and Ganguly (1996).

(ii) STUDIES ON THE EFFECT OF HEAVY METALS ON THE HATCHING AND MORTALITY OF *ROTYLENCHULUS RENIFORMIS* IN VITRO:

The different concentration viz. 0, 25, 50, 100, 200 and 400 ppm of chromium and nickel were prepared in distilled water as described in 3.12.

(a) NEMATODE HATCHING:

For determining, the effect of heavy metals viz. Cr and Ni on the cumulative larval hatch of reniform nematode, five freshly picked eggmasses of nearby uniform size were sterilized and transferred to Petri dishes (40mm diameter) containing 5 ml solution of different concentration (25, 50, 100, 200 and 400 ppm) of Cr or Ni. Eggmasses kept in distilled water served as control and all the treatments were replicated thrice. These Petri dishes were incubated at 25°C in BOD incubator and the total number of hatched larvae were counted after 5 days with the help of nematode counting dish.

(b) NEMATODE MORTALITY:

For determining, the effect of heavy metals on mortality of *R. reniformis*, one hundred freshly hatched nematodes were transferred to Petri dishes (40 mm diameter) containing 5 ml solution of different concentrations (25, 50, 100, 200 and 400 ppm) of Cr or Ni, following the procedure described by Alam (1985). An aqueous suspension containing about 100 reniform
nematode was poured over a metallic sieve of 350 meshes (1.5 cm diameter and 1.0 cm height, fitted with a flat handle of 5 cm). Thus the nematodes remained over the mesh and the water was drained off. Then the sieve was inverted over a Petri dish and the nematodes were washed down with 5 ml solution of the heavy metal. In this way, the nematodes were transferred to the solutions without changing its concentration. The same procedure was repeated for different concentrations with washed sieve. Each treatment including distilled water as control was replicated thrice. After 12, 24, 48, 72 and 96 hours, the number of immobilized nematodes was first transferred to distilled water for an hour to ascertain their mortality. If they failed to regain mobility, they were considered as dead and then percent mortality was determined.

(iii) TO STUDY THE GROWTH AND SPORULATION OF ROOT-ROT FUNGUS, *Fusarium solani* IN PRESENCE OF HEAVY METALS:

Desired amounts of Cr and Ni were added into the modified Richard’s medium to obtain desired concentrations (25, 50, 100, 200 and 400 ppm) of each heavy metal in the medium. One hundred ml of modified Richard’s medium containing heavy metal of desired concentration was poured into 250 ml Erlenmeyer flasks. Modified Richard’s medium without heavy metal served as control and each treatment was replicated thrice. These flasks were then autoclaved at 15 PSI pressure for 15 minutes. After cooling, the flasks were inoculated with *F. solani* and incubated at 28 ± 2°C.

After 15 days of inoculation the mycelial mats were gently taken out from the flasks. These mycelial mats were kept in Petri plates. For estimation of sporulation of *F. solani*, the conidia were harvested by adding 20 ml sterilized distilled water per Petri plate and carefully scrapping the conidia present on the surface of the mycelial mat with a soft paint brush. Finally the distilled water along with conidia was decanted from each Petri plate. The number of microconidia, macroconidia and chlamydospores / mycelial mat
were determined with the help of a Haemocytometer. For determining the dry mycelial weight of *F. solani*, the mycelial mats were kept in an oven for about 24 hours running at 60°C temperature and dry mycelial weight was recorded.

In order to determine the uptake of heavy metals by *F. solani*, the dried and weighed mycelial mat was ashed in a muffle furnace at 450°C for 12 hours and digested with 1 ml of mixture of analar grade nitric acid and hydrogen peroxide (1:1). Then it was centrifuged at 3000 rpm and the clear supernatant was made up to 10 ml with distilled water and analyzed for metal by Flame-atOMIC absorption spectrophotometer (Varian Techtran AA 1475).

(iv) **DETERMINATION OF POTENTIAL PATHOGENIC LEVEL OF *ROTYLENCHULUS RENIFORMIS* AND *FUSARIUM SOLANI* ON CHICKPEA:**

In order to determine the potential pathogenic level of *R. reniformis*, capable of causing significant damage, one week old seedlings were inoculated with 250, 500, 1000, 2000, 4000 and 8000 immature females of reniform nematode. Similarly, for determination of potential pathogenic level of *F. solani*, the chickpea seedlings were inoculated with 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 g of fungus. Since inoculation of plants with 1000 immature females of *R. reniformis* or with 3 g of *F. solani* caused significant reduction in plant growth, these inoculum levels were used in subsequent studies.

(v) **EFFECT OF CHROMIUM AND NICKEL ON PATHOGENIC POTENTIAL OF *ROTYLENCHULUS RENIFORMIS* AND *FUSARIUM SOLANI* ON CHICKPEA:**

In order to study the effect of heavy metals (Cr and Ni) on pathogenic potential of either *R. reniformis* or *F. solani*, appropriate amounts of stock solutions of Cr/Ni were added to the pots in such a way to get the different concentrations (25, 50, 100 and 400 ppm) as per procedure described earlier. The seedlings raised in these pots were later inoculated with either 1000 immature females of *R. reniformis* or 3.0 g of *F. solani*. Untreated and
uninoculated plants served as control. Each treatment was replicated three times. The schedule of the treatments was as follows:

1. Untreated – Uninoculated
2. Untreated – Inoculated with *R. reniformis*
3. Untreated – Inoculated with *F. solani*
4. Treated with Cr / Ni 25 ppm
5. Cr / Ni 25 ppm + *R. reniformis*
6. Cr / Ni 25 ppm + *F. solani*
7. Cr / Ni 50 ppm
8. Cr / Ni 50 ppm + *R. reniformis*
9. Cr / Ni 50 ppm + *F. solani*
10. Cr / Ni 100 ppm
11. Cr / Ni 100 ppm + *R. reniformis*
12. Cr / Ni 100 ppm + *F. solani*
13. Cr / Ni 200 ppm
14. Cr / Ni 200 ppm + *R. reniformis*
15. Cr / Ni 200 ppm + *F. solani*

(vi) **ESTIMATION OF HEAVY METALS BY ATOMIC ABSORPTION SPECTROPHOTOMETRY (AAS) IN CHICKPEA PLANTS INFECTED WITH ROTYLENCHULUS RENIFORMIS / FUSARIUM SOLANI:**

After determining the dry weight of shoot and root of plants related to the experiment number (v), these plants were further dried in an oven at 105°C for 24 h. The dried material was grounded into the fine powder with the help of mortar and pestle. Five gram of the dried powder of each sample was digested in the 20 ml boiling Analar HNO₃ in a 50 ml Kjeldahl flask. Digestion usually completed within about half an hour. The digests were made up to 25 ml by adding required quantity of HNO₃. The prepared solution was used for the

The instrument (AAS) was calibrated by using a series of standards Cr solutions of varying concentrations (0.21 to 1.50 ppm). Absorbance of standards was measured at 358.2 nm wavelength using air-acetylene flame. The calibration curve was plotted and then concentration of Cr in the samples was determined.

Similarly, instrument (AAS) was calibrated by using a series of standards Ni solutions of varying concentrations (0.21 to 1.50 ppm). Absorbance of standards was measured at 232.3 nm wavelength using air-acetylene flame. The calibration curve was plotted and then concentration of Ni in the samples was determined.

(vii) EFFECT OF HEAVY METALS ON THE LIFE CYCLE OF ROTYLENCHULUS RENIFORMIS ON CHICKPEA:

In order to study the effect of heavy metals on the life cycle of reniform nematode, one week old and well established seedlings grown in the soil treated with 100 ppm of chromium or nickel were inoculated with 1000 immature females of *R. reniformis*. The seedlings grown in untreated soil and inoculated with *R. reniformis* served as control. Thus there were three set of treatments and each set having 120 plants.

Observations on the development of reniform nematode were recorded from three plants of each set after every 24 hours and continued up to the completion of life cycle. Roots were stained with 0.01% acid fuchsin in lactophenol to study unswollen, slightly swollen or fully swollen females with matrix and eggmasses. The eggmasses were picked up randomly and pressed under a cover slip on glass slide and the number of eggs per eggmass were counted. The soil was also processed to isolate the different stages of nematode present in the soil from the day of observation of egg laying females. The
temperature during the experimentation was in the range of $28 \pm 2^0 \text{C}$ under glasshouse conditions.

(viii) **EFFECT OF CHROMIUM AND NICKEL ON THE EFFICACY OF OIL-SEED CAKES, BIOCONTROL AGENTS AND BAVISTIN IN THE MANAGEMENT OF *ROTYLENCHULUS RENIFORMIS* AND *FUSARIUM SOLANI* INFECTING CHICKPEA:**

The powdered oil cakes of neem, mustard, mahua, castor, linseed and sesame were added and thoroughly mixed with soil @ 20g oil-cake /Kg autoclaved soil. The biocontrol agents viz. *T. harzianum* and *P. lilacinus* @ 2 g mycelia mat + spores were also separately added and thoroughly mixed with 1 Kg autoclaved soil and kept in 6 inch earthen pots. Appropriate quantities of Cr/Ni were also added to the pots to obtain concentrations of 100 ppm of the heavy metal in the soil. These pots were kept moist for three weeks to facilitate the proper decomposition of the oil-cakes. After a waiting period of three weeks, seeds of chickpea (5 seeds/ pot) were sown in these pots and after their germination seedlings were thinned to one seedling per pot.

For comparison of effect of oil-cakes and biocontrol agents, a generally recommended fungicide viz. carbendazim / Bavistin (2-(methoxy-carbamoyl)-benzimidazole) was also used for the management of fungus and nematode diseases. Seeds of chickpea were treated separately with Bavistin prior to sowing. A slurry coating containing 0.2 g Bavistin and 15 g talc powder (used as inert material) was made in 20 ml synthetic neutral gum (used as a sticker). Seeds weighing 100 g were added and the container was shaken to have coating of slurry over the seeds. A care was taken to have a uniform coating over the seeds. The treated seeds were then spread in a tray and allowed to dry in shade at room temperature. These treated seeds were then sown @5 seeds per pot in earthen pots containing heavy metals (100 ppm) viz. Cr or Ni and were thinned to one seedling per pot soon after germination.
The seedlings were later inoculated with either 1000 immature females of *R. reniformis* or 3.0 g of *F. solani*. Untreated and uninoculated plants served as control. Each treatment was replicated three times. The schedule of the treatments was as follows:

1. Untreated-Uninoculated control
2. *R. reniformis* (*Rr*)
3. *F. solani* (*Fs*)
4. Chromium (*Cr*)
5. Nickel (*Ni*)
6. *Rr* + *Cr*
7. *Fs* + *Cr*
8. *Rr* + *Ni*
9. *Fs* + *Ni*
10. Mustard cake (*Mc*)
11. *Mc* + *Rr*
12. *Mc* + *Fs*
13. *Mc* + *Cr*
14. *Mc* + *Ni*
15. *Mc* + *Rr* + *Cr*
16. *Mc* + *Fs* + *Cr*
17. *Mc* + *Rr* + *Ni*
18. *Mc* + *Fs* + *Ni*
19. Castor cake (*Cc*)
20. *Cc* + *Rr*
21. *Cc* + *Fs*
22. *Cc* + *Cr*
23. *Cc* + *Ni*
24. *Cc* + *Rr* + *Cr*
25. *Cc* + *Fs* + *Cr*
26. *Cc* + *Rr* + *Ni*
27. *Cc* + *Fs* + *Ni*
28. Mahua cake (*M*)
29. *M* + *Rr*
30. *M* + *Fs*
31. *M* + *Cr*
32. *M* + *Ni*
33. *M* + *Rr* + *Cr*
34. *M* + *Fs* + *Cr*
35. *M* + *Rr* + *Ni*
36. *M* + *Fs* + *Ni*
37. Linseed cake (*Lc*)
38. *Lc* + *Rr*
39. *Lc* + *Fs*
40. *Lc* + *Cr*
41. *Lc* + *Ni*
42. *Lc* + *Rr* + *Cr*
43. *Lc* + *Fs* + *Cr*
44. *Lc* + *Rr* + *Ni*
45. *Lc* + *Fs* + *Ni*
46. Sesame cake (*Sc*)
47. *Sc* + *Rr*
48. *Sc* + *Fs*
49. *Sc* + *Cr*
50. *Sc* + *Ni*
51. *Sc* + *Rr* + *Cr*
52. *Sc* + *Fs* + *Cr*
53. *Sc* + *Rr* + *Ni*
54. *Sc* + *Fs* + *Ni*
55. Neem cake (*Nc*)
56. *Nc* + *Rr*
57. *Nc* + *Fs*
58. *Nc* + *Cr*
59. *Nc* + *Ni*
60. *Nc* + *Rr* + *Cr*
61. *Nc* + *Fs* + *Cr*
62. *Nc* + *Rr* + *Ni*
63. *Nc* + *Fs* + *Ni*
64. *Paecilomyces lilacinus* (*Pl*)
65. *Pl* + *Rr*
66. *Pl* + *Fs*
67. *Pl* + *Cr*
68. *Pl* + *Ni*
69. *Pl* + *Rr* + *Cr*
70. *Pl* + *Fs* + *Cr*
71. *Pl* + *Rr* + *Ni*
72. *Pl* + *Fs* + *Ni*
73. *Trichoderma harzianum* (*Th*)
74. *Th* + *Rr*
75. *Th* + *Fs*
76. *Th* + *Cr*
77. *Th* + *Ni*
78. *Th* + *Rr* + *Cr*
79. *Th* + *Fs* + *Cr*
80. *Th* + *Rr* + *Ni*
81. *Th* + *Fs* + *Ni*
82. Bavistin (*B*)
83. *B* + *Rr*
84. *B* + *Fs*
85. *B* + *Cr*
86. *B* + *Ni*
87. *B* + *Rr* + *Cr*
88. *B* + *Fs* + *Cr*
89. *B* + *Rr* + *Ni*
90. *B* + *Fs* + *Ni*
To determine the infection of biocontrol agents *viz.* *P. lilacinus* and *T. harzianum* in eggmasses, eggs and females of *R. reniformis*, fifty females and eggmasses of reniform nematode were collected from the roots of plants inoculated with either *P. lilacinus* or *T. harzianum* and *R. reniformis*. These females and eggmasses were transferred separately into Petri dishes containing 1.0 % water agar and inoculated at 28±2°C in BOD incubator. After 7 days incubation, the percentage of fungus infected females and eggmasses was calculated. For determining the percentage of infected eggs in each eggmass, the eggmasses were stained with cotton blue in lactophenol and gently pressed over a glass slide to separate the eggs. The numbers of eggs infected were counted under the microscopic fields and the percentage of infected eggs was calculated.

(ix) SCREENING OF DIFFERENT VARIETIES OF CHICKPEA AGAINST *ROTYLENCHULUS RENIFORMIS, FUSARIUM SOLANI, CHROMIUM AND NICKEL:*

Twenty five chickpea varieties *viz.* Annegiri-1, Avarodhi, CSJD, JG-74, Gauraw, Gaut, Gulab, K-850, KGD-1168, KUSCR-2, KWR-108, Pant 186, Phule-G 8602, Phule G 92028, Phule G 96020, Pragati, Pusa-1103, Pusa-120, Pusa-1060, Radhey, Sadabahar, Vardan, Vijay, WCG-2 (Surya) and XVSCR-2 were screened for resistance to reniform nematode (*R. reniformis*), root-rot fungus (*F. solani*) and heavy metals (Cr and Ni). Five bacterised and sterilized seeds of each chickpea variety were sown in 6 inch earthen pots containing 1 kg autoclaved soil either untreated or treated with 100 ppm Cr or Ni. Soon after germination the seedlings were thinned to one plant per pot. One week well established seedlings were inoculated with either 1000 immature females of *R. reniformis* or 3g of *F. solani*. The untreated and uninoculated plants of each variety served as control and each treatment was replicated thrice. After three months the crop was terminated and plant dry weight (root and shoot), reproduction factor of nematode and disease index (%) of *F. solani* were determined. The degree of resistance and susceptibility of different chickpea
varieties to *R. reniformis, F. solani* and heavy metals was determined by using Resistance – Susceptibility Indices as given below.

Resistance – Susceptibility Indices:

(a) **FOR RENIFORM NEMATODE:**

1. Reproduction factor of nematode < 1.0; no significant reduction in plant growth = Resistant (R).
2. Reproduction factor of nematode 1.1-2.0; significant reduction in plant growth < 10.0 % = Moderately Resistant (MR).
3. Reproduction factor of nematode 2.1-4.0; significant reduction in plant growth 10.1-15.0 % = Tolerant (T).
4. Reproduction factor of nematode 4.1-7.0; significant reduction in plant growth 15.1-25.0 % = Susceptible (S).
5. Reproduction factor of nematode >7.00; significant reduction in plant growth > 25 % = Highly Susceptible (HS).

(b) **FOR ROOR-ROT FUNGUS:**

1. Disease index < 10.0%; no significant reduction in plant growth = Resistant (R).
2. Disease index 10.1 – 20.0%; significant reduction in plant growth < 10.0 % = Moderately Resistant (MR).
3. Disease index 20.1-30.0%; significant reduction in plant growth 10.1-15.0 % = Tolerant (T).
4. Disease index 30.1-50.0%; significant reduction in plant growth 15.1-25.0 % = Susceptible (S).
5. Disease index > 50.0%; significant reduction in plant growth > 25 % = Highly Susceptible (HS).
MATERIALS AND METHODS

(c) FOR HEAVY METALS:

Resistance rating against heavy metals (Cr and Ni) were determined by taking into account the percentage reduction in plant growth only as follows:

1. No significant reduction in plant growth = Resistant (R).
2. Significant reduction in plant growth < 10.0 % = Moderately Resistant (MR).
4. Significant reduction in plant growth 15.1-25.0 % = Susceptible (S).
5. Significant reduction in plant growth > 25 % = Highly Susceptible (HS).

3.14: RECORDING OF OBSERVATIONS:

(i) PLANT GROWTH DETERMINATION:

Plants were uprooted after 90 days of inoculation. Roots were washed thoroughly in slow running tap water. Utmost care was taken to avoid loss or injury of root system during the entire operation. For measuring length and weight, the plants were cut with a sharp knife just above the base of root emergence. Length of shoot and root was recorded in centimeters from the cut end to the tip of first leaf and the longest root, respectively. The excess water of plants was removed by putting them between the two folds of blotting sheets for some time before recording the fresh weight of shoots and roots. For measuring dry weight, the shoot and root were kept in envelops separately for drying in an oven running at 80°C for 24 hours and after this the dry weight of both root and shoot was taken separately. For interpretation of results, the reduction in plant growth was calculated in terms of percentage dry weight reduction.
MATERIALS AND METHODS

(ii) ROOT-NODULE ESTIMATION:

Nodulation was estimated by counting the number of nodules per root system.

(iii) SEED YIELD PER PLANT:

The pods from each plant, representing each treatment, were threshed and cleaned to assess the seeds weight per plant.

(iv) POPULATION ESTIMATION OF ROTYLENCHULUS RENIFORMIS:

For extraction of reniform nematode, the soil from each treatment was mixed thoroughly and a sub-sample of 200g soil was processed through sieves according to Cobb’s sieving and gravity method followed by Baermann funnel technique. However, in the experiment pertaining to life cycle, the extraction of reniform nematode from the soil was done according to Centrifugal – Floatation technique (Jenkins, 1964).

The nematode suspension was collected in a beaker and volume made upto 100ml. For proper distribution of nematodes, the suspension was bubbled with the help of pipette and 10ml suspension of each sample was drawn and transferred to a counting dish. The number of nematodes were counted in three replicates for each sample. Mean of three such counting’s was calculated and the final population of nematode / Kg soil was determined.

To estimate the nematode population in roots, 1.0g root from each replicate was macerated with enough water in an electrically operated waring blender for about 30-40 seconds. The macerate was collected in a beaker and volume made upto 100ml. The nematode population was counted as described above. Reproduction factor (R) of the nematode was calculated by the formula 
\[ R = \frac{P_f}{P_i} \]
where Pf represented the final and Pi the initial population of the nematode.
(v) Estimation of Disease index (%):

In order to determine the disease index in terms of *Fusarium solani* infection, washed roots of inoculated plants were cut into 1.0 cm pieces, then treated with 10% KOH solution and finally kept at 90 °C for 1 h. These root segments were washed again with distilled water, then acidified and stained with trypan blue (0.5% *V/V* in lactophenol) as described by Philips and Hayman (1970). Five stained pieces of each taproot were mounted on slides in lactophenol and presence of mycelium of the fungus was estimated. The disease index (%) was calculated by measuring the infected portion in relation to total length of root pieces.

(vi) DETERMINATION OF WATER ABSORPTION CAPACITY OF CHICKPEA ROOTS:

After 90 days of inoculation plants were uprooted and washed gently to avoid any damage to plant tissues. These plants were kept singly in 500 ml Erlenmeyer flasks containing a known amount of water (300 ml) with the support of cotton plugs. Flasks having cotton plugs and only water served as control. These flasks were kept on glasshouse bench with temperature ranging from 28±2°C. The plants were taken out of the flasks after 24 hours and the remaining quantity of water was measured. Amount of water lost from the unplanted flasks was deducted from the amount of water lost from the flasks having plants and the difference gave the actual amount of water lost by the plants or in other words amount of water absorbed by the roots.

(vii) ESTIMATION OF CHLOROPHYLL CONTENT:

The chlorophyll content in fresh leaf was estimated following the method worked out by Mackinney (1941).

One gram of finely cut fresh leaves and 20 ml of 80% acetone was grounded into a fine pulp using a mortar and pestle. The mixture was centrifuged at 5,000 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 the mark, using 80% acetone. The absorbance was read at 645 and 663 nm against the blank (80% acetone) on spectrophotometer. The chlorophyll content present in the extract (mg g⁻¹ tissue) was calculated using the following equations:

\[
\text{Chlorophyll a} = 12.7 \, (A_{663}) - 2.69 \, (A_{645}) \times \frac{V}{1000 \times W} \quad (\text{mg g}^{-1} \text{ fresh mass})
\]

\[
\text{Chlorophyll b} = 22.9 \, ((A_{645}) - 4.68 \, (A_{663})) \times \frac{V}{1000 \times W} \quad (\text{mg g}^{-1} \text{ fresh mass})
\]

Where

- \( A \) = absorbance at specific wavelengths
- \( V \) = final volume of chlorophyll extract in 80% acetone
- \( W \) = fresh mass of tissue used for extraction

(viii) ESTIMATION OF PROTEIN CONTENT:

The total protein content in seeds was estimated by adopting the methodology of Lowry et al. (1951). Fifty milligram (50 mg) of the oven dried seed powder was transferred to a mortar. The sample was ground with the addition of 1 ml of 5% trichloroacetic acid. The pulp was transferred to a glass centrifuge tube with repeated washing with 5% TCA to make the final volume 5 ml. The mixture was centrifuged at 4000 rpm for 15 minutes and the supernatant was discarded. Five ml of IN NaOH was added to the residue. The tube was kept in a water bath at 60° C for 30 minutes. After cooling for 15 minutes, the mixture was centrifuged at 4000 rpm for 15 minutes. The supernatant collected in 25 ml volumetric flask with repeated washings. Volume was made up to the mark by using IN NaOH and used to estimate total protein content. One ml of the above extract was transferred to a test tube and 5 ml of reagent C (Table-B) was added to it. The solution was shaken well and
Table- B: Reagents for the estimation of protein content of seeds.

<table>
<thead>
<tr>
<th>Reagent A</th>
<th>2% sodium carbonate (2 g dissolved in 100 cm³ DDW) and 0.1N NaOH (4g NaOH dissolved in 1000 cm³ DDW) were mixed in the ratio of 1:1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B</td>
<td>0.5% copper sulphate (500 mg CuSO₄ dissolved in 100 cm³ DDW) and 1% sodium tartarate (1g sodium tartarate dissolved in 100 cm³ DDW), were mixed in the ratio of 1:1.</td>
</tr>
<tr>
<td>Reagent C</td>
<td>50 cm³ of reagent A was mixed with 1cm³ of reagent B, except omission of sodium hydroxide</td>
</tr>
</tbody>
</table>
allowed to stand at room temperature for 15 minutes. Folin phenol reagent (0.5 ml) was added rapidly with immediate mixing. The blue colour developed. The absorbance of this solution was read at 660 nm using spectrophotometer. A blank was run with each set of samples. The total protein content was calculated by comparing the absorbance of each sample with a calibration curve plotted by taking known graded concentrations of bovine albumin.

(ix) STATISTICAL ANALYSIS:

The experimental data was analyzed following the standard procedures described by Gomez and Gomez (1984). Each treatment was replicated three times. The ‘F’ test was applied to assess the significance of the data at 5% and 1% level of probabilities. Least Significant Difference (L.S.D.) was calculated to compare the effect of various components by putting the values on the following formula.

\[
\text{L.S.D. (at 5\% level)} = \sqrt{\text{Standard Error} \times 2 \over \text{Replicates}} \times t(\text{value}) 5\%
\]

\[
\text{L.S.D. (at 1\% level)} = \sqrt{\text{Standard Error} \times 2 \over \text{Replicates}} \times t(\text{value}) 1\%
\]