EXPERIMENTAL INVESTIGATIONS
4. **Experimental Investigations:**

The present investigations on “Molecular characterization of *Pochonia chlamydosporia*, Zare and bio-management of disease complex in Okra (*Abelmoschus esculentus* L. Moench) using microbial antagonists” were carried out at Nematology laboratory of IIHR (Indian Institute of Horticultural Research), Hessarghatta, Bangalore North- 560 089.

The glasswares used in the experimental investigations were procured from Borosil; the plastic wares used were obtained from Tarsons. Reagents and chemicals used for the experiment were procured from Hi-media laboratories. Chemical composition of media, frequently used techniques such as sterilization of media, picking up of nematode egg-masses, inoculation of nematodes, serial dilution method and enrichment of Neem cake/ FYM or Vermicompost with the combination formulation of bio-pesticides are mentioned in the appendicies.

The strains of bio-agents used for the research work *viz.*, *P.chlamydosporia* and *P. fluorescens* were isolated in the nematology laboratory of IIHR (Indian Institute of Horticultural Research), Bangalore-89. The second stage juveniles (J2) of *M. incognita* were collected from egg-masses of okra culture stocks. *F. oxysporum f.sp.vasinfectum* strain which was isolated from okra by the scientist of Division of Plant Pathology, IIHR, Bangalore was used in the experiments.
In these investigations we developed the combination formulation of *P. chlamydosporia* and *P. fluorescens*. The efficacy of this combination formulation was evaluated in Nematology field, IIHR, Bangalore-89.

4.1 **Molecular characterization of strains of *P. chlamydosporia***

4.1.1 **Bio-agent isolation**

4.1.1.1 **Isolation of bio-control agent *P. chlamydosporia* through surveys**

Intensive surveys were conducted at various regions such as Kolar, Tumkur, Dharwad, Doddaballapur, Shimoga (Karnataka) and Nelliampatti (Tamil Nadu) to collect root and soil samples from the fields of okra affected with root-knot nematodes. Soil samples were also collected from undisturbed forest regions as they are rich in microbial flora and fauna. All the properly labeled root and soil samples collected were brought to carry out the investigations in the laboratory. Details regarding the nature of soil, present age of the crop and the types of chemicals, fertilizers applied were noted down.

In order to achieve the isolation of native bio-agents, roots of okra infested with root-knot nematode were selected for isolation of *P. chlamydosporia*. The fungus *P. chlamydosporia* was also isolated from soil using the semi-selective medium (appendices) as described by Kerry *et al.* (1993).
4.1.1.2 Bio-agent \textit{P. chlamydosporia} isolation from infected nematode egg-masses

Heavily galled roots of okra were selected for picking up of nematode egg-masses. The galled roots were thoroughly washed with tap water to remove all the soil adhering to the root system. The washed roots were placed in three 500ml beakers containing distilled water (200ml). The egg-masses adhering to the galled regions of the root system were picked up with the help of forceps and were placed in petri plates containing distilled water. Only the egg-masses which were blackened were selectively collected. Three petri plates were taken; in the first petri plate 0.1% mercuric chloride (2 ml) was placed followed by two petri plates of distilled water. These egg-masses were surface sterilized with mercuric chloride for 45 seconds later followed by washing with distilled water for 60 second respectively. The surface sterilized egg-masses were then placed in petri plates containing semi selective media. The plates were incubated for 5-7 days to observe the growth of the fungal culture \textit{P. chlamydosporia}.

4.1.1.3 Bio-agent isolation from soil samples:

In order to isolate the bio-agents from soil, two grams of soil sample was weighed and dissolved in sterile distilled water present in the test tube (18 cm x 150 cm). Serial dilution was carried out up to $10^{-7}$ dilutions. Then one ml of the serial diluted sample was pipetted out from $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilution tubes respectively. The pipetted dilutions were placed in petri
plates. To each plate sterilized semiselective media of about 15 ml was poured. The plates were then incubated for 5 to 7 days at 25°C ± 4°C.

4.1.2 Confirmation of *P. chlamydosporia* based on chlamydospore production.

The fungal mycelium was observed under microscope at 10X, 20X and 40X magnifications in stereo binocular microscope. Colonies were checked for the presence of verticilliate nature of the fungal mycelium (three phialides, bearing chlamydospores originating from the same point). Chlamydospore production was tested using the following method:

Protocol

1. Milled barley (50g) was washed on a 53µm sieve and was blot dried.

2. This was mixed with equal volumes of coarse sand and air dried.

3. Mixture was added with 15% of moisture and sterilized at 120°C for 20 minutes.

4. After cooling, four to five discs (5mm) of 15 days old culture of *P. chlamydosporia* grown on potato dextrose agar were inoculated into this substrate and were incubated for 3 weeks at 25 °C.

5. Contents were manually shaken for 5-10 minutes. This was repeated 2-3 times a day at a regular interval till the incubation period was over. This facilitates the aeration and helps in proper growth of the fungus.
6. Colonized media was tipped on to series of 250 µm sieve, over a 53µm sieve over a sieve tray.

7. Suspension was collected and poured on to 10 µm sieve and excess water was squeezed off using sponge.

8. Sieve was air dried and the chlamydospores were scrapped off carefully using smooth thin rubber pads.

9. Mixed with fine sand at 1:10 ratio and chlamydospore count/g substrate was calculated using haemocytometer.

4.1.3 Characterization of the bio-agent based on morphology:

The fungal cultures grown on the semi selective media were transferred onto solidified PDA (Potato Dextrose Agar) plates as well as CMA (Corn meal agar) plates in order to observe their sporulation patterns under aseptic conditions. The isolates differed markedly in their growth and sporulation in vitro (Irving & Kerry, 1986). The growth and sporulation patterns of the newly isolated strains of *P. chlamydosporia* on PDA (Potato Dextrose Agar) plates and CMA (Corn meal agar) plates were observed. The growth and sporulation patterns of the stains of *P. chlamydosporia* were also observed on PDB (Potato Dextrose Broth) placed in 250ml conical flasks. The plates and conical flasks were incubated for seven days at 25°C ± 4°C. After 24hrs on a daily basis the observations on their growth and sporulation were recorded.
The colonies of the culture which were identified as cultures of *P. chlamydosporia* were further sub-cultured on PDA (Potato Dextrose Agar) plates and stored for experimental investigations at around 4°C.

The observations on the rate of growth and the different characteristics of the colonies and sporulation pattern were taken at two different temperatures *viz.*, 24°C and 32°C. These observations were taken for one week. By measuring the radial growth of the culture, the rate of growth was estimated.

**4.1.4 Characterization of bio-agent based on Phenotype:**

The phenotypic characterizations of the fungal cultures were made on two different medium *viz.*, PDA (Potato Dextrose Agar) and CMA (Corn Meal Agar). 5 ml of molten and cooled PDA (Potato Dextrose Agar) as well as CMA (Corn Meal Agr) were amended with two antibiotic solutions which are chloramphenicol and streptomycin sulphate. Glass slides with three cavities were taken and into each cavity PDA media was poured and left to solidify. Similarly CMA media was also poured into the cavities of the glass slide and allowed to solidify. Once the glass slides with molten media were solidified fungal cultures were inoculated on to the media with the help of sterilized needles. These glass slides were later maintained in two big petri plates. One plate containing glass slides with PDA (Potato Dextrose Agar) media and the other plate containing glass slides with CMA (Corn Meal Agar). In order to maintain moisture for the growing cultures blotting sheets were cut in circular manner to fit the diameter of the petri plates.
and placed on the inner surface both on the top and bottom of the petri plates and sprinkled with some distilled water. The petriplates were incubated at 25°C± 4°C for three days.

Microscopic observations were carried out under the inverted microscope OLYMPUS 1X70 in order to estimate the sporulation pattern of the fungal cultures at all the stages. Based on the the orientation of the conidiophores, mycelium formation, and conidia as observed under the microscopic field the sporulation pattern was estimated. The characteristic feature of the fungal culture of this bio-control agent is verticillate.

4.1.5 Identification and conformation of *P. chlamydosporia* through Molecular studies:

To confirm the results obtained from characterization of the bio-agent through morphological and phenotypical means these molecular studies were conducted.

Molecular studies were conducted by exploiting the variations in ribosomal DNA. These studies were used to analyze the diversity of ribosomal DNA repeat units containing highly conserved DNA sequence regions as well as variable sequence regions such as Internal Transcribed spacers (ITS). Non- transcribed sequences like the intergenic spacers (IGS) also show sufficient variability to discriminate closely related fungi. Universal primers ITS-4 and ITS-5 were used to for analysis by Polymerase chain reaction. These primers were used in Polymerase chain reaction for
species level of identification of the newly isolated strains of 
*P. chlamydosporia*.

### 4.1.5.1 DNA isolation from the strains of *P. chlamydosporia:*

Genomic DNA was extracted from the 7 day old culture of 
*P. chlamydosporia* grown on potato dextrose broth by following the Raeder and Broda, 1985 method.

Protocol:

1. 0.5 gram of the mycelia powdered using liquid nitrogen was 
suspended in 1.5ml eppondorf tube containing 500µl of extraction 
buffer vortexed and homogenized throughly.

2. 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was amended 
into the above eppondorf tubes. The tubes were incubated at 60°C for 
20 minutes.

3. The tubes were centrifuged at 4°C and 10,000 rpm. Formation of 
three layers occurred after centrifugation.

4. The first aqueous upper layer formed was pipette out into a fresh 
1.5 ml eppendorf tube.

5. To the above eppondorf tubes 4µl of ribonuclease was amended and 
the tubes were incubated at 37°C for 10 minutes.
6. 500 µl of chloroform: isoamyl alcohol (24:1) was added and the tubes were centrifuged at 4°C and 10,000 rpm for two minutes. Formation of two layers occurred after centrifugation.

7. The first aqueous upper layer formed was later pipetted out very carefully into a fresh eppendorf tube.

8. In order to precipitate the DNA three fourth volume of ice cold isopropanol was added to the eppendorf tubes. These tubes were later centrifuged at 4°C and 10,000 rpm for two minutes.

9. Supernatant formed was discarded and to the pellet left behind in the tubes 100µl of 70% ethanol was amended, thoroughly mixed and again centrifuged for one minute.

10. The lid of the eppendorf tubes were open and left over night drying.

11. DNA pellet was dried and dissolved with 30-90µl of TE buffer after the complete evaporation of ethanol. This was stored for further use at 20°C.

12. The concentration of DNA was measured using the nano drop instrument.

4.1.5.2 Amplification of β- tubulin gene by Polymerase Chain Reaction using tub1f and tub1r primers.

All the primers used for the polymerase chain reaction were ordered and got synthesized from Bio-serve, Hyderabd. The template DNA used for
the Polymerase Chain Reaction analysis was the DNA isolated from the new strains of *P. chlamydosporia*.

Standard polymerase chain reaction of 25µl reaction was carried out by amending the following:

1. Template DNA : 1.0µl
2. 10xPCR buffer : 2.5µl
3. dNTP mix : 0.5µl
4. forward primer : 0.5µl
5. reverse primer : 0.5µl
6. Taq : 0.3µl
7. Nuclease free water : 19.7µl

Polymerase Chain Reaction amplification conditions included an initial denaturation for two minutes followed by 40 cycles of denaturation at 94°C for 40 seconds, primer annealing temperature at 50°C for two minutes and extension at 72°C for two minutes followed by final extension at 72°C for eight minutes. Primers recognize an intron that is present in the *P. chlamydosporia* – β tubulin gene.
4.1.5.3 Analysis of Polymerase chain reaction Product by agarose gel electrophoresis:

The Polymerase chain reaction product was analysed by running 5µl of the product mixed with three µl bromophenol blue loading dye on two% agarose gel (appendicies) placed in 0.5x TAE buffer at 100 volts. For reference 1kb ladder of Fermantas™ was used. The bands obtained after the Polymerase Chain Reaction were visualized under UV illumination.

4.1.5.3.1 Sequencing and analysis of β-tubulin gene.

The resulting Polymerase Chain Reaction product was purified using the Polymerase Chain Reaction purification kit supplied by Bioserve. Later the product was sent for sequencing to Bioserve, Hyderabad, India. The sequence results were obtained and analysed at NCBI server.

Sequence analysis of ITS regions, IGS regions and β-tubulin gene was done by converting the sequence results into FASTA format and later analysed using the blast tool at the NCBI server.

4.2 To study the compatibility of P. chlamydosporia and P. fluorescens under in vitro and in vivo conditions.

4.2.1 Evaluating the Compatibility of P. chlamydosporia and P. fluorescens under in vitro conditions.

100 ml of Kings’s B broth (appendicies) was prepared in a 250ml conical flask. To this broth the bacterial culture of P. fluorescens (PF-7) was
inoculated a day before the actual experiment was initiated using a sterilized loop. The 250 ml conical flask was incubated at room.

With the help of a cork borer disc shaped cultures of \textit{P. chlamydosporia} were obtained. The discs of culture were about 5 mm in diameter. These discs were later inoculated onto solidified PDA (Potato Dextrose Agar) petri plates. The petri plates were incubated at 25°C±4°C.

In order to evaluate the compatibility of the newly isolated strains of \textit{P. chlamydosporia} (PC-3 and PC-2) with \textit{P. fluorescens} (PF-7) there were 3 different set of experiments which were conducted under \textit{in vitro} conditions. Three replicates were maintained for all the three sets of experiments that were conducted. All the petri plates were incubated at 25°C±4°C. Observations on the radial growth of the fungal cultures \textit{P. chlamydosporia} (PC-3 and PC-2) and their interaction with the bacterial culture \textit{P. fluorescens} were recorded every day till the termination of the experiment.

\textbf{4.2.1.1 I SET}

About half of the petri plate was streaked with the bacterial culture \textit{P. fluorescens} (PF-7) at one end with help of a sterilized loop. A fresh fungal culture disc of \textit{P. chlamydosporia} of about 5 mm in diameter was placed in the petri plate after 8 hours of inoculation of the bacterial culture at the other end of the plate on the same day. This set of experiment was conducted separately with the newly isolated strains of \textit{P. chlamydosporia} (PC-3 and PC-2).
The pertri plates seperately inoculated with only *P. chlamydosporia* (PC-3 and PC-2) without the inoculation of the bacterial culture *P. fluorescens* (PF-7) and the pertri plates inoculated with only the bacterial culture *P. fluorescens* (PF-7) without the fungal culture were maintained as control.

4.2.1.2 II SET

With the help of a sterilized loop fresh bacterial culture of *P. fluorescens* (PF-7) was streaked right at the centre of the petri plate. Later after 8 hours two fresh fungal culture discs of *P. chlamydosporia* of about 5mm in diameter were placed on either side of the streaked bacterial culture. This set of experiment was conducted separately for each strain of the newly isolated strains of *P. chlamydosporia* (PC-3 and PC-2).

The pertri plates seperately inoculated with only *P. chlamydosporia* (PC-3 and PC-2) without the inoculation of the bacterial culture *P. fluorescens* (PF-7) and the pertri plates inoculated with only the bacterial culture *P. fluorescens* (PF-7) without the fungal culture were maintained as control.

4.2.1. III SET

With the help of a sterilized loop fresh bacterial culture of *P. fluorescens* (PF-7) was streaked at one end of the petri plate in a zig zag pattern. After about 8 hours two fresh fungal culture disc of *P. chlamydosporia* of about 5mm in diameter was placed at other end of the
petri plate. This set of experiment was conducted separately for each strain of the newly isolated strains of *P. chlamydosporia* (PC-3 and PC-2).

The pertri plates seperately inoculated with only *P. chlamydosporia* (PC-3 and PC-2) without the inoculation of the bacterial culture *P. fluorescens* (PF-7) and the pertri plates inoculated with only the bacterial culture *P. fluorescens* (PF-7) without the fungal culture were maintained as control.

Percentage compatibility between the fungal culture *P. chlamydosporia* (PC-3 and PC-2) and the bacterial culture *P. fluorescens* (PF-7) was calculated based on the following formula:

\[
\% \text{ compatibility} = 100 - \% 'I' \quad (\text{Where } I \text{ is } \% \text{ Inhibition or } \% \text{ Increase})
\]

The percentage increase in the radial growth of the cultures was calculated by using the formulation

\[
\% \text{ increase} = \frac{\text{Growth weight in dual culture} - \text{Growth weight in control}}{\text{Growth weight in control}} \times 100
\]

The percentage inhibition in the radial growth of the colony is calculated by using the formulation

\[
\% \text{ inhibition} = \frac{\text{Growth weight in control} - \text{Growth weight in dual culture}}{\text{Growth weight in control}} \times 100
\]
4.2.2 Evaluating the compatibility of *P. fluorescens* and *P. chlamydosporia* by mycelial dry method:

The compatibility of *P. chlamydosporia* and *P. fluorescens* was also evaluated by conducting mycelial dry weight experiment. This particular experiment was carried out in order to confirm the results of the dual culture method.

100 ml of Kings’s B (appendices) broth was prepared in a 250ml conical flask. To this broth the bacterial culture of *P. fluorescens* (PF-7) was inoculated a day before the actual experiment was initiated using a sterilized loop. The 250 ml conical flask was incubated at room temperature.

In a 500ml conical 250 ml of PDB (Potato Dextrose Broth) was prepared. To this flask 0.5 ml of the bacterial culture of *P. fluorescens* (PF-7) and fresh fungal culture disc of *P. chlamydosporia* were inoculated. The newly isolated strains of *P.chlamydosporia* (PC-3 and PC-2) were evaluated for their compatibility separately with *P. fluorescens* (PF-7). For the experiment 3 replicates were maintained.

Conical flask inoculated with only *P. chlamydosporia* (PC-3 and PC-2) separately without inoculation of *P. fluorescens* were treated as controls. Conical flask inoculated with only *P. fluorescens* separately without inoculation *P. chlamydosporia* (PC-3 and PC-2) were treated as control. The conical flasks were incubated at 25°C±4°C.
Observations on the radial growth of the fungal cultures *P. chlamydosporia* (PC-3 and PC-2) and their interaction with the bacterial culture *P. fluorescens* were recorded for a period of fifteen days.

After fifteen days the contents in the conical flask were nicely mixed by shaking and one ml was pipetted out from the flask to check the colony forming units (CFU) by serial dilution method on both PDA (Potato Dextrose Agar) plates and King’s B plates.

Since this experiment is mycelial dry weight experiment the contents in each flask were allowed to get filtered through whatman filter paper no.1 which was placed in a funnel. The fungal mat collected over the filter paper was dried completely by placing the filter papers in Hot Air Oven at 36ºC for about 3-4 hours. The filter paper weight alone was taken at the beginning of the experiment and later the dry weight of the fungal culture mat was also recorded.

Percentage compatibility between the fungal culture *P. chlamydosporia* (PC-3 and PC-2) and the bacterial culture *P. fluorescens* (PF-7) was calculated based on the following formula:

\[
\text{% compatibility} = 100 - \% I \ (\text{where I is Inhibition or Increase})
\]

The percentage increase in the weight of the culture is calculated by using the formulation

\[
\text{% increase} = \frac{\text{Growth weight in dual culture} - \text{Growth weight in control}}{\text{Growth weight in control}} \times 100
\]
The percentage inhibition in the weight of the culture is calculated by using the formulation

\[
\% \text{ inhibition} = \frac{\text{Growth weight in control} - \text{Growth weight in dual culture}}{\text{Growth weight in control}} \times 100
\]

4.2.3 Evaluating the efficacy of *P. chlamydosporia* and *P. fluorescens* on the target fungal pathogen *F. oxysporum* f.sp. *vasinfectum*

In order to evaluate the efficacy of the newly isolated strains of *P. chlamydosporia* (PC-3 and PC-2) and *P. fluorescens* (PF-7) against *F. oxysporum* f.sp.*vasinfectum* there were 3 different set of experiments which were conducted under *in vitro* conditions. Three replicates were maintained for all the three sets of experiments that were conducted. All the petri plates were incubated at 25°C±4°C. Observations on the radial growth of the fungal cultures *P. chlamydosporia* (PC-3 and PC-2) and their interaction with the bacterial culture *P. fluorescens* in inhibiting the growth of target fungal pathogen *F. oxysporum* f.sp.*vasinfectum* were recorded every day till the termination of the experiment.

4.2.3. 1 SET

With the help of a sterilized cork borer and needle a 5mm culture disc of *P. chlamydosporia* was placed at one end of the petri plate. At the other end of the plate a 5mm culture disc of the target fungal pathogen *F. oxysporum* f.sp.*vasinfectum* was placed. This set of experiment was
conducted separately for each strain of the newly isolated strains of *P. chlamydosporia* (PC-3 and PC-2).

The petri plate’s seperately inoculated with only *P. chlamydosporia* (PC-3 and PC-2) without the inoculation of the target fungal pathogen *F. oxysporum f.sp. vasinfectum* were treated as control. The perti plates inoculated only with the fungal pathogen *F. oxysporum f.sp. vasinfectum* were treated as control.

**4.2.3. I ISET**

With the help of a sterilized loop fresh bacterial culture of *P. fluorescens* (PF-7) was streaked at one end of the petri plate in a zig zag pattern. After about 8 hours a 5mm culture disc of the target fungal pathogen *F. oxysporum f.sp. vasinfectum* was placed at the other end of the petri plate.

The perti plates inoculated only with the fungal pathogen *F. oxysporum f.sp. vasinfectum* were treated as control. The perti plates streaked only with the bacterial culture *P. fluorescens* (PF-7) were treated as control.

**4.2.3. III SET**

With the help of a sterilized loop fresh bacterial culture of *P. fluorescens* (PF-7) was streaked at one end of the petri plate in a zig zag pattern. After about 8 hours a fresh fungal culture disc of the target pathogen *F. oxysporum f.sp. vasinfectum* of about 5 mm was
placed at middle of the petri plate, followed by inoculation of
*P. chlamydosporia* (PC-3 and PC-2) at the other end of the plate. This set of
experiment was conducted separately for each strain of the newly isolated
strains of *P. chlamydosporia* (PC-3 and PC-2).

The petri plates inoculated only with the fungal pathogen
*F. oxysporum* f.sp.*vasinfectum* were treated as control. The petri plates
streaked only with the bacterial culture *P. fluorescens* (PF-7) were treated
as control. The petri plates inoculated only with the fungal culture
*P. chlamydosporia* (PC-3 and PC-2) were treated as control.

Percentage inhibition in the radial growth of the cultures was
calculated by using the formula:

\[
\text{% inhibition} = \frac{\text{Growth weight in control} - \text{Growth weight in dual culture}}{\text{Growth weight in control}} \times 100
\]

**4.2.4 Evaluating the efficacy of *P. chlamydotosporia* and *P. fluorescens* on the target fungal pathogen *F. oxysporum* f.sp. *vasinfectum* by dry mycelia method:**

The efficacy of *P. chlamydotosporia* and *P. fluorescens* in inhibiting the
growth of the target fungal pathogen *F. oxysporum* f.sp.*vasinfectum* was
evaluated by conducting mycelial dry weight experiment. This particular
experiment was carried out in order to confirm the results of the above
experiments.
100 ml of Kings’s B broth (appendicies) was prepared in a 250ml conical flask. To this broth the bacterial culture of *P. fluorescens* (PF-7) was inoculated a day before the actual experiment was initiated using a sterilized loop. The 250 ml conical flask was incubated at room temperature.

In a 500ml conical flask 250 ml of King’s B broth was prepared and 0.5 ml of the bacterial culture of *P. fluorescens* (PF-7) was inoculated into the flask. To the same conical flask a fresh fungal culture disc of the target pathogen *F. oxysporum* f.sp.*vasinfectum* was also added. The conical flasks were incubated at room temperature. Three replicates were maintained.

Conical flasks inoculated only with the bacterial culture of *P. fluorescens* (PF-7) and the conical flasks inoculated only with the target fungal pathogen *F. oxysporum* f.sp.*vasinfectum* were treated as control.

In a 500ml conical flask 250 ml of PDB (Potato Dextrose Broth) was prepared and fresh fungal culture disc of *P. chlamydosporia* was inoculated. To the same flask a fresh fungal culture disc of the target pathogen *F. oxysporum* f.sp.*vasinfectum* was also added. The newly isolated strains of *P. chlamydosporia* (PC-3 and PC-2) were evaluated for their efficacy in inhibiting the growth of the target pathogen *F. oxysporum* f.sp.*vasinfectum* separately. For the experiment 3 replicates were maintained and the flasks were incubated at room temperature.

Conical flask inoculated with only with *P. chlamydosporia* (PC-3 and PC-2) the conical flasks inoculated only with the target fungal pathogen *F. oxysporum* f.sp.*vasinfectum* were treated as control.
In a 500ml conical flask 250 ml of PDB (Potato Dextrose Broth) was prepared and fresh fungal culture disc of *P. chlamydosporia* was inoculated. To the same flask a fresh fungal culture disc of the target pathogen *F. oxysporum f.sp.vasinfectum* as well as 0.5 ml of the bacterial culture of *P. fluorescens* (PF-7) was inoculated. The newly isolated each strains of *P. chlamydosporia* (PC-3 and PC-2) along with the bacterial culture of *P. fluorescens* (PF-7) were evaluated for their efficacy in inhibiting the growth of the target pathogen *F. oxysporum f.sp.vasinfectum* separately. For the experiment 3 replicates were maintained. The conical flasks were maintained at room temperature.

Conical flask inoculated with only with *P. chlamydosporia* (PC-3 and PC-2) and the conical flasks inoculated only with the target fungal pathogen *F. oxysporum f.sp.vasinfectum* were treated as control as well as conical flasks inoculated only with the bacterial culture of *P. fluorescens* (PF-7) were treated as control.

Observations on the radial growth of the fungal cultures *P. chlamydosporia* (PC-3 and PC-2) and their interaction with the bacterial culture *P. fluorescens* in inhibiting the growth of the target fungal pathogen *F. oxysporum f.sp.vasinfectum* were recorded for a period of fifteen days.

After fifteendays the contents in the conical flask were nicely mixed by shaking and one ml was pipetted out from the flask to check the colony forming units (CFU) by serial dilution method on both PDA (Potato Dextrose Agar) plates and King’s B plates.
Since this experiment is mycelial dry weight experiment the contents in each flask were allowed to get filtered over whatman filter paper no.1 which was placed in a funnel. The fungal mat collected over the filter paper was dried completely by placing the filter papers in Hot Air Oven at 36ºC for about 3-4 hours. The filter paper weight alone was taken at the beginning of the experiment and later the dry weight of the fungal culture mat was also recorded.

Percentage inhibition in the growth of the target fungal pathogen cultures was calculated in terms of weight by using the formula:

$$\% \text{ inhibition} = \frac{\text{Growth weight in control} - \text{Growth weight in dual culture}}{\text{Growth weight in control}} \times 100$$

4.2.5 Evaluating the compatibility of P. chlamydosporia and P. fluorescens under in vivo conditions.

To evaluate the compatibility of P. chlamydosporia and P. fluorescens a vegetable crop tomato was chosen, as the growth of tomato is faster when compared to other vegetable crops and accurate data in terms of root length, root weight, shoot length and shoot weight can be obtained.

Mass production of the two strains of P. chlamydosporia (PC-3 and PC-2) and P. fluorescens (PF-7) were taken up. Each strain of P. chlamydosporia (PC-3 and PC-2) and P. fluorescens (PF-7) was tested individually as well as in combination by means of seed treatment and
substrate treatment. Tomato seeds were treated with talc based formulation of *P. chlamydosporia* and *P. fluorescens* individually as well as in combination. The substrate taken for raising the seedlings of tomato was cocopeat. Five grams of each of *P. chlamydosporia* and *P. fluorescens* were mixed in 1.5kg of cocopeat and filled into seedling trays. A total of 6 treatments were planned.

T1: Seeds of Tomato as well as the substrate-cocopeat were treated with *P. chlamydosporia* (PC-3) talc based formulation: PC-3 (SD+SB)

T2: Seeds of Tomato as well as the substrate-cocopeat were treated with *P. chlamydosporia* (PC-2) talc based formulation: PC-2 (SD+SB)

T3: Seeds of Tomato as well as the substrate-cocopeat were treated with *P. fluorescens* (PF-7) talc based formulation: PF-7(SD+SB)

T4: Seeds of Tomato as well as the substrate-cocopeat were treated with talc based combination formulation of *P. chlamydosporia* (PC-3) and *P. fluorescens* (PF-7). PC-3+PF-7(SD+SB)

T5: Seeds of Tomato as well as the substrate-cocopeat were treated with talc based combination formulation of *P. chlamydosporia* (PC-2) and *P. fluorescens* (PF-7).PC-2+PF-7(SD+SB)

T6: Control. Individual treatments were replicated five times in a CRBD (Completely Randomized Block Design).
4.2.5.1 Estimating the plant growth parameters:

5 seedlings from each treatment were randomly picked and growth parameters such as shoot length, shoot weight, root length, and root weight were recorded.

4.2.5.2 Estimating the extent of root colonization:

In order to study the extent of root colonization by both *P. chlamydosporia* and *P. fluorescens* around 1g of the root was surface sterilized and finely grounded using mortar and pestle. Serial dilution was carried out and 1ml from dilutions $10^{-4}$, $10^{-5}$, $10^{-6}$ were placed in petri plates. Molten Potato Dextrose Agar (PDA) was later poured into each plate. 3 replicates were maintained for each dilution. Dilution of $10^{-5}$, $10^{-6}$ and $10^{-7}$ were pipetted out into petri plates and molten King’s B agar was poured into these plates. The contents in the plates were thoroughly mixed by moving the petri plates in a circular fashion. All the plates were incubated at 25 ±4°C for 5 days. Observations on the colony forming units were recorded.

4.3 Development of the combination formulation of *P. fluorescens* + *P. chlamydosporia* and evaluate the shelf life.

From the compatibility, mycelial dry weight experiment the newly isolated strain of *P. chlamydosporia* (PC-3) proved effective in inhibiting the growth of the target fungal pathogen *F. oxysporum* f.sp. *vasinfectum* was selected for mass production.
1 ml of \textit{P. fluorescens} was inoculated into 1000ml conical flask containing King's B Broth. A 5mm fresh culture disc of \textit{P. chlamydosporia} (PC-3) was inoculated into a conical flask containing Potato dextrose broth. The culture was allowed to grow for a period of one week. After a week 400ml of \textit{P. fluorescens} was mixed into 1kg of talc powder and to this 50g of CMC was also added. The resulting mixture was thoroughly mixed and left for drying in trays for five days. Similarly the formulation of \textit{P. chlamydosporia} was also prepared in the same manner.

500g of \textit{P. chlamydosporia} talc based formulation and 500g of \textit{P. fluorescens} were mixed together and sealed in polypropylene packets. In this manner 15 packets were made and labeled. Each month one packet was taken and 1g of the formulation was taken and CFU was carried out by dilution plating method on PDA and Kings B agar media. Serial dilutions up to $10^{-6}$ concentration were prepared. 1ml from each of $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions were pipetted into the Petri dishes and spread completely in the plate. Freshly prepared PDA or Kings B agar media was poured into each plate and made to spread evenly by pour plate method and allowed for solidification. Three replicates for each dilution were maintained with controls and CFU was recorded. The CFUs were estimated for a period of 15 months in the similar way.
4.4 Evaluation of the bio-efficacy of *P. fluorescens* and *P. chlamydosporia* against *M. incognita* and *F. oxysporum* f.sp. *vasinfectum* under screen house conditions.

4.4.1(A) Experimental investigation was conducted under screen house conditions using vermicompost enriched with *P. fluorescens* and *P. chlamydosporia*. The enrichment process was done by mixing 1kg of combination formulation of bio-pesticides with 1 ton of vermicompost. The mixture was thoroughly mixed and left in shade for 15 days for the multiplication of the bio-agents in the substrate – vermicompost. The treatments were as follows:

T1: *P. fluorescens* (Colony forming units of $2 \times 10^9$/g) treated seeds of okra were sown in pots filled with 2500g of soil. [PF -SD]

T2: 25g of vermicompost enriched with *P. fluorescens* bio-pesticide was mixed with 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PF+VER-SB]

T3: *P. fluorescens* (Colony forming units of $2 \times 10^9$/g) treated seeds of okra were sown in 2500g of soil pre-treated with 25g of vermicompost enriched with *P. fluorescens*.[PF +VER-SD+SB]

T4: *P.chlamydosporia* (Colony forming units of $2 \times 10^7$/g) treated seeds of okra were sown in pots filled with 2500g of soil.[PC –SD]

T5: 25g of vermicompost enriched with *P.chlamydosporia* bio-nematicide was mixed with 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PC+VER -SB]
T6: *P. chlamydosporia* (Colony forming units of $2 \times 10^7$/g) treated seeds of okra were sown in 2500g of soil pre-treated with 25g of vermicompost enriched with *P. chlamydosporia*. [PC+VER-SD+SB]

T7: *P. fluorescens*+*P. chlamydosporia* treated seeds of okra were sown in pots with 2500g of soil. [PC+PF-SD]

T8: 25g of vermicompost enriched with *P. fluorescens*+*P. chlamydosporia* bio-pesticide was mixed in 2500g of soil in the pots and the seeds of okra which were untreated were sown. [PC+PF+VER-SB]

T9: *P. fluorescens*+*P. chlamydosporia* treated seeds of okra were sown in 2500g of soil pre-treated with 25g of vermicompost enriched with *P. fluorescens*+*P. chlamydosporia*. [PC+PF+VER-(SD+SB)]

T10: Control or check was maintained without giving any treatment.

**Inoculum preparation of pathogenic fungi *viz* F. oxysporum f.sp. vasinfectum:**

*F. oxysporum f.sp. vasinfectum:* Spore suspensions were prepared from week-old cultures grown in potato dextrose broth. Spores were filtered through two layers of cheesecloth and blended with distilled water to $1 \times 10^5$ conidia per ml and inoculated into 20 day old plants. Tamara Zakayyah Scott (2012).
**Nematode inoculum:**

*M. incognita* was collected from okra field soil and multiplied on eggplant (*Solanum melongena*) using a single egg mass. Egg masses were hand-picked using sterilized forceps and placed in 9cm-diameter sieves of 1-mm pore size lined with cross-layered tissue paper. The sieves were placed in petri dishes with distilled water and incubated at 27 ºC. The nematode suspension was collected and freshly hatched second stage juveniles were inoculated after 20 days of planting.

All the treatments had fifteen replicates. Five plants (twenty days old) of each treatment were inoculated with *M. incognita* J₂ (one juvenile/g), 5 replicates were inoculated with *F. oxysporum* f.sp. *vasinfectum* (@1× 10⁵ conidia per ml) and 5 replicates were inoculated with both *M. incognita* J₂ (one juvenile/g) and *F. oxysporum* f.sp. *vasinfectum* (@1× 10⁵ conidia per ml) pathogens which produce disease complex.

After 3 months plants were depotted and the roots were washed with water. *Meliodogyne incognita* egg-masses were treated with 0.5% NaOCl and eggs were extracted by following the protocol of Hussey and Barker 1973. Each egg-mass was kept in 0.1 ml of 0.1% NaOCl in between a slide and cover slip. It was mechanically crushed and further examined at 40x magnification of microscope. Infected and uninfected eggs were observed Fatemy 1998. Observations were made on the root colonization by these bio-agents in controlled conditions (screen house conditions). The extent of root colonization was calculated by estimating the colony forming units.
formed on semi-selective medium using the protocol of Kerry et al. 1993. Observations of the root-knot index on a 1-10 scale (Bridge and Page, 1980), root and soil nematode population densities, percent disease infection on okra and reduction in disease complex severity due to the bio-agent application were also recorded.

4.4.1(B) Conducted another experiment in the Screen house conditions with the following treatments mentioned below:

T1:  *P. fluorescens* (Colony forming units of $2 \times 10^9/g$) treated seeds of okra sown in pots filled with 2500g of soil.[PF -SD]

T2:  25g of FYM enriched with *P. fluorescens* bio-pesticide was mixed with 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PF+FYM-SB]

T3:  *P. fluorescens* (Colony forming units of $2 \times 10^9/g$) treated seeds of okra sown in 2500g of soil pre-treated with 25g of FYM enriched with *P. fluorescens*. [PF +FYM-SD+SB]

T4:  *P. chlamydosporia* (Colony forming units of $2 \times 10^7/g$) treated seeds of okra sown in pots filled with 2500g of soil.[PC –SD]

T5:  25g of FYM enriched with *P. chlamydosporia* bio-nematicide was mixed with 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PC+FYM-SB]
T6: *P. chlamydosporia* (Colony forming units of \(2 \times 10^7/g\)) treated seeds of okra sown in 2500g of soil pre-treated with 25g of FYM enriched with *P. chlamydosporia*. [PC+FYM-SD+SB]

T7: *P. fluorescens*+*P. chlamydosporia* treated seeds of okra were sown in pots with 2500g of soil.[PC+PF-SD]

T8: 25g of FYM enriched with *P. fluorescens*+*P. chlamydosporia* bio-pesticide was mixed in 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PC+PF+FYM-SB]

T9: *P. fluorescens*+*P. chlamydosporia* treated seeds of okra were sown in 2500g of soil pre-treated with 25g of FYM enriched with *P. fluorescens*+*P. chlamydosporia.*[PC+PF+FYM-(SD+SB)]

T10: Control or check was maintained without giving any treatment.

All the treatments had fifteen replicates. Five plants (twenty days old) of each treatment were inoculated with *M. incognita* J\(_2\) (one juvenile/g), 5 replicates were inoculated with *F. oxysporum* f.sp.*vasinfectum* (@ \(1 \times 10^5\) conidia per ml) and 5 replicates were inoculated with both *M. incognita* J\(_2\) (one juvenile/g) and *F. oxysporum* f.sp.*vasinfectum* (@\(1 \times 10^5\) conidia per ml) pathogens which produce disease complex.

After 3 months plants were depotted and the roots were washed with water. *M. incognita* egg-masses were treated with 0.5% NaOCl and eggs were extracted by following the protocol of Hussey and Barker
1973. Each egg-mass was kept in 0.1 ml of NaOCl 0.1% in between a slide and cover slip. It was mechanically crushed and further examined at 40x magnification of microscope. Infected and uninfected eggs were observed (Fatemy, 1998). Observations were made on the root colonization by these bio-agents in controlled conditions (screen house conditions). The extent of root colonization was calculated by estimating the colony forming units formed on semi-selective medium using the protocol of Kerry et al. 1993. Observations of the root-knot index on a 1-10 scale (Bridge and Page, 1980), root and soil nematode population densities, percent disease infection on okra and reduction in disease complex severity due to the bio-agent application were recorded.

4.4.1(C) Conducted one more experiment in the Screen house conditions with the following treatments mentioned below:

T1: *P. fluorescens* (Colony forming units of $2 \times 10^9$/g) treated seeds of okra sown in pots filled with 2500g of soil.[PF -SD]

T2: 25g of Neem cake enriched with *P. fluorescens* was mixed with 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PF+NC-SB]

T3: *P. fluorescens* (Colony forming units of $2 \times 10^9$/g) treated seeds of okra sown in 2500g of soil pre-treated with 25g of Neem cake enriched with *P. fluorescens*. [PF +NC-SD+SB]
T4: *P. chlamydosporia* (Colony forming units of $2 \times 10^7$/g) treated seeds of okra sown in pots filled with 2500g of soil.[PC –SD]

T5: 25g of Neem cake enriched with *P. chlamydosporia* was mixed with 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PC+NC-SB]

T6: *P. chlamydosporia* (Colony forming units of $2 \times 10^7$/g) treated seeds of okra sown in 2500g of soil pre-treated with 25g of Neem cake enriched with *P. chlamydosporia*. [PC+NC-SD+SB]

T7: *P. fluorescens*+*P. chlamydosporia* treated seeds of okra were sown in pots with 2500g of soil.[PC+PF-SD]

T8: 25g of Neem cake enriched with *P. fluorescens*+*P. chlamydosporia* bio-pesticide was mixed in 2500g of soil in the pots and the seeds of okra which were untreated were sown.[PC+PF+NC-SB]

T9: *P. fluorescens*+*P. chlamydosporia* treated seeds of okra were sown in 2500g of soil pre-treated with 25g of Neem cake enriched with *P. fluorescens*+*P. chlamydosporia.*[PC+PF+NC- (SD+SB)]

T10: Control or check was maintained without giving any treatment.

All the treatments had fifteen replicates. Five plants (twenty days old) of each treatment were inoculated with *M. incognita J₂* (one juvenile/g), 5 replicates were inoculated with *F. oxysporum f.sp.vasinfectum*, (@$1 \times 10^5$ conidia per ml) and 5 replicates were inoculated with both *M. incognita J₂* (one juvenile/g) and
*F. oxysporum* f.sp.*vasinfectum* (@1× 10^5 conidia per ml) pathogens which produce disease complex.

After 3 months plants were depotted and the roots were washed with water. *M. incognita* egg-masses were treated with 0.5% NaOCl and eggs were extracted by following the protocol of Hussey and Barker 1973. Each egg-mass was kept in 0.1 ml of NaOCl 0.1% in between a slide and cover slip. It was mechanically crushed and further examined at 40x magnification of microscope. Infected and uninfected eggs were observed (Fatemy, 1998). Observations were made on the root colonization by these bio-agents in controlled conditions (screen house conditions). The extent of root colonization was calculated by estimating the colony forming units formed on semi-selective medium using the protocol of Kerry *et al.* 1993. Observations of the root-knot index on a 1-10 scale (Bridge and Page, 1980), root and soil nematode population densities, percent disease infection on okra and reduction in disease complex severity due to the bio-agent application were recorded.

**4.5 Standardization of methods for the management of disease complex caused by *F. oxysporum* f.sp.*vasinfectum* + *M. incognita* on okra under field conditions.**

The experiments were carried out in the plots of Indian Institute of Horticulture Research (IIHR), Hessarghatta Lake P.O., Bangalore – 560 089.

Experiments related to the bio-management of nematode induced disease complex were carried out using the combination
formulations of *P. chlamydosporia* with *P. fluorescens* developed in Nematology Laboratory of Division of Entomology and Nematology, Indian Institute of Horticulture Research, Bangalore.

**4.5.1 To study the effect of delivery system of combination formulation of *P. chlamydosporia* and *P. fluorescens* on the suppression of disease complex produced by *M. incognita* and *F. oxysporum f.sp.vasinfectum* in field.**

4.5.1(A). Field trial was taken up using neem cake enriched with bio-control agents in the field with plot size of 2x2m. In each plot 20 plants were maintained with a spacing of 35cm in a row and a spacing of 50cm from row to row. One quintal of Neem cake was enriched with 2500g of *P. chlamydosporia* or *P. fluorescens*. The okra seeds were either treated with talc based formulation of *P. fluorescens* (20g/kg) or *P. chlamydosporia* (20g/kg) or combination formulation of *P. fluorescens* + *P. chlamydosporia* (20g/kg). The following treatments were given:

T1: Okra seeds treated with *P. fluorescens* were sown in plots treated with Neem cake @ 50g/m².[PF -SD]

T2: Seeds of okra (untreated) were sown in plots treated with neem cake enriched *P. fluorescens* @50g/m².[PF+NC-SB]

T3: Seeds treated with *P. fluorescens* were sown in plots treated with neem cake enriched *P. fluorescens* @50g/m².[PF +NC-SD+SB]

T4: Seeds treated with *P. chlamydosporia* were sown in plots treated with Neem cake @ 50g/m².[PC –SD]
T5: Seeds of okra (untreated) were sown in plots treated with neem cake enriched with *P. chlamydosporia* @ 50g/m².[PC+NC-SB]

T6: Seeds treated with *P. chlamydosporia* were sown in plots treated with neem cake enriched with *P. chlamydosporia* @ 50g/m².[PC+NC-SD+SB]

T7: Seeds treated with combination formulation of *P. chlamydosporia* + *P. fluorescens* were sown in plots treated with Neem cake @ 50g/m².[PC+PF-SD]

T8: Seeds of okra (untreated) were sown in plots treated with neem cake enriched with combination formulation of *P. chlamydosporia* + *P. fluorescens* talc @ 50g/m².[PC+PF+NC-SB]

T9: Seeds treated with combination formulation of *P. chlamydosporia* + *P. fluorescens* were sown in plots treated with neem cake enriched with combination formulation of *P. chlamydosporia* + *P. fluorescens* talc @ 50g/m².[PC+PF+NC-(SD+SB)]

T10: Seeds of okra (untreated) were sown in plots treated with Neem cake @ 50g/m².[NC]

T11: Seeds of okra (untreated) were sown in plots treated with carbofuran (5g/m²) + carbendazium (5g/m²).[CARBO+CARB]

T12: Untreated seeds sown in untreated plots were maintained as control.

Each treatment was replicated 5 times in a RBD (randomized block design). The experiment was carried out in nematode (96 ± 9) *J₂* and *F. oxysporum f.sp.vasinjectum* sick plots. Observations were recorded on, the height of the plant, nematode disease incidence and on the no. of fruits harvested at the regular
intervals. Root populations of the *M. incognita* were estimated from 10 g samples of roots from each plant. The root samples were stained using acid fuchsin following the method of Bridge *et al.* (1982), homogenised, and the numbers of nematodes in the roots were recorded. To estimate the soil population density of the nematode, the *J₂* of *M. incognita* were extracted from 100 cm³ soil per replicate by Cobb’s sieving and decanting method (Cobb, 1918) and counted.

4.5.1(B). Another field trail was taken up using FYM enriched with bio-control agents in the field with plot size of 2x2m. In each plot 20 plants were maintained with a spacing of 35cm in a row and a spacing of 50cm from row to row. One ton of FYM was enriched with 2500g of *P. chlamydosporia* or *P. fluorescens*. The okra seeds were either treated with talc based formulation of *P. fluorescens* (20g/kg) or *P. chlamydosporia* (20g/kg) or combination formulation of *P. fluorescens* +*P. chlamydosporia* (20g/kg). The following treatments were given:

T1: Okra seeds treated with *P. fluorescens* were sown in plots treated with FYM @ 250g/m².[PF-SD]

T2: Seeds of okra (untreated) were sown in plots treated with FYM enriched *P. fluorescens* @250g/m².[PF+FYM-SB]

T3: Seeds treated with *P. fluorescens* were sown in plots treated with FYM *P. fluorescens* @250g/m².[PF-SD+SB]
T4: Seeds treated with *P. chlamydosporia* were sown in plots treated with FYM @250g/m². [PC –SD]

T5: Seeds of okra (untreated) were sown in plots treated with FYM with *P. chlamydosporia* @250g/m².[PC+FYM -SB]

T6: Seeds treated with *P. chlamydosporia* were sown in plots treated with FYM enriched with *P. chlamydosporia* @250g/m².[PC+FYM -SD+SB]

T7: Seeds treated with combination formulation of *P. chlamydosporia* + *P. fluorescens* were sown in plots treated with FYM @ 250g/m². [PF+ PC -SD]

T8: Seeds of okra (untreated) were sown in plots treated with FYM enriched with combination formulation of *P. chlamydosporia* + *P. fluorescens* talc @250g/m².[PF+ PC+FYM - SB]

T9: Seeds treated with combination formulation of *P. chlamydosporia* + *P. fluorescens* were sown in plots treated with FYM enriched with combination formulation of *P. chlamydosporia* + *P. fluorescens* talc @250g/m².[PC+PF+FYM (SD+SB)]

T10: Seeds of okra (untreated) were sown in plots treated with FYM @250g/m². [FYM]

T11: Seeds of okra (untreated) were sown in plots treated with carbofuran (5g/m²) + carbendazium (5g/m²). [CARBO+CARB]

T12: Untreated seeds sown in untreated plots were maintained as control.

Each treatment was replicated 5 times in a RBD (randomized block design). The experiment was carried out in nematode (96 ± 9) J₂ and *F. oxysporum* f.sp. *vasinfectum* sick plots. Observations were recorded on, the height of the plant, nematode
disease incidence and on the no. of fruits harvested at the regular intervals. Root populations of the *M. incognita* were estimated from 10 g samples of roots from each plant. The root samples were stained using acid fuchsin following the method of Bridge *et al.* (1982), homogenised, and the numbers of nematodes in the roots were recorded. To estimate the soil population density of the nematode, the *J₂* of *M. incognita* were extracted from 100 cm³ soil per replicate by Cobb’s sieving and decanting method (Cobb, 1918) and counted.

**4.5.1 (C).** One more field trial was taken up using Vermicompost enriched with bio-control agents in the field with plot size of 2x2m. In each plot 20 plants were maintained with a spacing of 35cm in a row and a spacing of 50cm from row to row. One quintal of Vermicompost was enriched with 2500g of *P. chlamydosporia* or *P. fluorescens*. The okra seeds were either treated with talc based formulation of *P. fluorescens* (20g/kg) or *P. chlamydosporia* (20g/kg) or combination formulation of *P. fluorescens* + *P. chlamydosporia* (20g/kg). The following treatments were given:

T1: Okra seeds treated with *P. fluorescens* were sown in plots treated with Vermicompost @ 100g/m². [PF-SD]

T2: Seeds of okra (untreated) were sown in plots treated with Vermicompost enriched *P. fluorescens* @ 100g/m².[PF+VER-SB]

T3: Seeds treated with *P. fluorescens* were sown in plots treated with Vermicompost *P. fluorescens* @ 100g/m². [PF-SD+SB]
T4: Seeds treated with *P. chlamydosporia* were sown in plots treated with Vermicompost@100g/m². [PC –SD]

T5: Seeds of okra (untreated) were sown in plots treated with Vermicompost with *P. chlamydosporia*@100g/m². [PC+VER-SB]

T6: Seeds treated with *P. chlamydosporia* were sown in plots treated with Vermicompost enriched with *P. chlamydosporia*@100g/m². [PC+VER-SD+SB]

T7: Seeds treated with combination formulation of *P. chlamydosporia*+ *P. fluorescens* were sown in plots treated with Vermicompost@100g/m². [PF+ PC -SD]

T8: Seeds of okra (untreated) were sown in plots treated with Vermicompost enriched with combination formulation of *P. chlamydosporia* + *P. fluorescens* talc @100g/m². [PF+ PC+VER-SB]

T9: Seeds treated with combination formulation of *P. chlamydosporia* + *P. fluorescens* were sown in plots treated with Vermicompost enriched with combination formulation of *P. chlamydosporia*+*P. fluorescens* talc 100g/m².[PC+PF+VER(SD+SB)]

T10: Seeds of okra (untreated) were sown in plots treated with Vermicompost100g/m². [VER]

T11: Seeds of okra (untreated) were sown in plots treated with carbofuran (5g/m²) + carbendazium (5g/m²).[CARBO+CARB]

T12: Untreated seeds sown in untreated plots were maintained as control.

Each treatment was replicated 5 times in a RBD (randomized block design). The experiment was carried out in nematode (96 ± 9) J₂ and *F. oxysporum* f.sp. *vasinfectum* sick plots.
Observations were recorded on, the height of the plant, nematode disease incidence and on the no. of fruits harvested at the regular intervals. Root populations of the *M. incognita* were estimated from 10 g samples of roots from each plant. The root samples were stained using acid fuchsin following the method of Bridge *et al.* (1982), homogenised, and the numbers of nematodes in the roots were recorded. To estimate the soil population density of the nematode, the *J2* of *M. incognita* were extracted from 100 cm³ soil per replicate by Cobb’s sieving and decanting method (Cobb, 1918) and counted.