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RESEARCH ARTICLE

# Synergistic interaction between *Meloidogyne incognita* and *Fusarium solani* in black pepper, *Piper nigrum* L.

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## Abstract

The nematode-fungus disease complex is becoming a significant constraint in black pepper production in Kerala. Basic information on major nematodes and fungal pathogens associated with disease complex as well as histopathological and biochemical changes due to the interactive effects of both organisms, is essential for developing effective management strategies. Soil and root samples were collected from prominent black pepper-growing areas in Kannur, Idukki and Wayanad districts to document nematodes and fungal pathogens associated with black pepper. Plant parasitic nematodes, such as *Meloidogyne incognita, Rotylenchulus reniformis, Radopholus similis, Helicotylenchus dihystera* and *Macroposthonia* sp. and fungal pathogens such as *Fusarium solani, F. oxysporum*, *Phytophthora capsici, Rhizoctonia* sp. and *Pythium* sp., were associated with black pepper plants in surveyed areas. The disease severity was higher in regions where *M. incognita* co-existed with fungal pathogens. *F. solani* was the predominant pathogen along with *M. incognita* in the areas surveyed. Pot culture studies were conducted to investigate the interaction between nematodes and fungi. Plants inoculated with *M. incognita* 14 days before *F. solani* exhibited the highest (100%) and early (24 days) disease incidence, as evidenced by the increased number of giant cells, distortion of vascular bundles and plugging of fungal mycelia in xylem vessels. The highest defense enzyme activity and phenol content were observed in plants inoculated with *M. incognita* + *F. solani* 14 days after nematode inoculation. Results of the study highlighted that nematode infestation predisposes plants to fungal infection and synergistic interaction between nematode and fungal pathogen increases disease severity in field conditions.

## Keywords

biochemical changes; black pepper; defense enzymes; fungus; giant cell; histopathological changes; nematode-fungus interaction; root-knot nematode

## Introduction

Black pepper (*Piper nigrum* L.), known as "King of Spices" or "black gold" is an important spice crop cultivated in regions with hot and humid climates worldwide. In India, it is cultivated in an area of 278050 ha, producing 64000 tons (1). Karnataka is the largest black pepper cultivating state with an area of 180000 ha and production of 36000 tons. It is cultivat-

ed in 82000 ha with a production of 36000 tons in Kerala (1). Plant parasitic nematodes (PPNs) serve as a significant biotic factor causing substantial economic losses in the production of black pepper and slow wilt disease is a major production constraint. The losses due to nematodes in black pepper ranged from 30.50 to 64.60% in Kerala (2). They are obligate parasites that extract nutrients from the cytoplasm of plant cells and are recognized as significant pathogens affecting various crops. They cause damage to the plants by injuring root tissues and consuming nutrients from the root's epidermal, cortical and stelar tissues. They establish parasitism by making wounds in roots using stylet and introducing secreted effectors from oesophageal glands (3). They also serve as predisposing agents in disease complex development as the soil-borne fungal and bacterial pathogens cause huge losses to the farmers growing black pepper. PPNs belonging to 29 genera and 48 species have been reported in black pepper (4).

Slow decline (yellows) disease in black pepper was first reported in Wayanad area in Kerala in 1902 (5) and the symptoms include pale-yellow drooping leaves on the vines, shedding of leaves and spikes, reduced growth and dieback. The root system exhibits purple-coloured lesions and rotting in plants infested with burrowing nematodes (6). In the case of root-knot nematode (RKN) infestation, root system become heavily galled and females with egg masses can be seen deep within roots. Root-knot and burrowing nematodes in association with fungal pathogen *Phytophthora capsici* Leonian aggravated wilt symptoms in black pepper as the wounds caused by the nematode serve as entry points of the pathogen (7). More than 90% black pepper gardens in Kerala are infested with root-knot nematodes. *Meloidogyne* juveniles (J2) invade intercellularly, and the giant cells induced by the nematodes block the translocation of water and nutrients to the aerial parts of the plants.

Disease complexes involving nematodes and fungi have gained momentum in recent years, leading to considerable yield loss in black pepper. Although diseases and insect pests have long being recognized as important constraints in spice production, extensive research on plant parasitic nematodes and plant-pathogen interaction mainly soil borne fungi are lacking. The studies exploring the histopathological and biochemical aspects of nematodes and fungal co-infection in black pepper are meagre. These aspects are important in understanding the mechanisms of infection and the pathways involved in resistance of plants against these pathogens. Hence the present study was undertaken 1) to document the nematode fauna and fungal pathogens in the rhizosphere of black pepper growing areas in Kannur, Idukki and Wayanad districts; 2) to study the interaction between *M. incognita* and fungal pathogens; and 3) to study the histopathological and biochemical changes due to nematode-fungus disease complex in black pepper.

## Materials and Methods

### Documentation of nematodes and fungal pathogens in rhizosphere of black pepper

A survey was conducted in 3 Agro-Ecological Units (AEUs) such as in Kannur (AEU 15), Idukki (AEU 16) and Wayanad (AEU 21) districts during 2021-22, to assess the distribution of nematodes and fungal pathogens in black pepper. Soil and root samples were collected from the rhizosphere of black pepper plants at a depth of 10-30 cm using an auger. Samples were taken around the plants from 3 spots at a horizontal distance of 30 and 45 cm away from the base of the plant. The samples were bulked, and nematodes were extracted from the composite sample (200 cc) by Cobb's sieving and decanting method (8), followed by a modified Baermann funnel technique (9). The population of each species was counted under a stereo-zoom microscope and recorded using a tally counter. Permanent mounts were prepared for the identification of nematodes up to species level. Triethanolamine formalin (TAF) was used as a fixative. Live nematodes suspended in the soil solution were stirred in a water bath for 20-30 sec at 70-90 °C followed by adding equal volume of hot fixative (65-70 °C) to it. The fixed nematodes were processed by slow glycerol method (10) through stepwise transfer of the specimens to anhydrous glycerol. The processed specimens were mounted on glass slides using dehydrated glycerol. A drop of dehydrated glycerine was placed at the centre of a glass slide. Subsequently, 2-3 processed nematodes were carefully arranged on this droplet, allowing them to settle at the bottom. Three pieces of glass fibres, approximately one mm in length and of thickness equal to the nematodes, were placed around the specimen and a cover slip was placed over the drop to ensure no air bubbles were trapped in between. The coverslip edges were sealed with glyceel and the specimens were identified.

### Community analysis of nematodes in the rhizosphere of black pepper

The data on nematode population was subjected to community analysis. Absolute frequency, relative frequency, absolute density, relative density and prominence value for each species of nematodes obtained were calculated using formula of Norton (11).

$$\text{Absolute frequency} = \frac{\text{No.of samples containing the species}}{\text{Total no. samples collected}} \times 100$$

$$\text{Relative frequency} = \frac{\text{Frequency of species}}{\text{Sum of frequencies of all species}} \times 100$$

$$\text{Absolute density} = \frac{\text{No.of individuals of a species in sample}}{\text{Volume of the sample}} \times 100$$

$$\text{Relative density} = \frac{\text{No.of individuals in a sample}}{\text{Total of all individuals in a sample}} \times 100$$

$$\text{Prominence value} = \text{Density} \times \sqrt{\text{Frequency}}$$

### Isolation and identification of fungal pathogens

Fungal pathogens were isolated from soil samples by serial dilution technique followed by pour plate technique (12). 1 g of soil was added to a test tube containing 9 mL of sterile distilled water to obtain a 1/10 solution (stock solution). Subsequently, a series of dilutions, including 1/100, 1/1000, 1/10000 and 1/100000 were prepared by adding 1 mL of the solution to 9 mL of sterile distilled water respectively. 0.1 mL of the diluted sample was plated in sterile petri plates and then PDA media was poured over it. The medium was supplemented with 1% streptomycin solution to prevent bacterial growth. The plates were then incubated at room temperature (27 °C) for a period of 48 hr. Morphologically distinct colonies were selected and purified. The isolated fungi were purified by inoculating them onto plates containing PDA medium. The cultures were then sub-cultured on PDA slants and allowed to grow for a period of 5-7 days before being stored as stock cultures at 4 °C. The morphological and cultural characteristics of the fugal pathogens obtained were examined by culturing the fungus in PDA medium. The slide culture technique described (13) was utilized to observe spore characters.

The identity of the pathogens was confirmed by sending samples to ITCC, Division of Plant Pathology, IARI and further by molecular characterization (14). Mycelium from a 7 days old fungus culture, grown on PDA was collected using a sterile toothpick and transferred to 100 µL sterile water in a 1.5 mL microcentrifuge tube. The resulting mixture was thoroughly vortexed and then centrifuged at 10000 rpm for 1 min. Following the careful removal of the supernatant using a micropipette, 100 µL of lysis solution was added to the microcentrifuge tube. The mixture was subsequently incubated at 85 °C in a water bath for 30 min. The DNA sequence from the internal transcribed spacer regions (ITS) was amplified using the universal primers ITSI/ITS4.

ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4: 5'-TCCTCCGCTTATTGATATGC-3'

The PCR reaction mixture was composed of 2 µL of 10X PCR buffer, 1.2 µL of dNTP mixture (2.5 mmol each), 0.4 µL of $MgCl_2$ (25 mmol), 0.8 µL of each primer (10 mmol), 0.2 µL of Taq DNA polymerase (5 U/µL) and 1 µL of crude fungal genomic DNA, making a total volume of 20 µL.

PCR amplification conditions

| | | |
|---|---|---|
| Initial denaturation | : 94 °C for 5 min | |
| Denaturation | : 94 °C for 50 s | 35 cycles |
| Annealing | : 54 °C for 50 s | |
| Extension | : 72 °C for 50 s | |
| Final extension | : 72 °C for 10 min | |

PCR amplification products were analyzed by preparing 1% agarose gel using 0.5 X TAE buffers and were stained with ethidium bromide solution. The PCR amplified products and 100 base pair DNA ladder were run at 80 V for 2 hr in an electrophoresis unit. The gel profile was examined in an UV trans-illuminator. ITS sequences of the isolates were BLASTN against sequences in NCBI (National Centre for Biotechnology Information) database. Percentage of identity was used to verify the species.

### Maintenance of pure culture of M. incognita

The root knot nematode culture was maintained in the glass house of Department of Nematology, College of Agriculture, Vellayani. Egg masses were collected from infected black pepper plants and were sterilized using 0.1% sodium hypochlorite for 3 min. Then it was sterilized using 95% ethanol for 1 min and later washed in sterile water 3 times. This was placed in distilled water for hatching. The hatched second stage juveniles were inoculated at 1J2/g soil (15) in tomato seedlings (Variety -Vellayani Vijay) raised in pots containing sterilized potting mixture prepared by mixing soil, sand and farmyard manure in 2:1:1 ratio for maintenance of nematode culture. Periodic sub-culturing was done to ensure sufficient quantity of nematode inoculum. The identity of *M. incognita* was confirmed by preparing the perineal pattern of the adult female (16). Plant roots with galls containing females were carefully washed under a continuous flow of water to remove the adhering soil particles. The mature females were collected and preserved in 45% lactic acid. Initially, an anterior cut was made and squeezed, allowing for the removal of internal contents using a brush. Then a posterior cut was made and the perineal pattern was collected. The pattern was then carefully trimmed under a stereoscopic binocular microscope until it attained a square shape. These trimmed patterns were then transferred to a clean slide, where they were aligned with the internal side of the cuticle facing towards the glass slide and the anus pointing downwards. A cover glass was placed over the glycerine drop containing the perineal pattern, which was then sealed and labelled accordingly. The identification of the perineal patterns of the collected nematodes as *M. incognita* was done based on characteristics in identification keys *viz.,* the lateral field, striae, dorsal arch and tail terminus (17).

### Interaction studies between M. incognita and fungal pathogen

A pot culture experiment was designed using pots of size 35 × 20 × 20 inches with 6 treatments and 3 replications in Complete Randomized Design to study the interaction effect of *M. incognita* and *F. solani* in black pepper variety Panniyur 2. Black pepper cuttings were planted in denematized potting mixture containing soil, sand and dry cow dung mixed in 2:1:1 ratio. The fungal pathogen was mass multiplied in sand maize mixture (9:1) (18). The plants were inoculated with newly hatched second stage juveniles of *M. incognita* at 1 J2/g soil and the fungal pathogen multiplied in sand maize media at a concentration of 0.5% w/w either alone, simultaneously or sequentially. The treatments were $T_1$- *M. incognita* alone; $T_2$- Fungal pathogen alone; $T_3$- Both pathogens simultaneously; $T_4$- Nematode first and fungus 2 weeks after nematode inoculation; $T_5$- Fungus first and nematode 2

weeks after fungus inoculation; and $T_6$- Untreated control. Forty-five days after nematode inoculation (DAI), observations on nematode population characteristics *viz*. nematode population in soil (200 cc), root (5 g), number of galls (5 g), number of egg masses in root (5 g), number of eggs in egg mass, number of females in root (5 g), number of infected plants, nature of symptom, days taken for symptom development and disease intensity were recorded. Number of females in 5 g root was estimated by heating the root bits (1 cm) in a test tube containing lactophenol over a flame for differential staining (19). After removing the excess stain and rinsing with water, the stained root bits were placed in plain lactophenol overnight for de-staining. Reproduction factor (RF) was calculated by the formula RF = Pf/Pi (Pf- final nematode population; Pi- initial population) (20). Number of egg masses in root (5 g) was estimated by staining with Phloxine B prepared by mixing 0.15 g of Phloxine B in 1 L water. Disease incidence (21) and severity scoring was done using score scale of 0-4; where 0 - no disease/healthy seedling, 1 - yellow leaves, 2 - yellow leaves and slightly wilted, 3 - severe wilt and 4 - dead seedling (22). Biometric characters such as shoot length (cm), root length (cm), fresh weight of shoot and root (g) were recorded after uprooting plants 45 days after nematode inoculation.

## Histopathological changes during the establishment of nematode-fungus disease complex in black pepper

Histopathological changes in the root tissues of black pepper plants inoculated with *M. incognita* and fungal pathogen (independent, simultaneous and sequential) were done after uprooting the plants 45 DAI. The root bits were cut into small pieces (1 to 2 cm length) and transferred into distilled water. Galled root bits were selected, excised and fixed in formalin-aceto alcohol solution (FAA). Then, the root bits were dehydrated in a graded series of ethanol (35, 50, 75 and 100%, 1 hr in each series of concentration) infiltrated and embedded in paraffin wax (50-55 °C). Transverse cross sections of thickness 10 µm were cut using microtome. The sections were carefully transferred to glass slides and left for 2 min to dry. Paraffin wax was removed by soaking the sections in series of xylene and rinsed with ethanol followed by distilled water (23). Sections were stained with Safranin and Toluidine blue. The stained sections are then viewed under a research microscope at 40X magnification.

## Biochemical response in black pepper during the establishment of nematode-fungus disease complex

### *Estimation of total phenols*

1 g of leaf material was ground in 10 mL of 80% ethanol. The resulting homogenate was centrifuged at 10000 rpm for 20 min. and the supernatant was retained. The residue was then extracted using 5 times the volume of 80% ethanol and centrifuged for 20 min. at 10000 rpm. After pooling the supernatant, it evaporated until completely dry. The dried residue was dissolved in 5 mL of distilled water. An aliquot of 0.3 mL was pipetted out and made up to 3 mL with distilled water. Two mL of 20% sodium carbonate solution was added to each tube, along with 0.5 mL Folin-Ciocalteau reagent after 3 min. This was thoroughly mixed and then placed in a boiling water bath for one min. After cooling, the absorbance at 650 nm was measured in comparison to the reagent blank. Standard curve was prepared using different concentrations of catechol and total phenol which was expressed in catechol equivalents as mg $g^{-1}$ of leaf/root tissue on fresh weight basis (24).

### *Estimation of protein*

After homogenizing 1 g of leaf material in 10 mL of 0.1 M sodium acetate buffer (pH 4.7), the mixture was centrifuged at 5000 rpm for 15 min. at 4 °C and the supernatant was collected to estimate the soluble protein. The reaction mixture was prepared by mixing 0.5 mL of enzyme extract, 0.5 mL of distilled water and 5 mL of diluted dye solution (Coomassie Brilliant Blue G-250) which was diluted 5 times. The absorbance was measured at 595 nm using a reagent blank in a spectrophotometer. Bovine serum albumin was used as the standard protein. The protein was measured in terms of the amount of Bovine serum albumin (BSA) produced per g fresh $weight^{-1}$ of leaf /root samples (25).

### *Estimation of peroxidase (PO)*

A 400 mg leaf specimen was homogenized with a small amount of polyvinylpyrrolidone (PVP) in 2 mL of 0.1 M sodium phosphate buffer (pH - 6.5). Homogenization was carried out at 4 °C by using a pre-chilled mortar and pestle in an ice filled tray. The homogenate was subjected to centrifugation at 5000 rpm for 15 min. at 4 °C subsequently filtered through a muslin cloth. The supernatant was used as the enzyme extract for the estimation of peroxidase (PO) activity. 1 mL of 0.05 M pyrogallol and 50 µL of 1 mL enzyme extract were used in the reaction mixture, which was placed in reference and sample cuvette, stirred and then placed in a spectrophotometer, where the measurement was calibrated to zero at 420 nm. 1 mL hydrogen peroxide 1% was added to sample cuvette to initiate the enzyme reaction and changes in absorbance were documented every 30 sec for the first 180 sec (26).

### *Estimation of polyphenol oxidase*

Polyphenol oxidase activity was determined as per the procedure (27). The procedure for estimating peroxidase was followed in the preparation of the enzyme extract. The reaction mixture consisted of 50 µL of enzyme extract and 1 mL of 0.1 M sodium phosphate buffer (pH 6.5). The reaction was initiated by the addition of 1 mL of 0.01 M catechol. The measurements were taken using a spectrophotometer. The change in absorbance at 495 nm was recorded and the PPO activity was determined by calculating the change in absorbance $min^{-1}$ $g^{-1}$ of fresh weight.

### *Estimation of phenylalanine ammonia lyase (PAL)*

1 g of leaf sample was homogenized by using a cold mortar and pestle in 5 mL of 0.1 M sodium borate buffer (pH 8.8) containing a small amount of PVP to obtain the enzyme extract. The resulting homogenate was subjected

to centrifugation at 10000 rpm for 10 min. at a temperature of 4 °C. The PAL activity was determined using the supernatant. The reaction mixture consisted of 3 mL of 0.1 M sodium borate buffer (pH 8.8), 0.2 mL of enzyme extract and 0.1 mL of 12 mM L-phenylalanine prepared in the same buffer. The blank was composed of 0.2 mL of enzyme extract and 3 mL of 0.1 M sodium borate buffer (pH 8.8). The reaction mixture and blank were incubated at 40 °C for 30 min and reaction was stopped by adding 0.2 mL of 3 N hydrochloric acid. The absorbance at a wavelength of 290 nm was measured utilizing a spectrophotometer. The PAL activity was measured regarding the amount of cinnamic acid produced per g fresh weight of leaf /root samples (28).

### *Statistical analysis*

Data generated from the experiment were subjected to ANOVA (29). Those variables that did not satisfy the basic assumptions of ANOVA were subjected to square root transformation. Analysis was conducted using the statistical software KAU GRAPES 1.0.0.l.

## Results and Discussion

### Documentation of nematodes and fungal pathogens in rhizosphere of black pepper

Survey was conducted in black pepper growing areas of Kannur (AEU 15), Idukki (AEU 16) and Wayanad (AEU 21) districts and a total of 210 soil and 62 root samples were collected from rhizosphere of black pepper plants to document the plant parasitic nematodes and fungal pathogens. Analysis of the soil and root samples revealed the presence of 5 plant parasitic nematodes such as *M. incognita, Rotylenchulus reniformis, Helicotylenchus dihystera, R. similis, Macroposthonia* sp. and free-living nematodes. *Macroposthonia* sp. was obtained only from AEU 21 (Wayanad) (Fig. 1-5).

In Kannur district (AEU-15), the survey was conducted in Thaliparamba and Irikkur blocks, and soil and root samples (60) were collected from Kurumathur, Thaliparambu, Padiyoor, Malapattam, Ulikkal and Irikkur panchayths (Table 1). *M. incognita* was the most predominant nematode with highest prominence value (PV-14.99) in Padiyoor panchayath. High population of *M. incognita* was observed in Thaliparamba (PV-12.28), Kurumathur (PV-11.79), Ulikkal (PV-11.31) and Irikkur (13.29) panchayaths also. Plants exhibited symptoms *viz*., yellowing, wilting and galling in roots in these panchayaths. The second most prominent nematode was *R. reniformis* (PV - 12.29) in Malapattam panchayath. Highest population of *R. similis* was observed in Thaliparamba (average-147; PV - 7.33). The highest population of *H. dihystera* was found in Ulikkal panchayath with average of 198 and PV 9.41. It is in accordance with previous workers (30) who

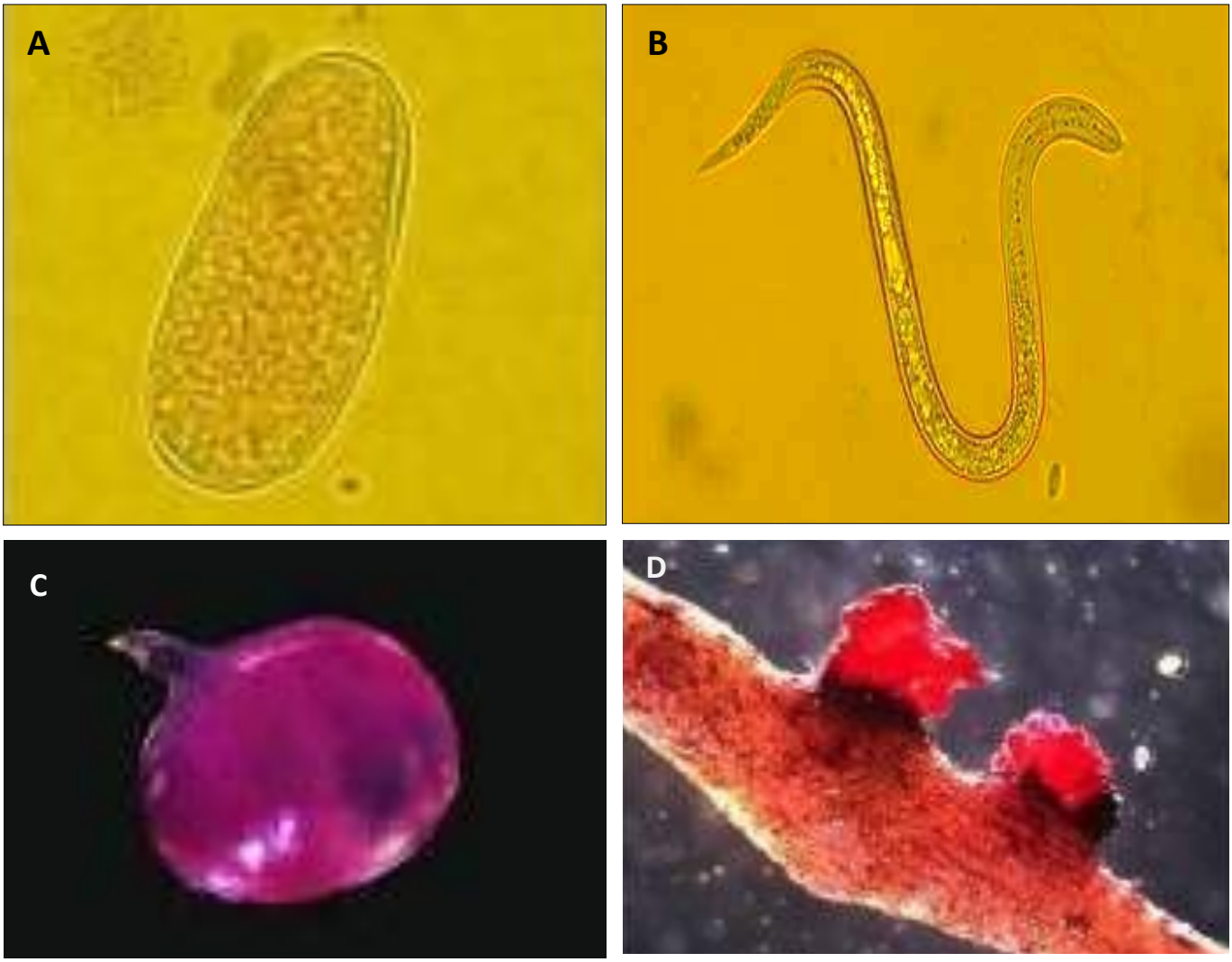


**Fig. 1.** Root-knot nematode- *M. incognita.* **A**-Egg, **B**- J2 stage, **C**- Adult female, **D**- Egg mass.

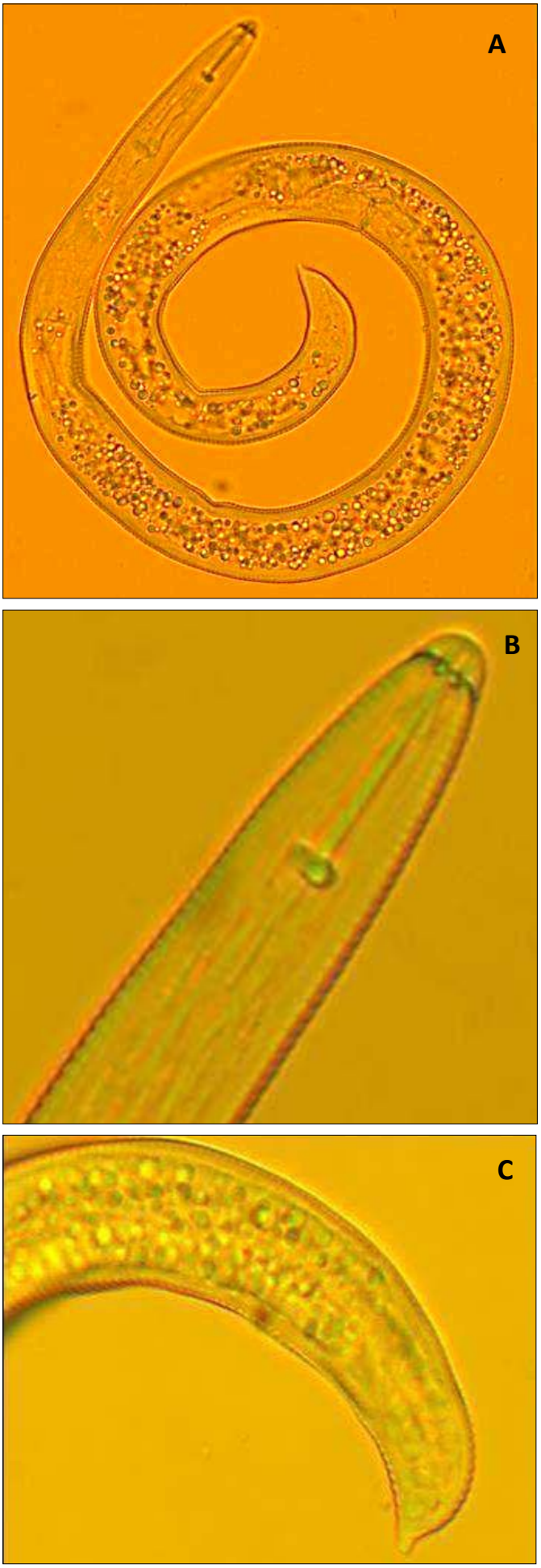


**Fig. 2.** Spiral nematode- *H. dihystera*. **A**-Female, **B**- Anterior region of female, **C**- Posterior region of female.

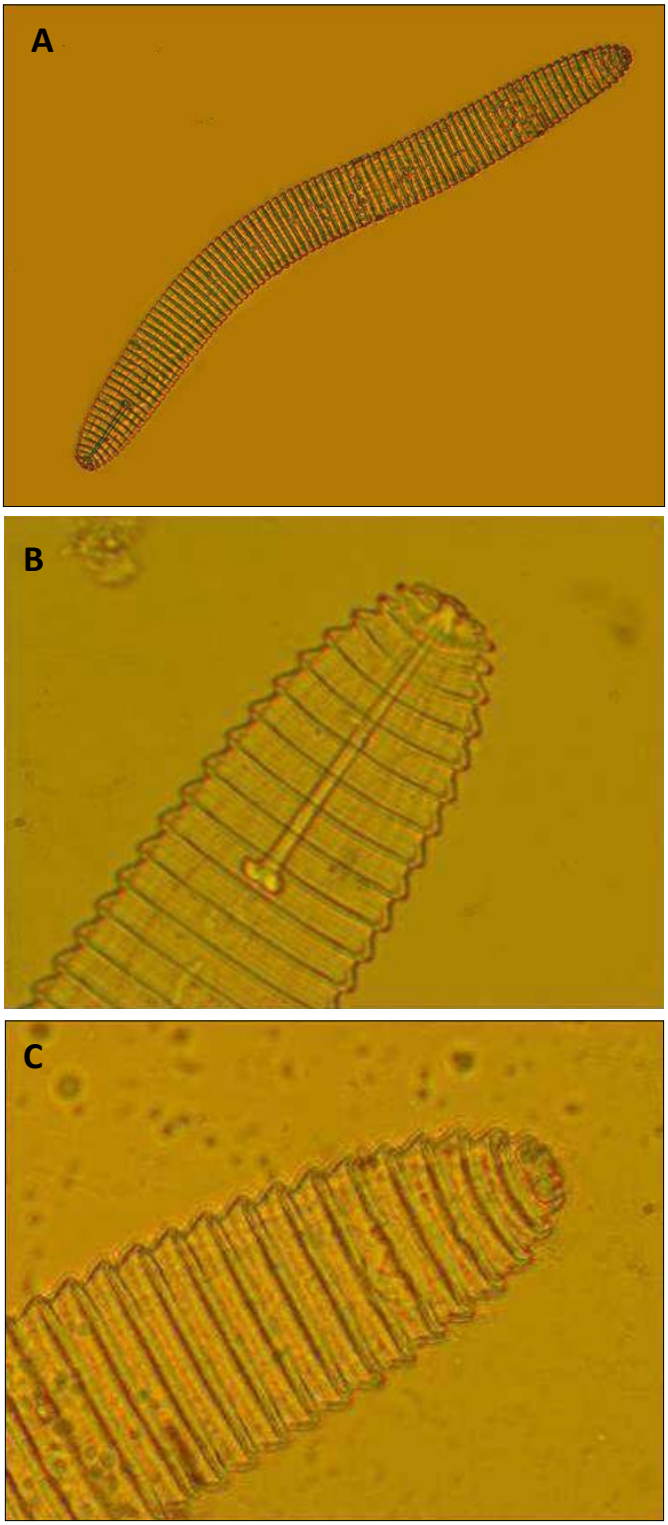


**Fig. 3.** Ring nematode- *Macroposthonia sp.* **A**- Female, **B**- Anterior region of female, **C**- Posterior region of female.

reported occurrence of *M. incognita*, *R. similis*, *Tropho-tylenchulus piperis* Raski, *R. reniformis* and *Helicotylen-chus* sp. in black pepper gardens of Kerala.

In Idukki district (AEU-16), survey was conducted in Kattappana, Nedumkandam and Devikulam blocks. A total of 90 samples were collected from different pan-chayaths *viz.,* Kattappana, Kanchiyar, Erattayar, Upputhara, Pampadumpara, Manjappara, Chakkupallam, Marayoor and Munnar (Table 2). *H. dihystera* was the most promi-nent nematode in Marayoor (PV-15.29), Kattappana (PV-8.75), Pampadumpara (PV-10.46), Erattayar (PV- 11.37),

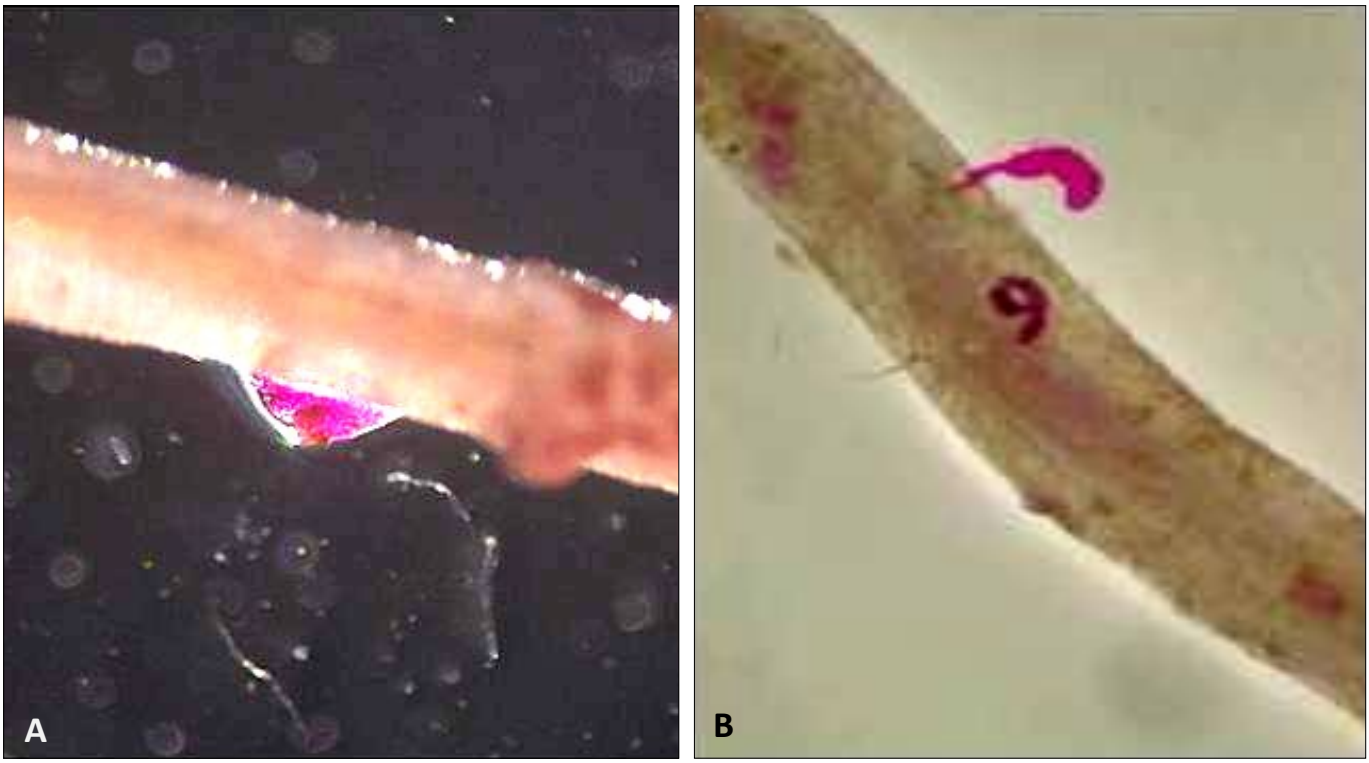


**Fig. 4.** Reniform nematode- *R. Reniformis.* **A**-Egg mass, **B**- Female.

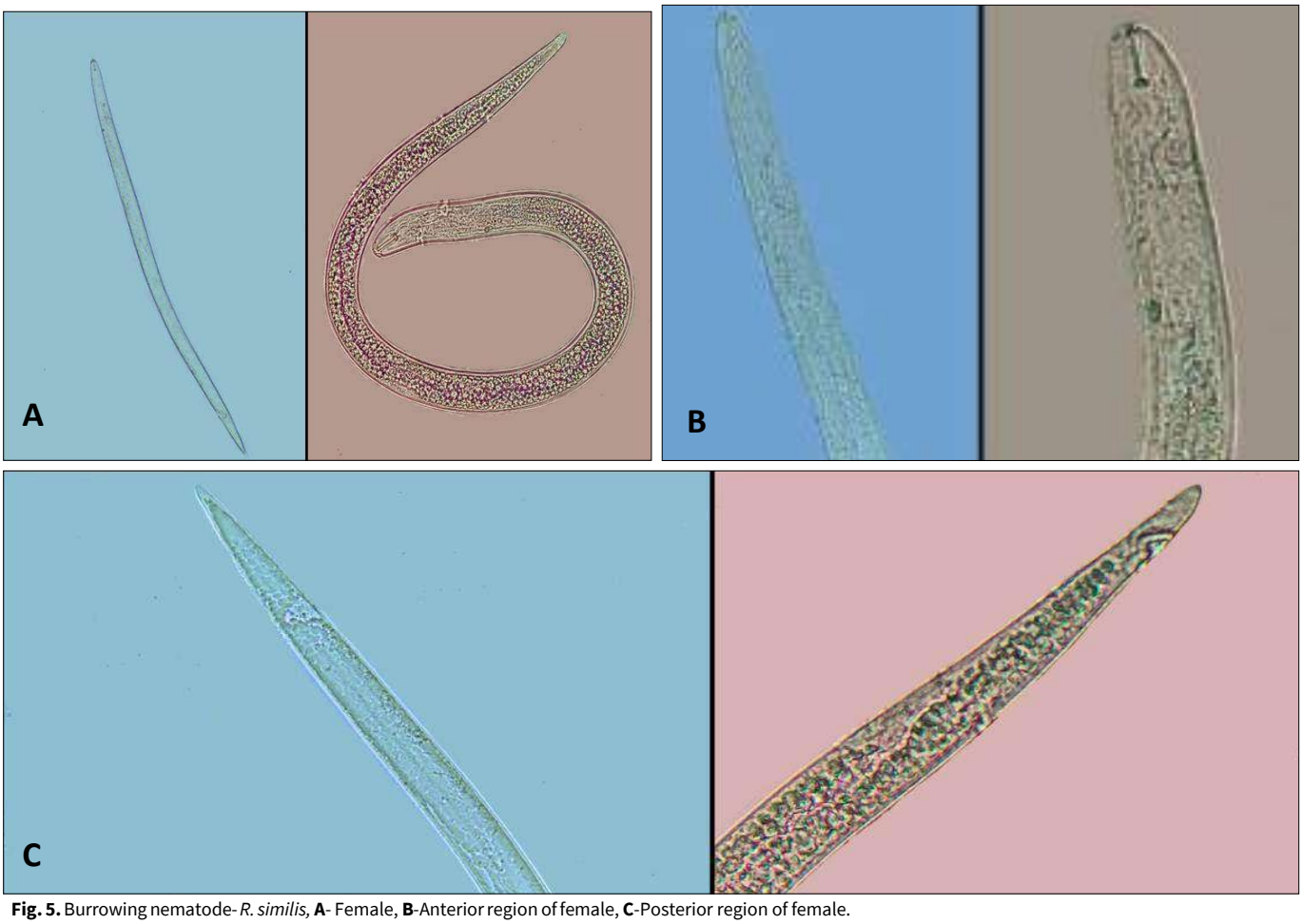


**Fig. 5.** Burrowing nematode- *R. similis,* **A**- Female, **B**-Anterior region of female, **C**-Posterior region of female.

Chakkupallam (PV-15.21) and Marayoor (PV-15.29). The highest population of *M. incognita* was recorded in Marayoor panchayath with average of 311 followed by Chakkupallam (254), Kattappana (200) and Pampadumpara (192). Similar findings were reported by previous workers (31).

Survey was conducted in 2 blocks (Mananthavady and Panamaram) in Wayanad district and a total of 60 samples were collected from 6 panchayaths *viz.,* Thirunelly, Vellamnunda, Mananthavady, Edavaka, Panamaram and Pulpally (Table 3). Presence of *M. incognita*, *R. reniformis*, *R. similis*, *H. dihystera, Macroposthonia* sp. and free-living nematodes were observed in the samp

**Table 1.** Community structure of plant parasitic nematodes distributed in black pepper growing areas in Kannur district (AEU- 15)

| District | Panchayath/ Municipality | Nematode species | Average | Absolute frequency (%) | Relative frequency (%) | Absolute density (No. of nematodes/200 cc soil) | Relative density (%) | Prominence value |
|---|---|---|---|---|---|---|---|---|
| Kannur | Kurumathur | *M. incognita* | 236 | 100 | 22.73 | 117.85 | 31.22 | 11.79 |
| | | *R. reniformis* | 103 | 70 | 15.91 | 51.71 | 13.70 | 4.33 |
| | | *R. similis* | 123 | 70 | 15.91 | 61.25 | 16.23 | 5.12 |
| | | *H.dihystera* | 137 | 100 | 22.73 | 68.70 | 18.20 | 6.87 |
| | | Free living | 156 | 100 | 22.73 | 77.95 | 20.65 | 7.80 |
| | Thaliparamba | *M. incognita* | 246 | 100 | 21.74 | 122.75 | 32.40 | 12.28 |
| | | *R. reniformis* | 84 | 70 | 15.22 | 41.85 | 11.05 | 3.50 |
| | | *R. similis* | 147 | 100 | 21.74 | 73.25 | 19.34 | 7.33 |
| | | *H.dihystera* | 136 | 90 | 19.57 | 67.85 | 17.91 | 6.44 |
| | | Free living | 146 | 100 | 21.74 | 73.10 | 19.30 | 7.31 |
| | Padiyoor | *M. incognita* | 316 | 90 | 20.00 | 158.10 | 35.51 | 14.99 |
| | | *R. reniformis* | 101 | 80 | 17.78 | 50.42 | 11.32 | 4.51 |
| | | *R. similis* | 143 | 80 | 17.78 | 71.66 | 16.09 | 6.41 |
| | | *H.dihystera* | 168 | 100 | 22.22 | 84.15 | 18.90 | 8.42 |
| | | Free living | 162 | 100 | 22.22 | 80.95 | 18.18 | 8.10 |
| | Malapattam | *M. incognita* | 214 | 90 | 19.57 | 106.88 | 22.15 | 10.14 |
| | | *R. reniformis* | 259 | 90 | 19.57 | 129.57 | 26.86 | 12.29 |
| | | *R. similis* | 124 | 90 | 19.57 | 61.84 | 12.82 | 5.87 |
| | | *H.dihystera* | 175 | 90 | 19.57 | 87.33 | 18.10 | 8.28 |
| | | Free living | 194 | 100 | 21.74 | 96.80 | 20.07 | 9.68 |
| | Ulikkal | *M. incognita* | 238 | 90 | 20.93 | 119.20 | 27.45 | 11.31 |
| | | *R. reniformis* | 147 | 70 | 16.28 | 73.35 | 16.89 | 6.14 |
| | | *R. similis* | 89 | 80 | 18.60 | 44.32 | 10.21 | 3.96 |
| | | *H.dihystera* | 198 | 90 | 20.93 | 99.16 | 22.84 | 9.41 |
| | | Free living | 196 | 100 | 23.26 | 98.16 | 22.61 | 9.82 |
| | Irikkur | *M. incognita* | 266 | 100 | 23.26 | 132.93 | 30.49 | 13.29 |
| | | *R. reniformis* | 101 | 80 | 18.60 | 50.62 | 11.61 | 4.53 |
| | | *R. similis* | 140 | 80 | 18.60 | 70.00 | 16.05 | 6.26 |
| | | *H.dihystera* | 191 | 70 | 16.28 | 95.35 | 21.86 | 7.98 |
| | | Free living | 174 | 100 | 23.26 | 87.10 | 19.97 | 8.71 |

**Table 2.** Community structure of plant parasitic nematodes distributed in black pepper growing areas in Idukki district (AEU-16)

| District | Panchayath | Nematode species | Average | Absolute frequency (%) | Relative frequency (%) | Absolute density (No. of nematodes/200 cc soil) | Relative density (%) | Prominence value |
|---|---|---|---|---|---|---|---|---|
| Idukki | Kattappana | *M. incognita* | 200 | 80 | 20.00 | 99.75 | 25.71 | 8.92 |
| | | *R. reniformis* | 50 | 50 | 12.50 | 24.90 | 6.42 | 1.76 |
| | | *R. similis* | 117 | 100 | 25.00 | 83.25 | 21.45 | 8.33 |
| | | *H.dihystera* | 209 | 70 | 17.50 | 104.64 | 26.97 | 8.75 |
| | | Free living | 151 | 100 | 25.00 | 75.50 | 19.46 | 7.55 |
| | Pampadumpara | *M. incognita* | 192 | 90 | 23.68 | 95.83 | 24.79 | 9.09 |
| | | *R. reniformis* | 43 | 50 | 13.16 | 21.30 | 5.51 | 1.51 |
| | | *R. similis* | 128 | 70 | 18.42 | 69.12 | 17.88 | 5.78 |
| | | *H.dihystera* | 250 | 70 | 18.42 | 125.00 | 32.34 | 10.46 |
| | | Free living | 151 | 100 | 26.32 | 75.30 | 19.48 | 7.53 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Manjappara | *M. incognita* | 90 | 70 | 25.00 | 44.85 | 28.28 | 3.75 |
| | | *R. similis* | 66 | 50 | 17.86 | 32.92 | 20.76 | 2.33 |
| | | *H.dihystera* | 50 | 60 | 21.43 | 24.91 | 15.71 | 1.93 |
| | | Free living | 112 | 100 | 35.71 | 55.90 | 35.25 | 5.59 |
| | Kanchiyar | *M. incognita* | 142 | 100 | 26.32 | 70.90 | 42.71 | 7.09 |
| | | *R. reniformis* | 34 | 40 | 10.53 | 17.00 | 10.24 | 1.08 |
| | | *R. similis* | 39 | 40 | 10.53 | 19.32 | 11.64 | 1.22 |
| | | *H.dihystera* | 61 | 100 | 26.32 | 30.30 | 18.25 | 3.03 |
| | | Free living | 57 | 100 | 26.32 | 28.50 | 17.17 | 2.85 |
| | Erattayar | *M. incognita* | 140 | 80 | 20.00 | 69.87 | 22.91 | 6.25 |
| | | *R. reniformis* | 46 | 60 | 15.00 | 22.75 | 7.46 | 1.76 |
| | | *R. similis* | 124 | 80 | 20.00 | 62.10 | 20.36 | 5.55 |
| | | *H.dihystera* | 254 | 80 | 20.00 | 127.12 | 41.68 | 11.37 |
| | | Free living | 46 | 100 | 25.00 | 23.17 | 7.60 | 2.32 |
| Idukki | Upputhara | *M. incognita* | 163 | 100 | 20.00 | 81.45 | 21.11 | 8.15 |
| | | *R. reniformis* | 86 | 100 | 20.00 | 42.90 | 11.12 | 4.29 |
| | | *R. similis* | 127 | 100 | 20.00 | 63.25 | 16.40 | 6.33 |
| | | *H.dihystera* | 277 | 100 | 20.00 | 138.35 | 35.87 | 13.84 |
| | | Free living | 120 | 100 | 20.00 | 59.80 | 15.50 | 5.98 |
| | Chakkupallam | *M. incognita* | 254 | 100 | 25.00 | 126.95 | 31.18 | 12.70 |
| | | *R. reniformis* | 15 | 30 | 7.50 | 7.50 | 1.84 | 0.41 |
| | | *R. similis* | 130 | 70 | 17.50 | 62.22 | 15.28 | 5.21 |
| | | *H.dihystera* | 304 | 100 | 25.00 | 152.05 | 37.35 | 15.21 |
| | | Free living | 117 | 100 | 25.00 | 58.40 | 14.34 | 5.84 |
| | Marayoor | *M. incognita* | 311 | 90 | 21.95 | 155.62 | 33.61 | 14.76 |
| | | *R. reniformis* | 77 | 50 | 12.20 | 38.30 | 8.27 | 2.71 |
| | | *R. similis* | 127 | 90 | 21.95 | 63.25 | 13.66 | 6.00 |
| | | *H.dihystera* | 342 | 80 | 19.51 | 170.93 | 36.92 | 15.29 |
| | | Free living | 70 | 100 | 24.39 | 34.85 | 7.53 | 3.49 |
| | Munnar | *H. dihystera* | 100 | 50 | 39.60 | 50.00 | 39.60 | 3.54 |
| | | Free living | 153 | 100 | 60.40 | 76.25 | 60.40 | 7.63 |

**Table 3.** Community structure of plant parasitic nematodes distributed in black pepper growing areas in Wayanad district (AEU-21)

| District | Panchayath/ Municipality | Nematode species | Average | Absolute frequency (%) | Relative frequency (%) | Absolute density (No. of nematodes/ 200 cc soil) | Relative Density (%) | Prominence value |
|---|---|---|---|---|---|---|---|---|
| Wayanad | Thirunelly | *M. incognita* | 228 | 100.00 | 22.22 | 114.00 | 38.61 | 11.40 |
| | | *R. reniformis* | 86 | 60.00 | 17.14 | 42.91 | 14.53 | 3.32 |
| | | *R. similis* | 94 | 90.00 | 20.00 | 46.78 | 15.84 | 4.44 |
| | | *H. dihystera* | 104 | 70.00 | 15.56 | 52.14 | 17.66 | 4.36 |
| | | *Macroposthonia* sp. | 3 | 30.00 | 6.67 | 1.33 | 0.45 | 0.07 |
| | | Free living | 76 | 100.00 | 22.22 | 38.12 | 12.91 | 3.81 |
| | Vellamunda | *M. incognita* | 231 | 100.00 | 21.28 | 115.35 | 34.42 | 11.54 |
| | | *R. reniformis* | 40 | 70.00 | 14.89 | 20.00 | 5.97 | 1.67 |
| | | *R. similis* | 130 | 70.00 | 14.89 | 65.22 | 19.46 | 5.46 |
| | | *H. dihystera* | 105 | 90.00 | 19.15 | 52.38 | 15.63 | 4.97 |
| | | *Macroposthonia* sp. | 2 | 40.00 | 8.51 | 0.50 | 0.15 | 0.03 |
| | | Free living | 163 | 100.00 | 21.28 | 81.65 | 24.37 | 8.17 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Wayanad | Mananthavady | *M. incognita* | 209 | 80.00 | 17.02 | 104.3 | 38.58 | 9.33 |
| | | *R. reniformis* | 86 | 80.00 | 17.02 | 43.12 | 15.95 | 3.86 |
| | | *R. similis* | 41 | 80.00 | 17.02 | 20.65 | 7.64 | 1.85 |
| | | *H. dihystera* | 91 | 80.00 | 17.02 | 45.50 | 16.83 | 4.07 |
| | | *Macroposthonia* sp. | 2 | 50.00 | 10.64 | 0.80 | 0.30 | 0.06 |
| | | Free living | 112 | 100.00 | 21.28 | 55.95 | 20.70 | 5.60 |
| | Edavaka | *M. incognita* | 258 | 90.00 | 18.24 | 129.16 | 36.28 | 12.25 |
| | | *R. reniformis* | 120 | 80.00 | 16.22 | 60.12 | 16.89 | 5.38 |
| | | *R. similis* | 124 | 90.00 | 18.24 | 61.99 | 17.41 | 5.88 |
| | | *H. dihystera* | 102 | 100.00 | 20.27 | 50.85 | 14.28 | 5.09 |
| | | *Macroposthonia* sp. | 1 | 33.33 | 6.67 | 0.50 | 0.14 | 0.03 |
| | | Free living | 107 | 100.00 | 20.27 | 53.35 | 14.99 | 5.34 |
| | Panamaram | *M. incognita* | 326 | 90.00 | 18.75 | 163.21 | 38.50 | 15.48 |
| | | *R. reniformis* | 36 | 90.00 | 18.75 | 18.20 | 4.29 | 1.73 |
| | | *R. similis* | 100 | 80.00 | 16.67 | 50.10 | 11.82 | 4.48 |
| | | *H. dihystera* | 207 | 100.00 | 20.83 | 103.70 | 24.46 | 10.37 |
| | | *Macroposthonia* sp. | 1 | 20.00 | 4.17 | 0.50 | 0.12 | 0.02 |
| | | Free living | 176 | 100.00 | 20.83 | 88.20 | 20.81 | 8.82 |
| | Pulpally | *M. incognita* | 321 | 70.00 | 15.11 | 160.25 | 26.15 | 13.41 |
| | | *R. reniformis* | 192 | 80.00 | 17.27 | 96.12 | 15.69 | 8.60 |
| | | *R. similis* | 141 | 100.00 | 21.58 | 70.29 | 11.47 | 7.03 |
| | | *H. dihystera* | 341 | 80.00 | 17.27 | 170.22 | 27.78 | 15.22 |
| | | *Macroposthonia* sp. | 1 | 33.33 | 7.19 | 0.60 | 0.10 | 0.03 |
| | | Free living | 231 | 100.00 | 21.58 | 115.26 | 18.81 | 11.53 |

les. The highest population of *M. incognita* was observed in Panamaram panchayath (average-326; PV-15.48). In Pulpally, population of both *H. dihystera* (PV-15.22) and *M. incognita* (PV-13.14) found prominent. Yellowing and galling in roots were common symptoms in black pepper vines in these panchayaths. *R. reniformis* was found highest in Pulpally with an average population of 192 and PV of 8.60. *R. similis* exhibited the highest average population (141) in Pulpally with PV of 7.03. The presence of *Macroposthonia* sp. was found in all panchayaths but the population was very low. The results of the present study highlighted occurrence of *M. incognita* in all the locations surveyed in the 3 AEUs in Kannur, Idukki and Wayand districts.

Fungal pathogens were also isolated from soil and root samples collected from AEU 15, 16 and 21 using the serial dilution technique. Based on morphological characteristics (Table 4) the pathogens were identified as *Fusarium oxysporum*, *F. solani, Phytophthora* sp., *Pythium* sp. and *Rhizoctonia* sp. *F. oxysporum* (Fig. 6) was isolated from the soil samples collected from Kurumathur and Padiyoor in Kannur; Manjappara and Pallanadu in Idukki; and Thazheyangadi in Wayanad. *F. solani* (Fig. 7) was isolated from Panniyur, Padiyoor, Malapattam and Perumannu in Kannur district; Vellilamkandam, Kochera and Pallanadu in Idukki district; and Koomankolly, Vandimoola, Karimbummal and Thazheyangadi in Wayanad district. *Phytophthora* sp. (Fig. 8) was isolated from Koomankolly, Vandimoola and Pallikkal in Wayanad district. *Pythium* sp. (Fig. 9) was isolated from Panniyoor and Pookkandam in Kannur, Chempakappara in Idukki and Palakkuli and Karimbummal in Wayanad district. *Rhizoctonia* sp. (Fig. 10) was isolated from Palakulangara and Malapattam in Kannur, Upputhara and Pampadumpara in Idukki and Pazhayngadi in Wayanad

**Table 4.** Morphological and cultural characteristics of fungal isolates

| Pathogen | Nature ofmycelia | Septation of hyphae | Spores/ Fruiting body | Shape ofspores/ Fruiting body | Size of spore/Fruiting body |
|---|---|---|---|---|---|
| *F. oxysporum* | Whitefluffy | Septate | Macroconidia | Falcate,pointedends | 37 µm × 3.5 µm |
| | | | Microconidia | Oval | 9 µm × 3.2 µm |
| *F. solani* | Whitefluffy | Septate | Macroconidia | Falcate tostraight, blunt ends | 26.3 µm × 3.2µm |
| | | | Microconidia | Ellipsoid | 9.8 µm × 2.6 µm |
| *Phytophthora* sp. | Hyaline | Aseptate | Sporangia | Lemonshaped | Pedicel length = 12 µmSporangia = 32 µm × 17 µm |
| *Pythium* sp. | White cottony | Aseptate | Oospores | Round,doublelayered | Diameter = 39.4µm |
| *Rhizoctonia* sp. | White cottony | Septate | Sclerotia | - | - |

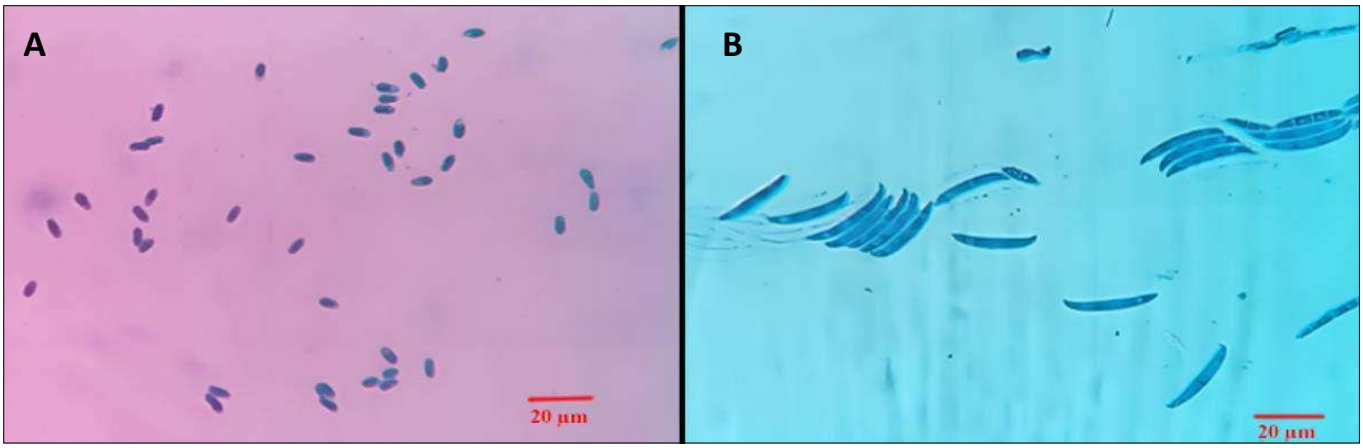


**Fig. 6.** Spore characters of *F. oxysporum.* **A**- Microconidia, **B**- Macroconidia.

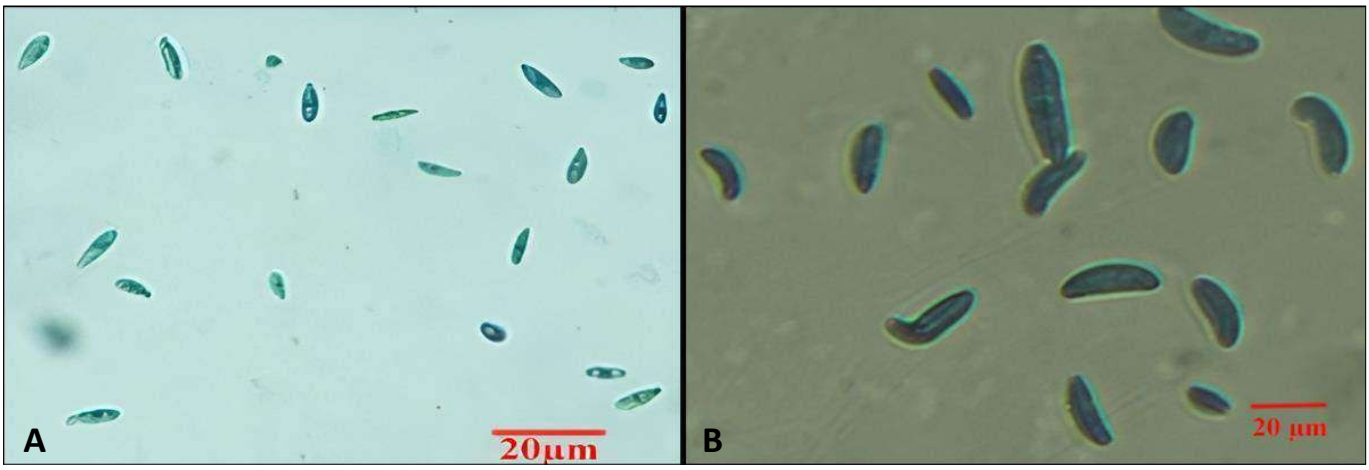


**Fig. 7.** Spore characters of *F. solani.* **A**- Microconidia, **B**- Macroconidia.

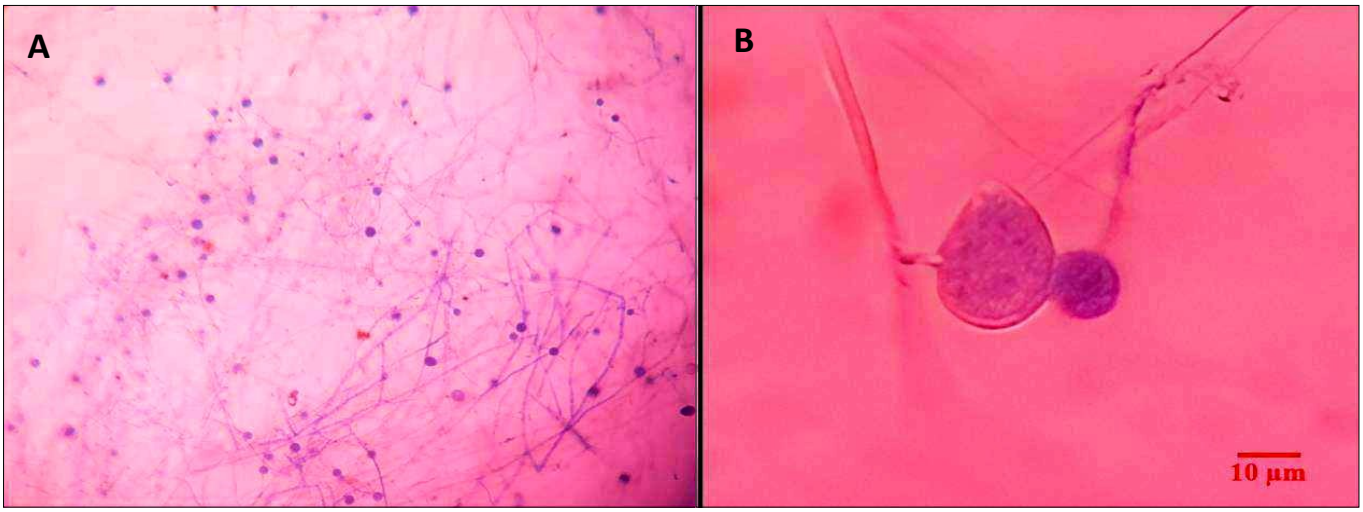


**Fig. 8.** Characteristics of *Phytophthora sp.* **A**- Fungal hyphae, **B**- Sporangium.

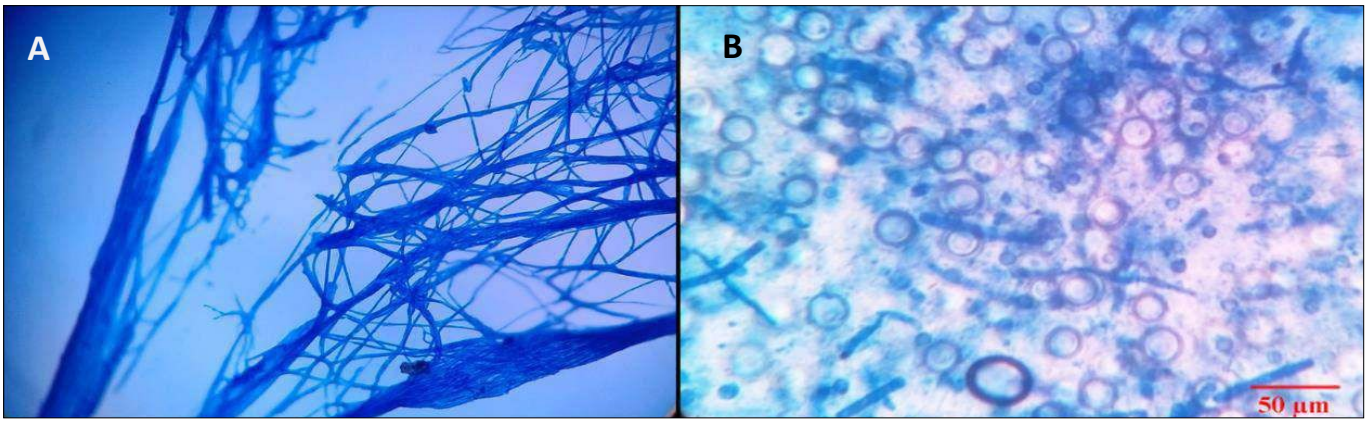


**Fig. 9.** Characteristics of *Pythium sp.* **A**- Large aseptate hyphae, **B**- Double walled oogonia.

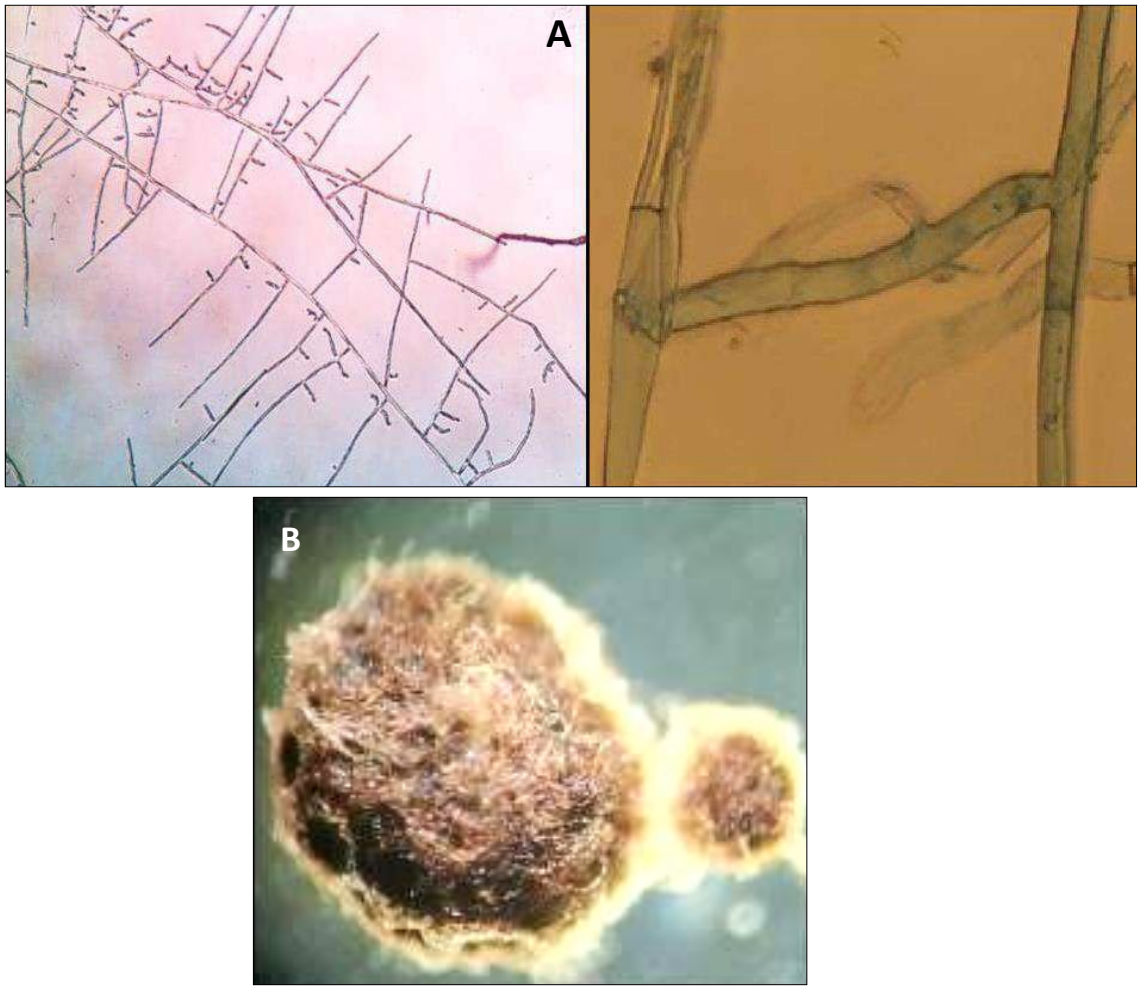


**Fig. 10.** Characteristics of *Rhizoctonia* sp. **A**-Right angled hyphae, **B**- Sclerotia.

**Table 5.** Fungal pathogens associated with nematode infested black pepper plants in surveyed locations

| AEU. | Block | Panchayath | Location | Latitude | Longitude | *F. oxysporum* | *F. solani* | *Phytophthora* sp. | *Rhizoctonia* sp. | *Pythium* sp. |
|---|---|---|---|---|---|---|---|---|---|---|
| 15 | Thaliparamba | Kurumathur | Kurumathur | 12.07732 | 75.40549 | + | - | - | - | - |
| | | | Palakulangara | 12.03641 | 75.37177 | - | - | - | + | - |
| | | | Panniyoor | 12.08114 | 75.39949 | - | + | - | - | + |
| | Irikkur | Padiyoor | Padiyoor | 12.00138 | 75.58176 | + | + | - | - | - |
| | | Malapattam | Pookkandam | 11.99628 | 75.62057 | - | - | - | - | + |
| | | | Malapattam | 11.98798 | 75.61496 | - | + | - | + | - |
| | | Irikkur | Perumannu | 11.99052 | 75.56371 | - | + | - | - | - |
| 16 | Kattappana | Mattukkatta | Vellilamkandam | 9.700285 | 77.06733 | - | + | - | - | - |
| | | Erattayar | Chempakappara | 9.794875 | 77.10759 | - | - | - | - | + |
| | | Upputhara | Upputhara | 9.710918 | 77.01439 | - | - | - | + | - |
| | Nedumkanadam | Pampadumpara | Pampadumpara | 9.794447 | 77.13414 | - | - | - | + | - |
| | | Manjappara | Manjappara | 9.874517 | 77.09044 | + | - | - | - | - |
| | | Anakkara | Kochera | 9.704891 | 77.18704 | - | + | - | - | - |
| | Devikulam | Marayoor | Pallanadu | 10.23238 | 77.13794 | + | + | - | - | - |
| 21 | Mananthavady | Thirunelly | Koomankolly | 11.90666 | 75.99558 | - | + | + | - | - |
| | | Vellamunda | Pazhayangadi | 11.73941 | 75.9405 | - | - | - | + | - |
| | | Manhthavady | Varadimoola | 11.81161 | 76.01644 | - | + | + | - | - |
| | | | Palakkuli | 11.81402 | 75.98538 | - | - | - | - | + |
| | | Edavaka | Pallikkal | 11.77599 | 76.07294 | - | - | + | - | - |
| | Panamaram | Panamaram | Karimbummal | 11.72855 | 76.07358 | - | + | - | - | + |
| | | Pulpally | Thazheyangadi | 11.78672 | 76.16639 | + | + | - | - | - |

(Table 5). The plants exhibited symptoms *viz.* yellowing, wilting, drying of immature berries, falling of berries, wilting and rotting in collar region. Slow wilt disease in black pepper was first reported from Wayanad district in Kerala in 1902 and mortality upto 10% of the vines due to the disease (32). *R. similis* and *Phytophthora capsici* alone or in association resulted in root rotting leading to slow decline disease (33).

From the above observations, it is clear that *F. solani* was the most occurring and major disease-causing pathogen in the nematode infested soils of the AEU-15, 16 and 21 in Kerala. *F. solani* was found occurring in the regions where *M. incognita* population was higher like Padiyoor region in Kannur (AEU-15), Pallanadu region in Marayur panchayath of Idukki district (AEU-16) and Karimbummal region in Panamaram panchayath of Wayanad (AEU-21). Thus *F. solani* was selected for the further interaction studies along with *M. incognita* in black pepper under pot culture conditions. The identity of *F. solani* was confirmed by morphological, cultural and molecular characterization. The DNA sequence from the internal transcribed spacer regions (ITS) were amplified using the universal primers ITS-1 (5'TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for molecular identification. An expected amplicon of size 572 bp was obtained on PCR analysis. The sequence obtained was submitted to NCBI with a submission number- PP106996. The result showed 100% similarity with *F. solani* on BLAST analysis.

## Interaction between *M. incognita* and *F. solani*

The results of pot culture study revealed significant difference in nematode population characteristics, disease parameters and growth parameters of black pepper plants when *M. incognita* and *F. solani* was inoculated individually, simultaneously and sequentially.

In the present study, the nematode population in the soil (616.00 *M. incognita* J2 in 200 cc) and root (64.33 J2 in 5 g) was found higher in the plants inoculated with *M. incognita* alone (Table 6). The population was lowest in the plants in which *F. solani* was inoculated 2 weeks prior to *M. incognita* inoculation (448.00). Effect of simultaneous inoculation of both pathogens (529.67) was statistically on par to inoculation of *M. incognita* 2 weeks prior to *F. solani* (540.67). Similar trend was observed in number of females (5 g root), total nematode population and reproduction factor. There was a noticeable decrease in the number of nematodes in sequential and simultaneous inoculation of both pathogens in comparison to plants inoculated with *M. incognita* alone (616.00). The infection of the fungus in plants resulted in reduction in the population of nematodes probably due to the unfavourable environment in the nematode feeding sites for nematode reproduction caused by the invasion of the fungal mycelia. The adverse condition led to sex reversal and the newly formed male nematodes departed roots without feeding (34). Lower number of juveniles and females in sequential inoculation of *F. oxysporum* prior to *M. incognita* followed by the concomitant inoculation of *M. incognita* and *F. oxysporum* was observed in roots of gerbera plants (35). In the present study, the reproduction factor (Rf) was found to be higher (4.12) in the plants in which the nematodes alone are inoculated and was followed by nematode 2 weeks prior to fungus (3.39), concomitant inoculation of both the pathogens (3.24) and

**Table 6.** Effect of *M. incognita* and *F. solani* on nematode population characteristics in black pepper under pot culture condition

| Treatments | Final nematode population | | | Total nematode population* | RF (Pf/Pi)* | No. of galls* (5 g root) | No. of eggmasses* (5 g root) | No. of eggs in egg masses* |
|---|---|---|---|---|---|---|---|---|
| | Soil* (200 cc) | Root*(5 g) | No. of females in root* (5 g) | | | | | |
| T1 | 616.00 (24.81)$^{a}$ | 64.33 (8.19)$^{a}$ | 111.33 (10.51)$^{a}$ | 791.67 (28.13)$^{a}$ | 4.12$^{a}$ | 77.00 (8.75)$^{a}$ | 66.67 (8.11)$^{a}$ | 194.67 (13.95)$^{a}$ |
| T2 | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{c}$ | 0.00 (1.00)$^{c}$ |
| T3 | 529.67 (23.01)$^{b}$ | 43.33 (6.65)$^{b}$ | 89.33 (9.45)$^{b}$ | 662.32 (25.73)$^{b}$ | 3.24$^{b}$ | 53.67 (7.31)$^{c}$ | 56.33 (7.50)$^{b}$ | 153.67 (12.39)$^{b}$ |
| T4 | 540.67 (23.25)$^{b}$ | 46.66 (6.81)$^{b}$ | 95.00 (9.74)$^{b}$ | 682.33 (26.12)$^{b}$ | 3.39$^{b}$ | 67.00 (8.18)$^{b}$ | 61.00 (7.81)$^{ab}$ | 191.67 (13.84)$^{a}$ |
| T5 | 448.00 (21.16)$^{c}$ | 26.00 (5.08)$^{c}$ | 75.16 (8.67)$^{c}$ | 549.16 (23.43)$^{c}$ | 2.72$^{c}$ | 49.34 (7.01)$^{c}$ | 54.34 (7.43)$^{b}$ | 144.67 (12.02)$^{b}$ |
| T6 | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00$^{d}$ | 0.00 (1.00)$^{d}$ | 0.00 (1.00)$^{c}$ | 0.00 (1.00)$^{c}$ |
| CD (0.05) | (0.623) | (0.793) | (0.585) | (0.588) | (0.315) | (0.521) | (0.452) | (0.408) |
| CV (%) | 2.230 | 9.300 | 4.889 | 1.887 | 7.880 | 5.277 | 4.640 | 2.538 |
| SE(m) | 0.202 | 0.257 | 0.190 | 0.225 | 0.102 | 0.152 | 0.174 | 0.212 |

**T1**: *M. incognita* alone; **T2**: *F. solani* alone; **T3**: Both pathogens simultaneously; **T4**: *M. incognita* first and *F. solani* 2 weeks after nematodeinoculation; **T5**: *F. solani* first and *M. incognita* 2 weeks after fungus inoculation; **T6**: Uninoculated Control; **CD**: Critical difference; **CV**: Coefficient of Variation; **SE(m)**: Standard Error of mean. *****Mean of 3 replications. Figures in the parentheses are square root transformed and transformed values ,**PF**-Final nematode population, **Pi**- Initial nematode population, **RF**- reproduction factor

fungus inoculated 2 weeks prior to nematode (2.72). The finding is in accordance with earlier workers (36) in vegetable cowpea where they reported high reproduction factor in *M. incognita* alone treatment (2.34) followed by *M. incognita* inoculated 7 days prior to *F. oxvsporum* inoculation (1.79).

Highest number of galls (77.00 in 5 g root) was recorded in plants inoculated with *M. incognita* alone. This was followed by plants inoculated with *M. incognita* 2 weeks prior to *F. solani* (67.00), both pathogens simultaneously (53.67) and inoculation of *F. solani* 2 weeks prior to *M. incognita* inoculation (49.34). The number of galls was reduced in plants inoculated with fungus 2 weeks prior to nematode inoculation compared to concomitant and sequential inoculation of nematode 14 days prior to fungus. The increased root galling in presence of *F. solani* in simultaneous inoculation and nematode inoculation 2 weeks prior to fungus inoculation may be due to increased penetration rate of *M. incognita* juveniles into plant roots and developing root galls associated with nematode feeding may serve as nutrient sink. Besides, inoculation of nematodes 2 weeks prior to fungus inoculation elevates carbohydrates and nitrogenous compounds in the root exudates which act as a nutrient source for the fungus to establish. Gall formation by nematode was affected in plants inoculated with *F. solani* 2 weeks prior to nematode due to the establishment of fungal mycelia and toxic metabolites produced by the fungus (37).

The data on number of egg masses in root (5 g) was highest in plants inoculated with *M. incognita* alone (66.67) and it was on par with *M. incognita* 2 weeks prior to *F. solani* (61.00). This was followed by plants inoculated with both the pathogens simultaneously (56.33) and *F. solani* 2 weeks prior to *M. incognita* (54.34). The same trend was observed in the case of number of eggs in egg mass also. The number of eggs in egg mass was found higher (194.67) in *M. incognita* alone treated plants and was followed by plants inoculated with *M. incognita* 2 weeks prior to *F. solani* (191.67). The lowest count of eggs in egg mass was observed in plants in which *F. solani* was inoculated 2 weeks prior to *M. incognita* inoculation (144.67). Similar type of reduced galling and reduction in egg mass in presence of *F. oxysporum* f. sp. *ciceri* was reported in chickpea (38).

Highest reduction in growth parameters (shoot length, root length, fresh weight of shoot and root) were observed at 45 days after inoculation (DAI) in plants inoculated with *M. incognita* 2 weeks prior to nematode inoculation (Table 7). It was followed by simultaneous inoculation of both pathogens and inoculation of *F. solani* 2 weeks prior to *M. incognita* inoculation. The percentage reduction of growth parameters over uninoculated control in these 3 treatments ranged from 30.89 to 70.21 (Fig. 11). Results of earlier studies revealed the synergistic effect of both organisms in suppressing growth parameters in black pepper (39, 40). The result obtained in this study demonstrated the role of the nematode as a primary invader and strengthening the ability of the fungus to aggravate disease and reduce the plant growth. Substantial decrease in plant growth parameters was observed in sequential and simultaneous inoculation of *M. incognita* and *F. solani*. This observation suggests that the synergistic effect of both pathogens may be responsible for the physiological and anatomical changes in the root tissues which make the plants more susceptible to

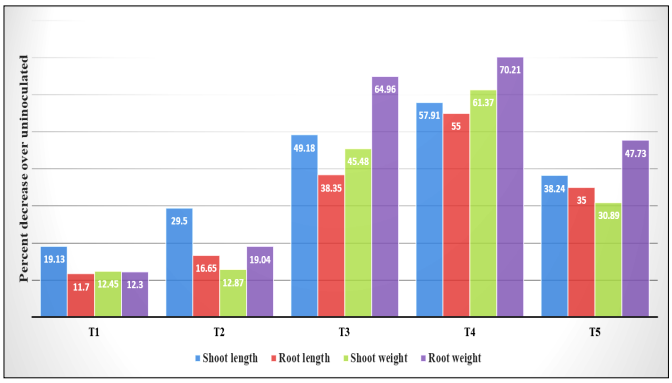


**Fig. 11.** Effect of *M. incognita* and *F. solani* on biometric characters of black pepper inpot culture condition. **T1**: *M. incognita* alone; **T2**: *F. solani* pathogen alone; **T3**: Both pathogens simultaneously; **T4**: *M. incognita* first and *F. solani* two weeks after nematode inoculation; **T5**: *F. solani* first and *M. incognita* 2 weeks after fungus inoculation.

**Table 7.** Effect of *M. incognita* and *F. solani* on growth parameters of black pepper under pot culture condition

| Treatments | Plant growth parameters | | | |
|---|---|---|---|---|
| | Length (cm)* | | Fresh weight (g)* | |
| | Shoot | Root | Shoot | Root |
| *M. incognita* alone | 49.33 ± 9.87 [b] | 15.00 ± 1.00 [bc] | 68.00 ± 4.35 [b] | 11.19 ± 1.13 [b] |
| *F. solani* alone | 43.00 ± 3.51 [bc] | 16.67 ± 1.52 [ab] | 67.67 ± 5.51 [b] | 10.33 ± 0.72 [b] |
| Both pathogens simultaneously | 31.00 ± 3.60 [de] | 12.33 ± 1.52 [cd] | 42.34 ± 3.51 [d] | 4.74 ± 0.74 [d] |
| *M. incognita* first and *F. solani* 2 weeks after nematode inoculation | 25.67 ± 3.05 [e] | 9.00 ± 1.00 [d] | 30.00 ± 5.00 [e] | 3.80 ± 0.66 [d] |
| *F. solani* first and *M. incognita* 2 weeks after fungus inoculation | 37.67 ± 4.58 [cd] | 13.00 ± 3.00 [c] | 53.67 ± 6.03 [c] | 6.67 ± 0.84 [c] |
| Uninoculated control | 61.00 ± 7.55 [a] | 20.00 ± 2.64 [a] | 77.67 ± 5.51 [a] | 12.76 ± 0.39 [a] |
| CD 0.05 | 8.53 | 3.458 | 8.993 | 1.387 |
| CV (%) | 11.618 | 13.560 | 8.939 | 9.453 |
| SE(m) | 2.769 | 1.122 | 2.919 | 0.450 |

Mean ± SD of 3 replications. *Mean of 3 replications, **CD**: Critical difference; **CV**: Coefficient of Variation; **SE(m)**: Standard Error of mean

fungal infection.

The days taken for disease initiation was considerably reduced (24 days) in plants inoculated with *M. incognita* 2 weeks prior to *F. solani* compared to other treatments (Table 8). The highest number of days for disease initiation (31 days) was observed in plants inoculated with *F. solani* alone. Highest disease incidence (100.00%) was also observed in plants inoculated with *M. incognita* 2 weeks prior to *F. solani* and lowest was observed in plants inoculated with *F. solani* alone (33.33 %). The Percentage Disease Index (PDI) was highest in plants inoculated with *M. incognita* 2 weeks prior to *F. solani* (90.00). The presence of the nematode seemed to contribute to the early onset of wilt symptoms, suggesting that nematodes predispose plants to fungal infection and exacerbate the overall disease incidence. In the present study, PDI in plants in which *F. solani* was inoculated 2 weeks prior to *M. incognita* inoculation was 65.00 while plants inoculated with both pathogens simultaneously recorded PDI of 37.50 followed by plants inoculated with *F. solani* alone (30.00). These results agree with the findings of interaction studies between *F. oxysporum* f. sp. *lycopercisi* and *M. incognita* in tomato (41, 42). The observation of reduced root infection in plants inoculated solely with *F. solani*, as opposed to treatments where the nematode was present along with the fungus, suggests that the delay in the entry of the fungus may be attributed to the absence of a predisposing agent that attracts the fungus to galled roots. In other words, the presence of the nematode might play a role in creating conditions that make the roots more susceptible to fungal infection.

**Table 8.** Effect of *M. incognita* and *F. solani* on disease incidence in black pepper under potculture condition

| Treatments | Days taken for symptom development | Disease incidence (%)(45 DAI)* | % Disease Index* |
|---|---|---|---|
| *M. incognita* alone | No disease | 0.00 | 0.00 |
| *F. solani* alone | 31 | 33.33 | 30.00 |
| Both pathogens simultaneously | 28 | 40.00 | 37.50 |
| *M. incognita* first and *F. solani* 2 weeks after nematode inoculation | 24 | 100.00 | 90.00 |
| *F. solani* first and *M. incognita* 2 weeks after fungus inoculation | 26 | 66.66 | 65.00 |
| Uninoculated control | No disease | 0.00 | 0.00 |

*Mean of 3 replications

The interaction of *M. incognita* with *F. solani* in the present study confirms the association between the nematode and fungal pathogens in developing disease complex in black pepper. The increased incidence of disease was observed when both pathogens were inoculated sequentially or concomitantly and it may be due to the superficial injury caused by nematode, which provides the fungus entry points into the host. The highest synergistic effect of both pathogens in nematode inoculation 14 days prior to fungus inoculation resulted in higher reduction in plant growth characters and increased disease severity than inoculation of pathogens individually.

## Histopathological changes during the establishment of *M. incognita* and *F. solani* disease complex in black pepper

No giant cell formation was observed in the uninoculated control, and the epidermis, cortex, xylem and phloem cells were intact devoid of any cavities (Fig. 12). The site of giant cell formation was found to be in the stelar region in plants inoculated with *M. incognita* alone, simultaneous inoculation of *M. incognita* and *F. solani* and *M. incognita* inoculated prior to *F. solani*. The formation of giant cells occurred in the cells in between the vascular bundles in plants inoculated with *F. solani* initially and *M. incognita* 2 weeks after fungal inoculation. It may be due to the restricted movement of *M. incognita* due to initial inoculation of *F. solani* (43). They observed similar finding in the formation of giant cells in the interaction study between *Fusarium* sp. and *M. incognita* in tobacco. The highest number of giant cells (5.00) were formed in *M. incognita* alone inoculated plants while in plants inoculated with *F. solani* 2 weeks prior to *M. incognita* inoculation the number of giant cells were lowest (2.00). The size of giant cells was higher (909.2 µm$^2$) in plants inoculated with *M. incognita* alone while in plants inoculated with *M. incognita* 2 weeks prior to *F. solani* was 461.3 µm$^2$. Hypertrophy and hyperplasia were present in vascular system and the cortex area. Hyperplasia was prominent in plants inoculated with *M. incognita* alone in which the number of giant cells also higher. As a result of hyperplasia, the cells became deformed in the cortex region and vascular tissues were disorganized and dispersed.

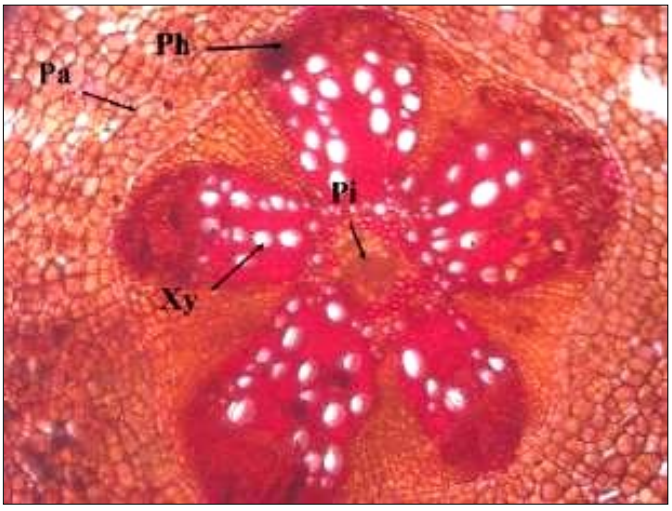


**Fig. 12**. Healthy root section of Panniyur 2**. Pa**: Parenchyma, **Ph**: Phloem, **Pi**: Pith, **Xy**: Xylem.

The fungal mycelium plugged the vascular bundles in all treatments with *F. solani.* Plugging of the xylem vessels were found to be severe in the presence of *M. incognita* and was comparatively lesser in plants inoculated with *F. solani* alone. In the present study, the formation of giant cells was found restricted or the giant cells were found to be invaded by the fungal mycelia dete-

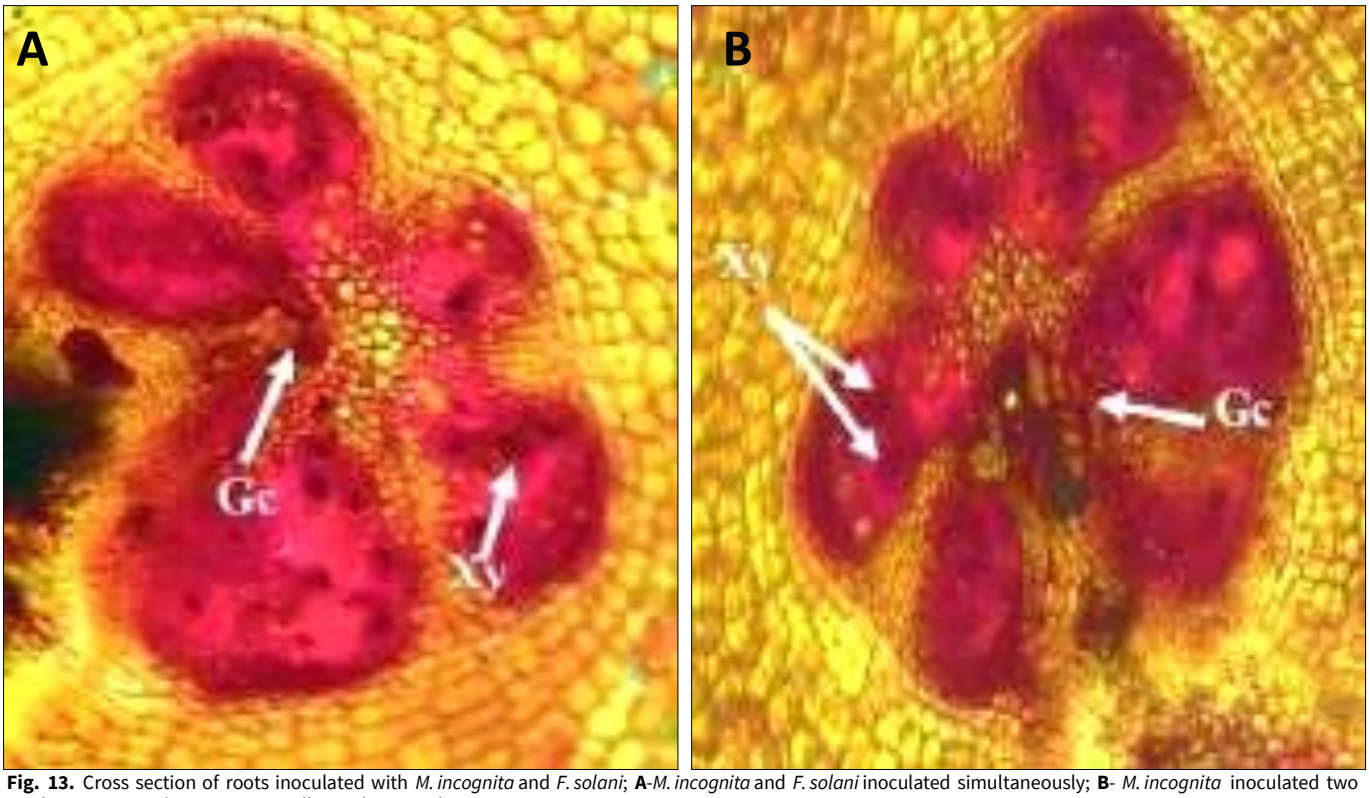


**Fig. 13.** Cross section of roots inoculated with *M. incognita* and *F. solani*; **A**-*M. incognita* and *F. solani* inoculated simultaneously; **B**- *M. incognita* inoculated two weeks prior to *F. solani ;* Gc- Giant cell; Xy- Obstructed.

riorating the giant cell complex (Fig. 13). In the histopathological studies, the size of female was found to be highest (720.23 µm) in *M. incognita* alone infected plants. This was followed by plants inoculated with *M. incognita* 2 weeks prior to *F. solani* inoculation (520.34 µm), both pathogens simultaneously (514.65 µm) and *F. solani* first and *M. incognita* 2 weeks after fungal inoculation (510.93 µm). The findings in the present study are in accordance with several workers (44, 45). The degradation of giant cells in *Fusarium* infected plants appears to be triggered by toxic metabolites released by the fungus outside the cell, rather than by the process of hyphal invasion and growth within the cell. Additionally, these giant cells were observed to be infected by multiple fungal hyphae, leading to the depletion of cytoplasm within these cells.

## Biochemical changes in black pepper due to nematode-fungus disease complex in black pepper

The biochemical changes that occurred during the interaction between *M. incognita* and *F. solani* showed significant increase in the phenol and defense enzymes *viz.,* peroxidase, phenylalanine ammonia lyase and polyphenol oxidase in the treatments with *M. incognita* and *F. solani* when compared to the uninoculated control. The roots exhibited comparatively higher increase in total phenols and the defense enzymes than the leaves. The highest phenolic content in leaves (0.49 mg $g^{-1}$ tissue) and roots (1.75 mg $g^{-1}$ tissue) were observed in plants inoculated with *M. incognita* prior to *F. solani* (Table 9). This was followed by plants in which both the pathogens are inoculated simultaneously, *F. solani* inoculated prior to *M. incognita*, *F. solani* alone and *M. incognita* alone inoculat-

**Table 9.** Effect of *M. incognita* and *F. solani* on protein, phenol and defense enzyme content in leaf and root of black pepper 45 days afterinoculation

| Treatments | Protein (µg of BSA g-1 fresh weight)* | | Phenol content (mg ofcatechol g-1 tissue)* | | Peroxidase (PO)* (min-1g-1 fresh weight) | | Phenylalanine AmmoniaLyase (PAL)* (µg of cinnamic acid g -1 fresh weight) | | Polyphenol oxidase(PPO) * (min-1g-1 fresh weight) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf | Root | Leaf | Root | Leaf | Root | Leaf | Root | Leaf | Root |
| T1 | $2.93 \pm 0.05^{b}$ | $3.06 \pm 0.16^{b}$ | $0.37 \pm 0.05^{b}$ | $0.93 \pm 0.03^{c}$ | $6.28 \pm 0.17^{c}$ | $10.01 \pm 0.16^{c}$ | $14.51 \pm 0.59^{b}$ | $14.86 \pm 0.16^{c}$ | $5.65 \pm 0.24^{b}$ | $4.31 \pm 0.17^{d}$ |
| T2 | $2.88 \pm 0.08^{bc}$ | $3.04 \pm 0.09^{bc}$ | $0.38 \pm 0.04^{b}$ | $0.93 \pm 0.08^{c}$ | $6.44 \pm 0.28^{c}$ | $9.95 \pm 0.39^{c}$ | $14.63 \pm 0.21^{b}$ | $14.99 \pm 0.21^{c}$ | $5.31 \pm 0.22^{b}$ | $4.61 \pm 0.17^{c}$ |
| T3 | $2.85 \pm 0.11^{bc}$ | $3.04 \pm 0.16^{bc}$ | $0.40 \pm 0.04^{b}$ | $1.47 \pm 0.09^{b}$ | $7.62 \pm 0.17^{b}$ | $11.01 \pm 0.09^{b}$ | $14.83 \pm 0.20^{b}$ | $15.61 \pm 0.25^{b}$ | $5.34 \pm 0.34^{b}$ | $5.08 \pm 0.16^{b}$ |
| T4 | $2.72 \pm 0.09^{c}$ | $2.82 \pm 0.17^{c}$ | $0.49 \pm 0.05^{a}$ | $1.75 \pm 0.07^{a}$ | $8.93 \pm 0.40^{a}$ | $12.56 \pm 0.32^{a}$ | $16.31 \pm 0.41^{a}$ | $16.61 \pm 0.38^{a}$ | $6.98 \pm 0.23^{a}$ | $6.99 \pm 0.18^{a}$ |
| T5 | $2.83 \pm 0.09^{bc}$ | $3.02 \pm 0.03^{bc}$ | $0.39 \pm 0.03^{b}$ | $1.43 \pm 0.13^{b}$ | $7.78 \pm 0.10^{b}$ | $11.31 \pm 0.06^{b}$ | $14.95 \pm 0.46^{b}$ | $15.57 \pm 0.29^{b}$ | $5.36 \pm 0.35^{b}$ | $5.28 \pm 0.10^{b}$ |
| T6 | $3.22 \pm 0.10^{a}$ | $3.46 \pm 0.32^{a}$ | $0.18 \pm 0.07^{c}$ | $0.20 \pm 0.40^{d}$ | $3.17 \pm 0.13^{d}$ | $9.40 \pm 0.21^{d}$ | $13.65 \pm 0.12^{c}$ | $12.97 \pm 0.33^{d}$ | $3.93 \pm 0.29^{c}$ | $3.83 \pm 0.12^{e}$ |
| CD 0.05 | 0.160 | 0.230 | 0.082 | 0.144 | 0.418 | 0.421 | 0.659 | 0.501 | 0.361 | 0.273 |
| CV (%) | 3.095 | 4.215 | 12.589 | 7.252 | 3.510 | 2.212 | 2.503 | 1.863 | 3.735 | 3.063 |
| SE(m) | 0.052 | 0.075 | 0.027 | 0.047 | 0.136 | 0.137 | 0.214 | 0.162 | 0.117 | 0.089 |

**T1**: *M. incognita* alone; **T2**: *F. solani* alone; **T3**: Both pathogens simultaneously; **T4**: *M. incognita* first and *F. solani* 2 weeks after nematodeinoculation; **T5**: *F. solani* first and *M. incognita* 2 weeks after fungus inoculation; **T6**: Uninoculated Control; **CD**: Critical difference; **CV**: Coefficient of Variation; **SE(m)**: Standard Error of mean. *****Mean of 3 replications, Mean ± SD of 3 replications

ed plants. Similar findings were reported by earlier workers in rice (46), who discovered that rice varieties infected with *Meloidogyne graminicola* (Golden and Birchfield) produced higher amount of phenolics compared to healthy plants. The accumulation of phenolic compounds was attributed to the activation of pathways like hexose monophosphate shunt and acetate pathways, along with the liberation of bound phenols by hydrolytic enzymes (47, 48).

The infection of *M. incognita* and *F. solani* resulted in a reduction in protein content, and the most significant decrease was observed in plants inoculated with *M. incognita* 2 weeks prior to *F. solani*. The highest protein content was found in the leaves of uninoculated control (3.22 µg of BSA $g^{-1}$ fresh weight) followed by plants inoculated with *M. incognita* alone (2.93 µg of BSA $g^{-1}$ fresh weight). Effect of these 2 treatments was statistically independent. Protein content in leaf samples of plants inoculated with *F. solani* alone (2.88 µg of BSA $g^{-1}$ fresh weight) found statistically on par with plants inoculated both pathogens simultaneously (2.85 µg of BSA $g^{-1}$ fresh weight), *M. incognita* alone and plants in which *F. solani* was inoculated first and *M. incognita* 2 weeks after fungus inoculation (2.83 µg of BSA $g^{-1}$ fresh weight). The lowest protein content was recorded by plants inoculated with *M. incognita* 2 weeks prior to *F. solani* (2.72 µg of BSA $g^{-1}$ fresh weight) and it was statistically similar to plants inoculated with *F. solani* alone, both pathogen simultaneously and *F. solani* first and *M. incognita* 2 weeks after fungus inoculation. Similar trend was observed in root also (Table 9). The decline in protein levels during the later stages of infection suggested that developing *M. incognita* continuously extracted significant amounts of nutrients from the giant cells (49).

PO, PAL and PPO in the leaves of plants inoculated with *M. incognita* 2 weeks prior to *F. solani* were 8.93 $min^{-1}g^{-1}$ fresh weight, 16.31 µg of cinnamic acid $g^{-1}$ fresh weight and 6.98 $min^{-1}g^{-1}$ fresh weight respectively and it showed significant superiority to all other treatments (Table 9). Similar trend was observed in root samples also. Higher activity of phenol and defense related enzymes can be attributed to the defense induced in plants due to the increased infection by nematode and fungus. Earlier studies documented that the initial rise in phenols resulting from pathogen invasion stimulated the transcription of messenger RNA responsible for encoding phenylalanine ammonia lyase (PAL). The heightened presence of PAL in the plant facilitated the synthesis of phenolic compounds (50).

## Conclusion

The investigations of the study highlighted that the plant parasitic nematodes such as, *M. incognita, R. reniformis, R. similis, H. dihystera* and *Macroposthonia* sp. and fungal pathogens *viz. F. solani*, *F. oxysporum*, *Phytophthora* sp. *Rhizoctonia* sp. and *Pythium* sp. were associated with black pepper grown in Kannur, Idukki and Wayanad districts of Kerala. The presence of nematodes not only predisposed the host to fungal infection but also shortened the incubation period for disease expression. Inoculation of *M. incognita* 14 days prior to *F. solani* enhanced the disease incidence and nematode multiplication as evidenced by increased number of giant cells and distortion of vascular bundles and plugging of fungal mycelia in xylem vessels. The highest synergistic effect between the *M. incognita* and *F. solani* occurred when *M. incognita* was inoculated 14 days prior to *F. solani* as evidenced from the increase in defense enzymes and phenols.

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## Authors' contributions

NMS conceived and designed the research work. AH carried out the experiments and analyzed the data. NMS wrote the manuscript and NR edited the manuscript. AR participated in biochemical analysis and editing the manuscript. RS edited the manuscript. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest**: Authors do not have any conflict of interests to declare.

**Ethical issues**: None

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