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Histological Interactions of *Paecilomyces lilacinus* with Root-Knot Nematode *Meloidogyne incognita* and Their Effect on the Growth of Tomato

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A glasshouse experiment was conducted to assess the effect of *Paecilomyces lilacinus* on the reproduction of root-knot nematode and on the growth of tomato. Data on the plant growth, leaf area, yield characteristics, root-knot and egg-mass indices were recorded. The histological interactions studies were also carried out to check the parasitism of root-knot nematode eggs by *P. lilacinus*. The results indicated that the use of *P. lilacinus* one week before nematode inoculation caused an increase in growth and yield characteristics of tomato, and also reduced the reproduction of nematode as compared to other treatments. The results of histological studies indicated that *P. lilacinus* parasitized on the *M. incognita* eggs through the formation of fungal hyphae and conidiophores and caused the disintegration of the eggshells, egg masses and juveniles of *M. incognita*.

KEYWORDS: Fungal Hyphae, *Lycopersicon esculentum*, *Meloidogyne incognita*, Parasitism, *P. lilacinus*.

1. INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is the world's largest vegetable crop and is the rich source of minerals, vitamins A, and C, amino acids and dietary fibers. In India, the estimated area under tomato cultivation is about 350,000 ha and the annual production is about 5,300,000 t. This may accounts about 12% of the vegetable crops of the world. The major tomato growing states in India are Uttar Pradesh, Karnataka, Maharashtra, Haryana, Punjab and Bihar. The global yield loss due to root-knot nematodes (*Meloidogyne* spp.) is about 12.3%,[1] while in India it is estimated about 15–60%.[2] The root-knot nematodes are tripoblastic, bilaterally symmetrical, un-segmented pseudocelomes belongs to highly diversified and ubiquitous group of invertebrates. The plant infected with *Meloidogyne* spp. showed poor and stunted plant growth and galling symptoms on roots. These may reduced water uptake, increased wilting and mineral deficiencies.[3] This disease becomes a major constraint in successful cultivation of this important crop.[4]

The most infective stage is the second stage juveniles (J_2). The J_2 penetrate from apical meristems sides and the mature female become sessile inside the infected root tissues. It forms small bi-nucleated giant cells, which later transformed into the large multinucleated cells due to rapid acytokinetic mitosis divisions and showing the typical characteristics of galls known as root-knots.[5] Robab et al.[6] found that infection due to the root-knot nematodes caused the disruption of xylem and phloem tissues, which may caused interference in transportation of mineral nutrients and water and also in the translocation of food materials inside the host plant. However, Niyaz et al.[7] reported that the damaged caused by *M. incognita* might be responsible for the induction of the giant cells inside the cortical tissues and proliferation of neighboring tissues.

Paecilomyces lilacinus (Thom) Samson, is one of the most widely tested biocontrol agents for the control of plant parasitic nematodes.[8–10] It is evident from the laboratory tests that this fungus had the ability to infect the eggs and female of *Meloidogyne* spp. and destroy their embryos within a week.[11] The production of secondary metabolites like leucinotoxins, chitinase, protease and acetic acid by *P. lilacinus* has been associated with the infection process which reduced the nematode population.[12–15] This fungus had the unique adaptability to grow on a wide range of soil pH, which makes it a competitive biocontrol agent in the most of the agricultural soil. It establishes himself in the soil very short span of time and become the dominant species in the introduced area. *P. lilacinus* is now a well

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recognized biocontrol agent the *Meloidogyne* spp. on various crop plants.[8, 10, 16, 17] It has the ability to improved the plant growth attributes and also reduced the nematode populations.[18, 19] The aim of the present investigation is to investigate the effect of *P. lilacinus* on the growth of tomato, population of root-knot nematode and also observe the histological change during interaction inside the host plant.

2. MATERIALS AND METHODS

2.1. Raising and Maintenance of Test Plant

Seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar Pusa Ruby were surface sterilized with 0.1% sodium hypochlorite (NaOCl) solutions for two minutes and then rinsed thrice with sterile distilled water. About hundred surface sterilized seeds were placed on a sterilized and moistened filter paper kept in a petri dish and allowed to germinate. After germination the seeds were transferred into 15 cm diameter clay pots filled with steam sterilized soil (7 clay:3 sand:1 farmyard manure).

2.2. Nematode Inoculums

Meloidogyne incognita was collected form highly infested tomato field and multiplied on egg plant (*Solanum melongena* L.) using single egg mass. Egg masses were hand-picked using the sterilized forceps and placed in 9 cm diameter sieves of 1 mm pore size, which were previously mounted with cross layered tissue papers. The sieves were placed in petri dishes with distilled water for hatching and incubated at 27 °C. Two thousand freshly hatched second stage juveniles (J_2) were used as inoculum.

2.3. Preparation of *P. lilacinus* Inoculums

The culture of *P. lilacinus* was obtained from Indian Agriculture Research Institute (IARI), New Delhi, India and maintained on potato dextrose agar (PDA). Fungal inoculum was prepared by culturing the fungus on the Richard's medium[20] for 15 days at 25 °C ± 2 °C. After enmeshing of fungus on Richard medium, the mycelium was collected on blotting sheets to remove the excess water and nutrients. About 100 g mycelium was blended in 1,000 ml of sterile distilled water in waring blender. Ten milliliter of this suspension (equivalent to 1 g) was inoculated to plants.

2.4. Inoculation Techniques

For the inoculation of *M. incognita* and *P. lilacinus*, soil around the root was carefully replaced without damaging the roots. The inoculums suspensions of these microorganisms were poured around the roots and the soil was replaced. An equal volume of sterile water was added to control treatments.

2.5. Experimental Design

The experiment was carried out in a complete randomized block design in the glass house bench at 22 °C ± 2 °C, belongs to Department of Botany, Aligarh Muslim University, Aligarh, India. There are seven sets of treatments:
(1) C = Un-inoculated control;
(2) T_1 = Inoculated with 2,000J_2 only;
(3) T_2 = Inoculated with 2,000J_2 + one week before inoculation of fungus;
(4) T_3 = Inoculated with 2,000J_2 + simultaneous inoculation of fungus;
(5) T_4 = Inoculated with 2,000J_2 + one week after inoculation of fungus;
(6) T_5 = Inoculated with 2,000J_2 + two weeks after inoculation of fungus;
(7) T_6 = Inoculated with 2,000J_2 + three weeks after inoculation of fungus.

Each replicates was treated time times and the plants were watered as needed.

2.6. Parameter Assessments

The plants were harvested 90 days after inoculation. Data were recorded on plant length, plant fresh weight, plant dry weight, leaf area, yield (no. of flower and fruits per plant) characterictics, root-knot and egg mass indices were recorded. The plants were gently uprooted and washed carefully to remove the adhering soil particles. After washing the plants were put on the blotting sheet to remove the excess moisture. The plants were cut at the margin of root and shoot the length of the root and the shoot was measured in centimeter with the help of meter scale. Fresh weight of the root and the shoot was determined. Roots and shoots were kept separately in bamboo paper envelopes and kept in an incubator maintained at 72 °C temperature for 3 days. Dry weight of the root and the shoot was also recorded. For the determination of leaf area five medium size mature leaves from each treatment were randomly selected and outline of the shape of each leaf drawn on graph paper and the area occupied by each leaf was recorded while the number of flowers and fruits per plant of each treatment was counted by visual observation. The gall and egg mass indices were rated on scale 0 = 0, 1 = 1–2; 2 = 3–10; 3 = 11–30; 4 = 31–100; 5 ≥ 100.

2.7. Histological Studies

For the histological studies, 90 days old infected severely roots were collected and thoroughly washed with distilled water and cut into pieces of 1–2 cm and fixed into formalin acetic acid alcohol. Selected root pieces were then dehydrated with *n*-butyl alcohol according to method of Johansen.[21] The transverse and longitudinal sections (10–12 μm thickness) of severely galled roots were cut with the help of rotary microtome and were stained with

safranin and fast green. After staining the sections were mounted in Canada balsam for microscopic examination and the necessary photograph also has been taken.

2.8. Statistical Analysis

The data were statistically analyzed by one way ANOVA by means of SPSS version 17.0. Duncan multiple range test (DMRT) was performed to denote the significance differences between the treatments. Standard errors were also calculated and graphs were prepared using sigma plot version 10.0.

3. RESULTS

3.1. Root and Shoot Length

The roots and shoots length of the tomato in control treatments had the maximum lengths compared to other treatments. The lengths of the roots and the shoots of plants in T_1 treatments were shortest and exhibited significant ($p \leq 0.01$) reduction over control. The reductions in the root and shoot length were significantly lower in all the treatments as compared to control except in T_2 treatments. There was gradual reduction in root and shoot length from T_2 to T_6 plants (Fig. 1).

In comparison to nematode inoculated plants (T_1) there was an significant ($p \leq 0.01$) increase in root and shoot lengths was noticed in T_2, T_3 and T_4 treatments. In T_5 and T_6 treatments the increase in root and shoot length was non-significant. Highest increase in root and shoot length was found in T_2 treatments and lowest in T_6 treatments as compared to T_1 treatments (Fig. 1).

3.2. Root and Shoot Weights

Fresh and dry root and shoot weights of plant from T_1 to T_6 treatments were decreased compared to control. Significant ($p \leq 0.01$) and highest reductions in fresh and dry weights of roots and the shoots were noticed in T_1 treatments. In T_3 treatments, very low but significant ($p \leq 0.01$) reduction over the control was observed, while, in T_4, T_5 and T_6 treatments the reductions were higher and significant ($p \leq 0.01$). However, the reduction in root and shoot was lower in T_1 treatments (Figs. 2 and 3).

Fresh and dry weights of the roots and the shoots were increased in *P. lilacinus* inoculated plants compared to T_1 treatments, but the highest and significant ($p \leq 0.01$) increase was observed in T_2 plants. In other treatments, from T_3 to T_6 increase in the root and shoot weight over T_1 treatments. The increase in root and shoot weight was significant ($p \leq 0.01$) in T_3 and T_4 treatments while it is non-significant in T_5 and T_6 treatments. The lowest and non-significant increase was noticed in T_6 treatments compared to T_1 treatments (Figs. 2 and 3).

3.3. Leaf Area

The size of the leaves was decreased in all the treatments compared to control. The leaf area decreased to the greatest extent and significantly ($p \leq 0.01$) different in T_1 treatments as compared to control. In T_2 and T_3 treatments, the reductions were non-significant. The leaf area was found significantly ($p \leq 0.01$) lower in T_4, T_5 and T_6 treatments at ($p \leq 0.05$) (Fig. 4).

The increased in the leaf area was recorded in all the treatments from T_2 to T_6 in compared to T_1 treatments,

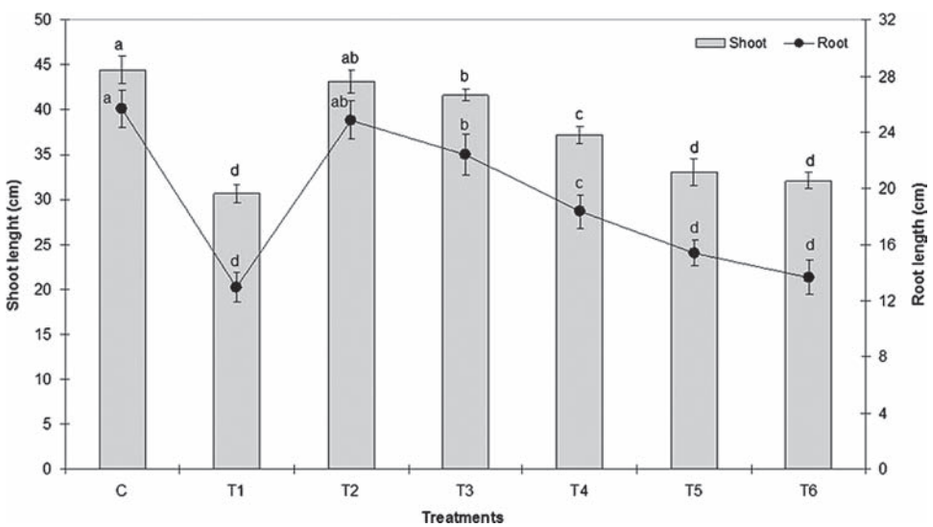

Fig. 1. Effect of *P. lilacinus* on the root and shoot length of tomato inoculated with *M. incognita*. Data represent ± standard error ($n = 5$). Different lower case letters above the bars indicate the significance differences ($p \leq 0.01$). (C = Un-inoculated control; T_1 = Inoculated with 2,000J_2 only; T_2 = Inoculated with 2,000J_2 + one week before inoculation of fungus; T_3 = Inoculated with 2,000J_2 + simultaneous inoculation of fungus; T_4 = Inoculated with 2,000J_2 + one week after inoculation of fungus; T_5 = Inoculated with 2,000J_2 + two weeks after inoculation of fungus; T_6 = Inoculated with 2,000J_2 + three weeks after inoculation of fungus).

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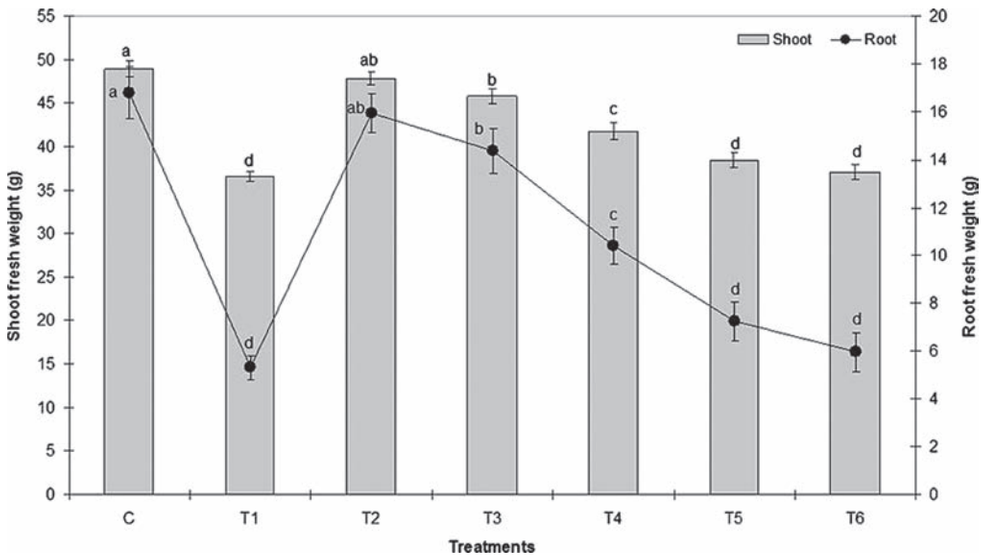

Fig. 2. Effect of *P. lilacinus* on the root and shoot fresh weight of tomato inoculated with *M. incognita*. Data represent ± standard error ($n = 5$). Different lower case letters above the bars indicate the significance differences ($p \leq 0.01$). (C = Un-inoculated control; T_1 = Inoculated with 2,000J_2 only; T_2 = Inoculated with 2,000J_2 + one week before inoculation of fungus; T_3 = Inoculated with 2,000J_2 + simultaneous inoculation of fungus; T_4 = Inoculated with 2,000J_2 + one week after inoculation of fungus; T_5 = Inoculated with 2,000J_2 + two weeks after inoculation of fungus; T_6 = Inoculated with 2,000J_2 + three weeks after inoculation of fungus).

while T_2 treatments have the highest increase in leaf area. Increase in leaf area of T_2 and T_3 treatments was significantly ($p \leq 0.01$) higher than other treatments. In T_4 and T_5 treatments, the leaf area was increased to a lesser extent but the increase was significant ($p \leq 0.05$). The values of leaf area of T_6 plants were at par with T_1 treatments (Fig. 4).

3.4. Yield (Flowers and Fruits) Characteristics

The highest reductions (significant at $p \leq 0.01$) in the yield characteristics were found in T_1 treatments in comparison to control. The yield characteristics of T_2 treatments were at par with the control. Significant ($p \leq 0.01$) reductions in both number of flowers and fruits, were observed in T_3,

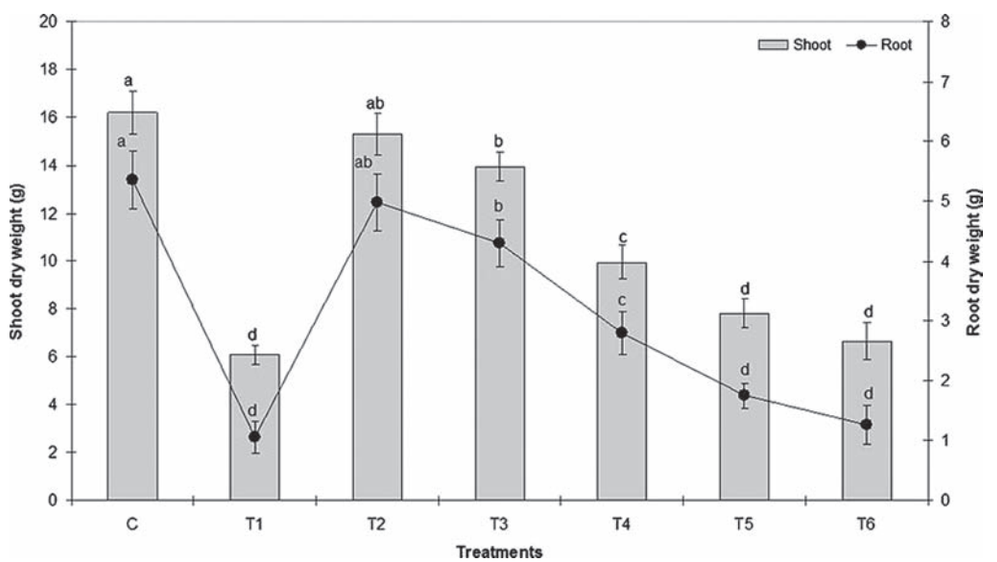

Fig. 3. Effect of *P. lilacinus* on the root and shoot dry weight of tomato inoculated with *M. incognita*. Data represent ± standard error ($n = 5$). Different lower case letters above the bars indicate the significance differences ($p \leq 0.01$). (C = Un-inoculated control; T_1 = Inoculated with 2,000J_2 only; T_2 = Inoculated with 2,000J_2 + one week before inoculation of fungus; T_3 = Inoculated with 2,000J_2 + simultaneous inoculation of fungus; T_4 = Inoculated with 2,000J_2 + one week after inoculation of fungus; T_5 = Inoculated with 2,000J_2 + two weeks after inoculation of fungus; T_6 = Inoculated with 2,000J_2 + three weeks after inoculation of fungus).

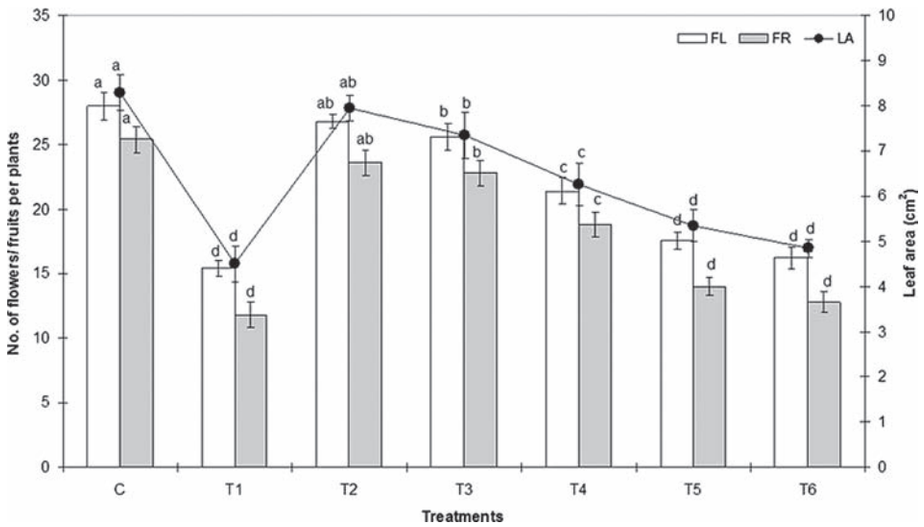

Fig. 4. Effect of *P. lilacinus* on the leaf area and yield characteristics (no. of flowers and fruits per plant) of tomato inoculated with *M. incognita*. Data represent ± standard error ($n = 5$). Different lower case letters above the bars indicate the significance differences ($p \leq 0.01$). (C = Un-inoculated control; T_1 = Inoculated with 2,000J_2 only; T_2 = Inoculated with 2,000J_2 + one week before inoculation of fungus; T_3 = Inoculated with 2,000J_2 + simultaneous inoculation of fungus; T_4 = Inoculated with 2,000J_2 + one week after inoculation of fungus; T_5 = Inoculated with 2,000J_2 + two weeks after inoculation of fungus; T_6 = Inoculated with 2,000J_2 + three weeks after inoculation of fungus).

T_4, T_5 and T_6 plants, over the control. Lowest reduction was noticed in T_2 treatments (Fig. 4).

The significant ($p \leq 0.01$) increase in yield characteristics over T_1 treated plants was encountered in T_2 treated plants in which *M. incognita* inoculated plants were treated with *P. lilacinus* one week prior to nematode inoculation. Increase in yield was also significantly ($p \leq 0.01$) high in T_3 and T_4 plants. Increase in number of flower in T_5 and T_6 treatments was non-significantly higher than T_1 treatments (Fig. 4).

3.5. Root-Knot and Egg Mass Indices

Highest number of galls and egg masses per root system were encountered in T_1 plants, where the plants were inoculated with *M. incognita* only. Number of galls and egg masses per root system (RKI and EMI) were greatly decreased in T_2 treatments, where *M. incognita* inoculated plants were treated with *P. lilacinus*. The values of RKI and EMI were found increased in other treatments (from T_3 to T_6). Root-knot and egg mass indices were lowest in

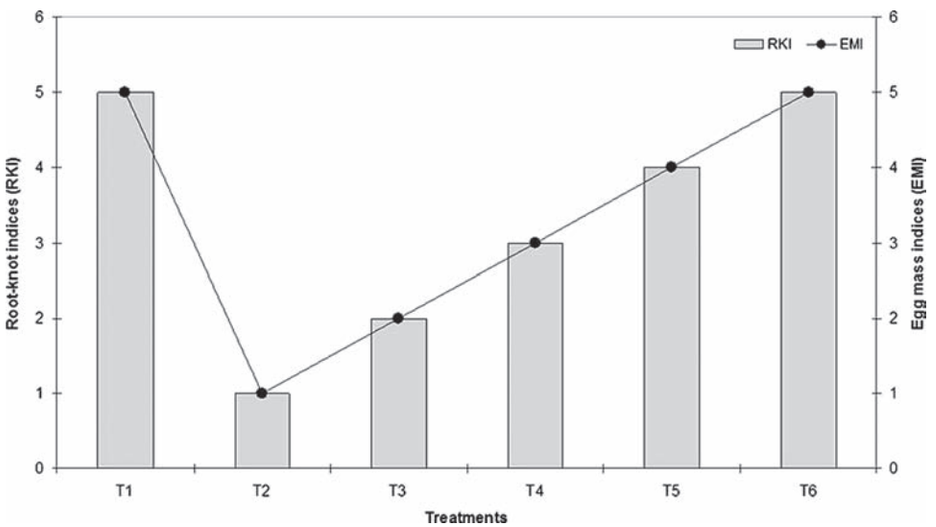

Fig. 5. Effect of *P. lilacinus* on the root-knot indices (RKI) and egg mass indices (EMI) of tomato inoculated with *M. incognita*. (T_1 = Inoculated with 2,000J_2 only; T_2 = Inoculated with 2,000J_2 + one week before inoculation of fungus; T_3 = Inoculated with 2,000J_2 + simultaneous inoculation of fungus; T_4 = Inoculated with 2,000J_2 + one week after inoculation of fungus; T_5 = Inoculated with 2,000J_2 + two weeks after inoculation of fungus; T_6 = Inoculated with 2,000J_2 + three weeks after inoculation of fungus).

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T_2 treatments, which gradually increased, up to the highest values in T_6 treatments (Fig. 5).

3.6. Histological Results

It was revealed from the morphological and cross sectional results that the level of infestation was high in tomato roots infected with the nematode (Figs. 6(A)–(D)). The second-stage juveniles (J_2) of root-knot nematode after penetration migrate towards the cortical region of the root and adopted the path of cortex and ray parenchyma. The parenchyma cells lying adjacent to the nematode, specifically near the head, were induced to become enlarged (Fig. 6(D)) and resulted into the severe galling due to host response towards the root-knot nematode infection inside the root. This may cause several time increased in the size of normal cells and transformed into the giant cells which later turns into the giant cell complexes (Figs. 6(C) and (D)). The hyperplastic and hypertrophic tissues were always present near to giant cell complex. When *P. lilacinus* was applied in the nematode infected plants, first the fungal hyphae develop rapidly and make luxuriant growth of hyphae inside the inner tissues of roots and then start parasitizing the nematode eggs (Figs. 7(A) and (B)). In later stage of infection, the hyphae produced chain of conidia in conidiophores on the surface of nematode egg

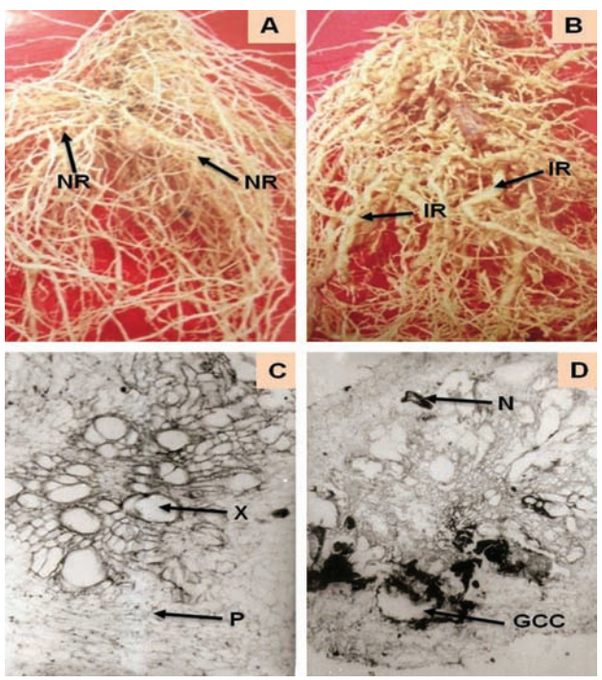

Fig. 6. Morphological and cross section comparisons between the normal and infected tomato roots. (A) Normal root (non infected); (B) Root infected with root-knot nematode (*Meloidogyne incognita*); (C) Normal root showing normal xylem and phloem tissues; (D) Infected root showing penetration of nematode and giant cell complex inside the root tissues (NR = Normal root; IR = Infected root; *X* = Xylem, *P* = Phloem; *N* = Nematode; GCC = Giant cell complex).

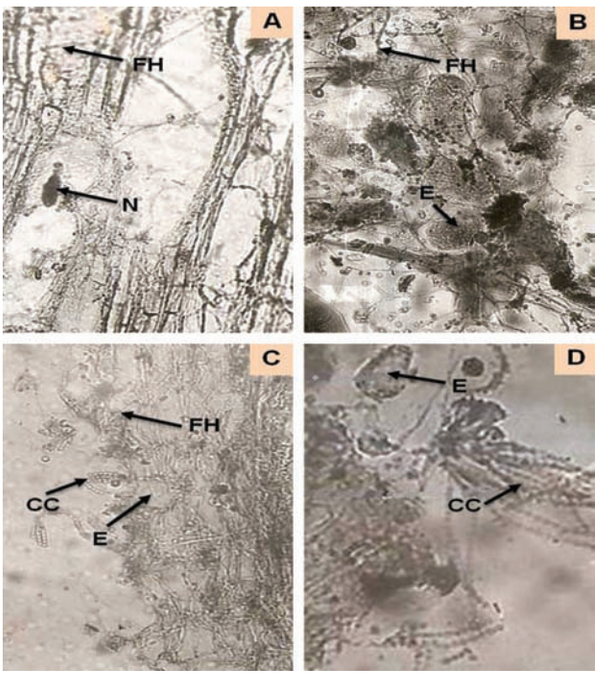

Fig. 7. Cross section of tomato roots infected with root-knot nematode (*Meloidogyne incognita*) and fungus *P. lilacinus*. (A) Nematodes engulfed with fungal hyphae; (B) Nematodes eggs parasitized by fungal hyphae; (C) formation of fungal conidia on the surface of nematode eggs; (D) magnified view of chain of fungal conidia (N = Nematode; FH = Fungal hyphae; CC = Chain of fungal conidia; E = Egg masses).

and penetrate inside the body of females and caused severe damage to the egg masses and juveniles inside the eggs (Figs. 7(C) and (D)).

4. DISCUSSION

It is evident from our results that the *M. incognita* caused significant reduction in growth of tomato. The main cause behind this reduction in tomato growth is the formation of galls which may deprive the plant ability to absorb the essential nutrients and water.[3] Our results are strengthened by the results of earlier workers.[22–24] However, Azam et al.[25] reported that the effect of root-knot disease was not only confined to plant length and weight but also affect the size of the leaf area. This might be reduced the photosynthetic surface area of plant and resulted in lesser number of flowers and fruits compared to un-inoculated plants.

Our results indicated that the plant length, plant weight, leaf area and yield characteristics of tomato were increased when nematode infected plants were treated with *P. lilacinus*. The application of the *P. lilacinus* one week before nematode inoculated plants, improved the plant growth compared to simultaneous post inoculation. The reason behind is that *P. lilacinus* had got sufficient time to enter into the root and developed fungal hyphae around and inside the eggs and finally enters inside the mature females. This may causes the distortion of the eggs and egg masses

in the infected plants. In general mechanisms the hyphae of *P. lilacinus* colonized on the outer surface of the roots and penetrate into the inner tissues of primary roots systems and develop below the epidermal layers of the cortex and in the stellar tissues. Due to saprophytic nature of *P. lilacinus* it is advisable that always apply this fungus prior to seedling transplantation, so, that the fungus established itself into the soil in absence of nematode and any other host.[26] This makes *P. lilacinus* a widely used fungi for the biocontrol of root-knot nematodes.[16, 18, 19] Beside this the management of root-knot nematode by *P. lilacinus* on is an effective and eco-friendly approach for sustainable environment.

Paecilomyces lilacinus has diverse modes of habits; basically it is a saprophytic fungus, but being able to compete for use a wide range of common substrates in soil.[10, 27] It is very clear from the histological results that the *P. lilacinus* was encountered frequently in and around normal and abnormal xylem. Probably the vessel elements provided favorable environment and sufficient space for the growth and development to the fungus. The growth of the fungus inside the root is inter- and intracellular. The earlier reports confirmed that *P. lilacinus* parasitized on the eggs and egg masses of root-knot nematodes.[11, 28] Cardona and Leguizamon[29] reported that 94% infection on *Meloidogyne incognita* egg and egg masses was by *P. lilacinus*. Similarly, Khan and Williams[30] found that *P. lilacinus* enters into the body of the mature females of *M. incognita* through natural openings.

Occurrence of smaller giant cells and reduction in amount of abnormal xylem and phloem indicated that nematode development was influenced by the presence of *P. lilacinus.* Larger giant cell and higher amount of abnormal tissues indicated that the nematodes enters prior to fungus were not affected. On the basis of these observations in might be concluded that the fungus cannot check primary infection of nematode when the plants have been attacked by the juveniles but it can be check secondary infection because it destroys eggshells of nematodes by the production of proteases and chitinases.[14, 31] Thus, our finding suggests that application of *P. lilacinus* one week prior to nematode inoculation is more effective in controlling the root-knot diseases caused by *M. incognita*, but further more trials are needed to confirm these results in field conditions.

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