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Intraction between root knot nematode with root rot and wilt fungi its effect on disease severity and soil population of fungus and nematode on tomato

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Abstract
A pot trial was conducted to determine the effect of single, sequential and concomitant inoculation with root-knot nematode (*Meloidogyne incognita*), root-rot fungus (*Rhizoctonia solani* and *Pythium aphanidermatum*) and wilt fungus (*Fusarium oxysporum* f sp. *lycopersici*) on gall, egg mass, wilt, root rot index and soil population of fungus and nematode on tomato cv. Local. The sequential inoculations comprised of nematode prior to fungus (N→F) and fungus prior to nematode (F→N). inoculation with *M. incognita*, *R. solani*, *F. oxysporum* f. sp. *lycopersici* and *P. aphanidermatum* singly caused severe galling, rotting and wilting on tomato root with 1.3, 2.3 and 3.3 indices on 0-5 scale, respectively. The severity of rotting (20-42%) and wilting (12-30%) significantly increased ($P \leq 0.05$) in the presence of root-knot nematode in both sequential and concomitant inoculations due to synergistic interaction between two pathogens. However, the galling in all combined treatments significantly decreased (31-46%) in comparisons to nematode alone. Among all the combinations, N→F was recorded most destructive, followed by F+N, F →N and fungus and nematode alone. Final soil population of nematode in alone treatment increased four times of their initial population while substantial variability in population of fungal pathogens were recorded with highest in *P. aphanidermatum* followed by *F. oxysporum* f. sp. *lycopersici* and lowest in *R. solani*. In combined treatments, the soil population of nematode significantly decreased (60-90%) in the presence of root-rotting and wilt inducing fungus while the population of fungal pathogens also influenced by the presence of nematode and significantly increased by 80-110%. Highest soil population of fungal pathogen was recorded with N→F, followed by (F+N), (F→N) and fungus and nematode alone.

Keywords: Interaction, severity, gall, giant cells, sedentary, antagonistic

Introduction
Among the horticulture crops, vegetables are most important, because of their high nutritive value. In India total vegetable production is 182 million tonnes (Anonymous, 2016-17) [1]. Tomato (*Solanum lycopersicum* L.) is the second most important vegetable crop after potato. The world annual production of tomato 177 million tonnes (FAO, 2016). Tomato is cultivated as an annual crop in most region of the world (Vossen *et al.*, 2004) [18]. India ranks fourth in the world in tomato production after China, United States and Turkey with an estimated production of 22 million tonnes in 2016-17 from about 7.3 per cent of the entire cropped land (Anonymous, 2016-17) [1]. In India, Andhra Pradesh is the leading state in both area and production.

The production of tomato is influenced by a number of factors which includes edaphic and environmental factors as well as pests and pathogens. Soil-borne pathogens, especially, *Rhizoctonia, Fusarium* and *Pythium* are highly destructive and cause tremendous yield loss to tomato crop. Root-rot caused by *R. solani*, is one of the dreaded diseases, prevalent throughout the tomato growing regions around the world and causes moderate to severe damage to the crop. Tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* occurs almost in every tomato growing area of world including India and other countries and causes up to 15% annual loss (Mandal *et al.*, 2009) [10]. Whereas, damping off caused by *P. aphanidermatum* is probably responsible for a larger proportion of seed bed failure and poor stands of tomato nursery in high humid and less aerated soil conditions. Root-knot disease of tomato caused by *Meloidogyne incognita* other major yield hampering pathogen and cause huge economic loss (24-61%) annually in India (Singh and Mathur, 2010a) [15].

Besides direct damage, root-knot nematodes possess great capability to synergise other soil-

borne pathogens, leading to development of disease complex (Khan, 1993) [6]. The nematode not only causes direct damage to plants at their own, but generally help fungi, bacteria and plant viruses to invade host plant. This leads to the development of a disease complex. The fungus also develops synergistic relationship with *Meloidogyne spp.* leading to root-knot wilt (Patel *et al*., 2000, Ganaie and Khan 2011), Root- knot and root rot disease complex (Khan 1993) [6]. It was also reported that, the root rot pathogen along with *M. incognita* could cause 37– 57 % yield loss in tomato (Kumar and Singh, 2006ab; Upadhyay and Dwivedi, 2008) [4].

In plants, phenolic compounds play very vital role in the induction of local and systemic disease resistance (Bari and Jones 2009). Major players in systemic induced defense are the salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and other phenolic compounds, which are produced in response to plant attack (Biere and Goverse, 2016). Root parasitisation by soil borne fungi and nematodes results in the production and accumulation of SA, JA, and ET at the site of infection (Kammerhofer *et al*., 2015). Consequently, transcriptional reprogramming results in the activation of the core defense system involving SA/JA/ET-mediated pathways in plants including tomato (Jayakumr and Ramakrishnaan, 2006).

Methods and Materials

Nursery culture of tomato

The seed of tomato cv. Local was procured from local market of Aligarh, The nursery was raised in earthen pots (30x30 cm) in the month of February. The pots were filled with 5 kg mixture of soil, sand and farm yard manure (3:1:1), and autoclaved at 15kg/cm^2 pressure at 121^{o}C for 15-20 minutes. Tomato seeds were sown in the pots. Pots were kept on a cemented platform and watered as and when considered necessary

Collection of root-knot nematode, *Meloidogyne incognita*

Infected root samples of tomato showing galls or knots were collected from cultivation unit in and around Aligarh. The root samples were collected in polythene bags and brought to the laboratory. Roots were rinsed under the slow stream of water thereafter females and egg masses from the galled tissue were excised. The species of *Meloidogyne incognita* (Kofoid and White) Chitwood was confirmed using perineal pattern technique (Fig. 1) of ten females from the each root system (Barker *et al*., 1985) [2]. To prepare inoculation of nematode, egg masses of *M. incognita* infected root swere excised from the root samples and placed on course sieve lined with two layer of tissue paper which was then put in a Baermann's funnel filled with adequate amount of water. Most of the larvae hatched out from the egg masses and migrated across the tissue paper reaching in the stem of the funnel during incubation of one week at 25+2 ^{o}C. Nematode suspension from the stems was collected and standardized by counting number of larvae/ml suspension in a counting dish under stereomicroscope.

Collection and mass culture of fungal pathogens

Pure culture of *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *Lycopersici* and *Pythium aphanidermatum*, were procured from the ITCC, Division of Mycology and Plant Pathology, IARI, New Delhi. The pure culture was maintained on potato dextrose agar (PDA) in culture tubes and Petri plates (fig.1) and stored in a refrigerator at 5^{o}C. For mass culturing of *R. solani*, *F. oxysporum* f. sp. *Lycopersici* and *P. aphanidermatum* the fungus was multiplied on sorghum grains. The seeds were soaked overnight in 5% sucrose and 0.0003% chloramphenicol solution (Whitehead, 1957). The seeds were transferred to conical flasks of 500 ml capacity and autoclaved twice at 15 kg/cm^2 pressure at 121^{o}C for 15-20 minutes. Thereafter, flasks were inoculated with the pure culture of *R. solani*, *F. oxysporum* f. sp. *Lycopersici* and *P. aphanidermatum* as separately, After inoculation, the flasks were incubated for 10 days in a BOD incubator at 25+2^{o}C. The contaminated flask, if any was removed immediately and discarded. During incubation, the flasks were shaken daily manually for a few minutes to promote uniform colonization on seeds. The incubation period, if required, was extended until the entire medium was fully colonized by fungus.

Inoculum level and doses

Sorghum seeds colonized by *R. solani*, *F. oxysporum* f. spp. *Lycopersici* and *P. aphanidermatum* were weighed macerated in the distilled water in an electric grinder to make fungus suspension. The suspension containing 2g colonized seeds (2 x 10^6) was applied to soil in each pot (1 kg soil)

Treatments

Earthen pots of 15 cm diameter were filled with 1 kg mixture of soil, sand and farm yard manure (3:1:1) and were autoclaved at 15 kg/cm^2 at 121.^{o}C for 15-20 minutes. Following 13 treatments were maintained.

T_1 = Plant alone (Control)
T_2 =*Rhizoctonia solani* alone
T_3 = *Fusarium oxysporum* f. sp. *lycopersici*alone
T_4 = *Pythium aphanidermatum* alone
T_5 = *M. incognita* alone
T_6 = *R. solani→M. incognita*
T_7 = *F. oxysporum* f. spp. *lycopersici→M. incognita*
T_8 = *P. aphanidermatum→M. incognita*
T_9 = *R. solani* + Nematode
T_{10} = *F. oxysporum* f. spp. *lycopersici* + Nematode
T_{11} = *P. aphanidermatum+M. incognita*
T_{12} = *M. incognita→R. solani*
T_{13} = *M. incognita→F. oxysporum* f. sp. *lycopersici*
T_{14} = *M. incognita→P. aphanidermatum*

Where
R→N = Rhizoctonia applied 3 day before the nematode
F→N = Fusarium applied 3 day before the nematode
P→N = Pythium applied 3 day before the nematode
R+N= Rhizoctonia & nematode applied simultaneously
F+N= Pythium applied 3 day before the nematode
P+N= Pythium & nematode applied simultaneously
N→R= Nematode applied 3 day before the Rhizoctonia
N→F= Nematode applied 3 day before the fusarium
N→P = Nematode applied 3 day before the Pythium

Following parameters were determined

1. Soil population of fungus
2. Soil population nematode
3. Gall and egg mass index
4. Root rot index
5. Wilt index

Root-knot index

At harvest roots were examined to count galls and egg masses on 0-5 scale (Taylor and Sasser, 1978).
0 =0 galls/egg masses/root system.

1 = 1-2 galls/egg masses/root system.
2 = 3-10 galls/egg masses/root system.
3 = 11-30 galls/egg masses/root system.
4 = 31-100 galls/egg masses/root system.
5 = >100 galls/egg masses/root system.

Root-rot index
Examined for root-rot disease severity scale on 0-5 scale (Chester, 1950; Chaube & Singh, 1991)
0 =No rotting
1 = 1-10% rotting
2 = 11- 25% rotting
3 = 26-60% rotting
4 = 61- 80% rotting
5 = 80-100% rotting

Wilt index
Scale (0-5) of evolution for wilt disease (Mayee and Datar, 1996)
0 =No rotting
1 = 1-20% necrosis
2 = 20- 40% necrosis
3 = 40-50% necrosis
4 = 50- 75% necrosis
5 = 80-100% necrosis

Soil population
***Meloidogyne incognita*:** Population of *M. incognita* was determined by Cobb's decanting and sieving method (modified) followed by Baermann's funnel technique (Southey, 1986). Soil was collected from each of three pots of a treatment. The soil sample was sifted through a coarse sieve and 200 g was mixed in 5 liters of water in a plastic bucket. The soil water mixture was stirred and then allowed to stand for 1-2 minutes. The suspension was decanted over a combination of 3 sieves (60, 200 and 400 mesh), the catch from the finest sieve was carefully washed and transferred to a beaker. A small coarse sieve with two layers of wet paper towels was kept in a Baermann's funnel filled with water. The nematode suspension from the beaker was gently poured onto the sieve and allowed to stand overnight. The nematode juveniles because of the wriggling movement migrated through the paper pores into the water and gradually settled down in the bottom of rubber tubing of the funnel. The nematode suspension recovered from the Baermann's funnel was taken into a beaker and counted in a dish under a stereomicroscope.

Soil population of fungal pathogens
Soil populations of fungal pathogens (*R. solani*, *F. oxysporum* f. sp. *Lycopersici* and *P. aphanidermatum*) in terms of colony forming units (CFU) of were determined by dilution plate method. Rhizospheric soil from the pots receiving different treatments was collected after harvesting the plant. A 10 gram of sample of the sieved soil was taken in a sterilized conical flask to which 100 ml distilled water was added. The suspension was stirred and poured into 1000 ml Erlenmeyer flask. The flask containing the suspension was subjected to mechanical shaking for 30 minutes followed by filtration through a coarse filter paper. One ml of the filtrate was added to a sterilized test tube containing 9 ml of distilled water. The procedure was repeated five times to obtain the dilution of 1:10,000000 (10^{-7}). One ml of the final dilution was pipetted over PDA in a Petri plate under laminar flow. Three plates were maintained for each treatment. Inoculated plates were incubated at 25±2 ºC for 72 hours in a BOD incubator. After incubation, the plates were examined and the colonies of the pathogens and fungi were counted.

Statistical analysis
Observations taken from three pots were averaged to calculate means. The data (3 replicates/ treatment) on the plant growth, yield parameter, total phenol content and salicylic acid was analyzed by analysis of variance (ANOVA) and least significance difference (LSD) was calculated at a probability level of data $P \leq 0.05$, to identify significant effect of a treatment (Dospekhov, 1984) [3]. Galls and egg masses index, root rot index, wilt index and final soil population of fungus and nematode was presented as figure using MS excel-2010. Percent variation over control was also calculated.

Results
Estimation gall and egg mass index
Meloidogyne incognita
Tomato cv. local grown in soil inoculated with 2000 juvenile of *M. incognita* in alone/ single inoculation treatment developed characteristic gall on the root system with 4gall index (GI) and 3.66egg mass index (EMI) at 0-5 scale. These galls varied in shape and size, oval pinhead to large and fleshy. However, the symptoms and number of gall produced by the nematode in combined treatment varied greatly.
Among the combined treatments, Inoculation of *P. aphanidermatum* 3 day before *M. incognita* (F→N) caused maximum (3 and 2.33GI and EMI, respectively, Fig. 1). Treatment of *R. solani* with *M. incognita* produced (2.66 GI and 2.0 EMI, Fig. 1). *F. oxysporum* f. sp. *Lycopersici* with *M. incognita* produced 2.66 GI and 2.0 EMI (Fig. 1). Among the sequential treatments (N→F), Inoculation of *M. incognita* 3 day before *R. solani*, caused maximum number 3.33 GI and 2.33 EMI (Fig.1) followed by *P. aphanidermatum* with *M. incognita*, which produced 3 and 2 GI and EMI, respectively (Fig. 1). Among the simultaneously inoculation of fungus and nematode (F+N), maximum galls were recorded in *F. oxysporum* f. sp. *Lycopersici* + *M. incognita* treatment with 3.66 GI and 2.66EMI (Fig. 1), followed by *R. solani* + *M. incognita* which produced3 GI and 2.33EMI (Fig. 1). *P. aphanidermatum*+ *M. incognita* produced least number of GI (2.66) and EMI 2, (Fig. 1).

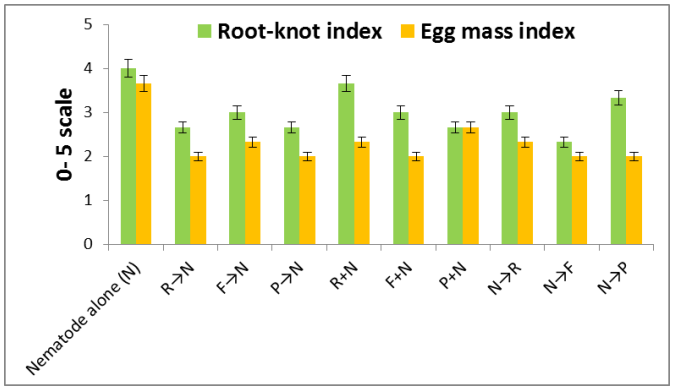

Fig 1: Effect of inoculation of *Meloidogyne incognita*, *Rhizoctonia solani*, *Pythium aphanidermatum* and *Fusarium oxysporum f. sp. Lycopersici* alone and different combination on root-knot and egg mass index.

Estimation of root rot index

Rhizoctonia solani

Roots of tomato plants inoculated with *R. solani* showed rotting in the form of browning of lateral as well as main root. However, the severity of rotting varied with the inoculation methods (Fig. 2). Inoculation of *R. solani* alone caused moderate rotting on tomato roots (1.66 at 0-5 scale) and the rotting significantly increased in presence nematode. Maximum root-rot index was recorded in simultaneous inoculation of *R. solani* + *M. incognita* (2.00 at 0-5 scale), followed by nematode prior to *R. solani* (1.66) and fungus prior to *M. incognita* (1.33, Fig. 2).

Pythium aphanidermatum

Inoculation of *P. aphanidermatum* alone caused severe rotting on tomato roots (3.33 at 0-5 scale). However, the severity of rotting significantly accelerated in presence *M. incognita* but varied with the inoculation combinations (Fig. 2).Highest root-rot index was recorded in inoculation of nematode prior to *P. aphanidermatum* (4.33 at 0-5 scale) followed by fungus prior to *M. incognita* (4.0) and concomitant inoculation of *P. aphanidermatum* + *M. incognita* (4.33, Fig. 2).

Estimation of wilt index

Fusarium oxysporum f.* sp. *lycopersici

Inoculation of *F. oxysporum f.* sp. *Lycopersici* alone caused moderate wilting on tomato plants with 2.33 at 0-5 scale (Fig. 2). However, the severity of wilting significantly increased in presence of *M. incognita* but varied with the inoculation combinations (Fig. 2).Highest wilt index was recorded in inoculation of *M. incognita* prior to *F. oxysporum f.* sp. *lycopersici*(3.33 at 0-5 scale). The effect of concomitant inoculation of *F. oxysporum f.* sp. *lycopersici*+ *M. incognita* and fungus prior to *M. incognita* was almost similar and caused 2.66 wilt index at 0-5 scale (Fig. 2).

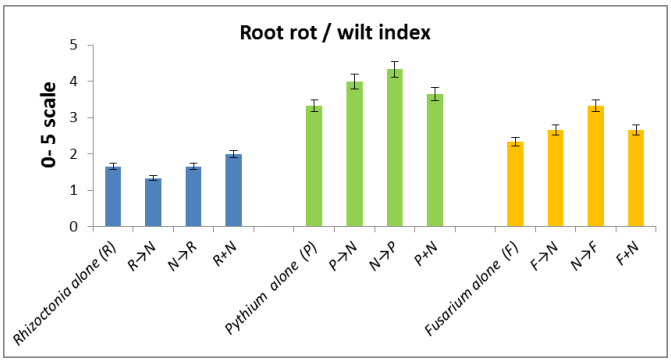

Fig 2: Effect of inoculation of *Meloidogyne incognita, Rhizoctonia solani, Pythium aphanidermatum* and *Fusarium oxysporum f. sp. Lycopersici* alone and different combination on root rot and wilt index.

Estimation Soil population of root knot nematode

Meloidogyne incognita

Final soil population of the nematode at the time of harvest increased around four times of its initial population (2000 J_2) in alone treatment (Fig. 3). However, the increase in nematode population in the presence of root-rot and wilt pathogen was significantly lower in comparison to the nematode alone. Among the combined treatments, inoculation of *F. oxysporum f.* sp. *Lycopersici* prior to *M. incognita* caused maximum increase (65%) in soil population of nematode (Fig. 3). Next treatment with respect to increase in nematode population was *P. aphanidermatum* prior to *M. incognita* (63%) followed by *R. solani* prior to *M. incognita* (40%, Fig. 3). Treatment of *M. incognita* prior to *P. aphanidermatum* caused least increase in number of nematode juvenile (31%, Fig. 3). Among all the treatments, highest increase in nematode population was recorded with nematode alone, followed by F→N, F+N and N→F (Fig. 3).

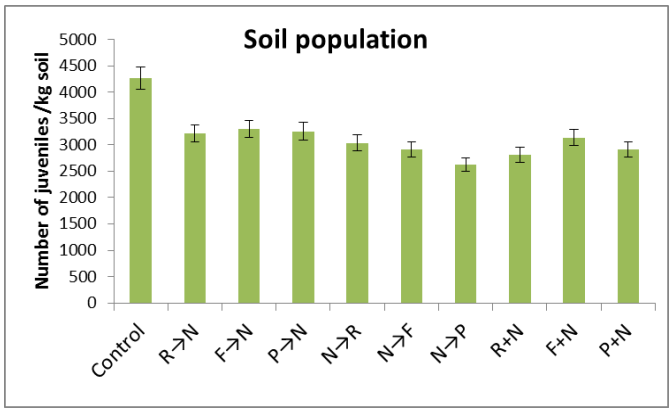

Fig 3: Effect of inoculation of *Meloidogyne incognita, Rhizoctonia solani, Pythium aphanidermatum* and *Fusarium oxysporum f. sp. Lycopersici* alone and different combination on soil population of root-knot nematode.

Estimation soil population of root rots fungi

Rhizoctonia solani

Soil population of *R. solani* increased by 110% at the time of harvest in alone treatment. In the presence of root-knot nematode, soil population of the fungus further increased but varied with treatment combination (Fig. 4). Highest increase (150%) in the population of root-rot fungus was recorded in the inoculation of *M. incognita* prior to *R. solani* followed by inoculation of *R. solani* prior to *M. incognita* (145%, Fig. 4) Among the combined treatments, simultaneous inoculation of *R. solani* + *M. incognita* caused least increase (115%) in soil population of the fungus (Fig. 4).

Pythium aphanidermatum

Soil population of *P. aphanidermatum* increased by four time of its initial population at the time of harvest in alone treatment. In the presence of root-knot nematode, soil population of *P. aphanidermatum* further significantly increased but varied with treatment combination (Fig. 4). Highest increase (310%) in soil population of *P. aphanidermatum* was recorded in the treatment where inoculation of *M. incognita* done prior to *P. aphanidermatum* (Fig. 4). Simultaneous inoculation of *P. aphanidermatum* + *M. incognita* also caused significant increase in soil population of *P. aphanidermatum* (290%). Inoculation of *P. aphanidermatum* prior to *M. incognita* caused least increase (220%) in soil population of the fungus (Fig. 4).

Estimation soil population of wilt fungi

Fusarium oxysporum f.* sp. *lycopersici

At harvests, soil population of *F. oxysporum f.* sp. *lycopersici* increased by three time of its initial population in alone treatment. In the presence of root-knot nematode, soil population of *F. oxysporum f.* sp. *lycopersici* further increased but varied with treatment combination (Fig. 4). Highest increase (225%) in soil population of *F. oxysporum f.* sp. *lycopersici* was recorded in the treatment where inoculation of *M. incognita* done prior to wilt fungus (Fig. 4). Simultaneous inoculation of *F. oxysporum f.* sp. *lycopersici* + *M. incognita*

also caused significant increase in soil population of the fungus (205%). Inoculation of *F. oxysporum f.* sp. *lycopersici* prior to *M. incognita* caused minimum increase (165%) in soil population of the fungus (Fig. 4).

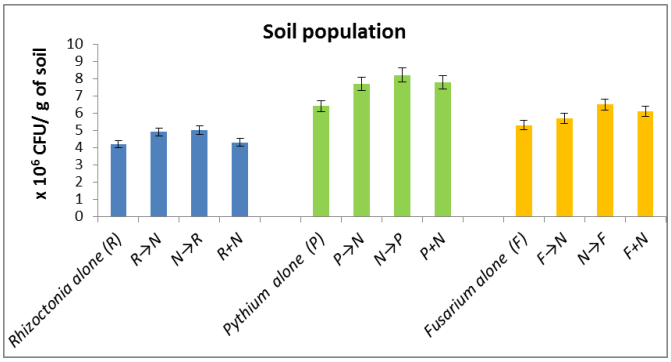

Fig 4: Effect of inoculation of *Meloidogyne incognita*, *Rhizoctonia solani*, *Pythium aphanidermatum* and *Fusarium oxysporum f. sp. lycopersici*alone and different combination on soil population of root rot and wilt fungus.

Tomato is one of the most susceptible crops to root-knot nematode and develop extensive galling as observed in present study. The severity of galling in all combined treatments (sequential or concomitant) significantly decreased in presence of root-rot (*R. solani* and *P. aphanidermatum*) and wilt (*F. oxysporum* f. sp. *lycopersici*) pathogen compared to nematode alone treatment. There are numerous reports which have demonstrated that the root-rot and wilt symptoms become severe in the presence of *Meloidogyne* spp. (Evans and Haydock, 1993). The nematode-fungus association is not always beneficial for the nematode as sometimes nematode may suffer a setback from such interaction as the secondary pathogen invade and feed on nematode feeding sites resulting in starvation and death of nematodes (Hasan, 1993) as observed in present study. Although nematode did not cause more damage to the roots or incited galls, as the number of galls decreases in combined treatments, but it accelerated the damage potential of the fungus. It seems that nematode activity in the root zone, probably its attempt to invade the roots somehow favored the pathogenesis of *R. solani*, *P. aphanidermatum* and *F. oxysporum* f. sp. *lycopersici*. This also indicating that the root-rot/wilt causing fungi destroy the galled tissue as well as feeding site of nematode and host could not provide nutrition to nematode because of a deteriorating status of the susceptible host. Similarly the egg mass production of nematode also reduced; indicating that the mature females may be invaded by *Fusarium* and *Rhizoctonia* spp. depriving female nematode to reach maturing stage, reproduce and form egg mass. Evidences exist in the literature which indicates that rhizosphere population of secondary pathogens increase in the presence of nematodes (Webster, 1971). Leakage of cell sap or greater root exudation terminates dormant state of fungal propagules and promotes their multiplication (Zackeo, 1993). However, in this way development and reproduction of the nematodes was interrupted and consequently their population decreases (Minton and Minton, 1963) as evidenced by their lower population in combined treatments than the nematode alone.

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