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Trichoderma viride- A powerful tool for managing Root knot nematode in Noni

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Root-knot nematodes (*Meloidogyne* spp.) are the most economically damaging species of plant-parasitic nematodes on horticultural and field crops. The fungal biocontrol agent *Trichoderma viride* was evaluated for its potential to control the root-knot nematode, *Meloidogyne incognita* infesting noni, *Morinda citrifolia*. Pot and field experiments were conducted to evaluate the effects *T. viride* and experimental results revealed that plants treated with *T. viride* reduced nematode infestation and increased the plant growth parameters and in terms of soil nematode population.*Trichoderma* showed the ability to colonize *M. incognita* eggs and second-stage juveniles (J2) collected from the *in vitro* assays. *Trichoderma* colonized eggs observed under scanning electron microscope showed attachment, complete colonization and penetration of conidia into the nematode eggs. Direct parasitic interactions between the fungus and the nematode revealed that *T. viride* was able to penetrate and colonize the eggs. The concentrated soil extracts from *Trichoderma* treated soils immobilized the infective J2. It is suggested that improved mycoparasitic activity of the antagonist may be important for the biological control of the nematodes.

Key words: Biological control, *Bacillus subtilis*, mycoparasitism, *M. incognita*, Noni. *Pseudomonas fluorescens*, *Trichoderma viride*

INTRODUCTION

Morinda citrifolia is one of the major medicinal plants belonging to the family Rubiacea. It is commercially known as Noni, and also known as Indian Mulberry,Cheese fruit, Yellow fruit, Pain killer, Nono etc. It is a large shrub or a dwarf tree and native to South East Asia but has extensively spread throughout India and the Pacific Islands extending up to the Hawaiian Islands. Noni succumb to outbreak of several pests viz., spider mites, grasshopper and scales. Added to this, an outbreak of a root-knot nematode *Meloidogyne incognita* becomes a main problem in this crop. *M. incognita* juvenile stages cause severe damage in the active fresh roots of noni and thereby affecting its yield and fruit quality.

The root knot nematode *M. incognita* is a sedentary endoparasite and is among the most damaging agricultural pests, attacking a wide range of crops (Katooli *et al*. 2010). The infections start with root penetration of second stage juveniles, hatched in soil from eggs stored in egg masses that have been laid by the female on the infected roots. Nematodes pass through an embryonic stage, four juvenile stages and an adult stage. Juvenile Meloidogynes hatch from eggs as vermiform second stage juveniles (J2), the first moult having occurred within the egg. Newly-hatched juveniles have a short free-living stage in the soil, in the rhizosphere of the host plant. They may reinvade the host plant of their parent or migrate through the soil to find a new host root. J2 larvae do not feed during the free living stage, but use lipids stored in the gut. These nematodes burrow into the soft tissues of root tips and young root and cause the nearby root cells to divide and enlarge. Root knot galls damage the vascular tissues of roots and thus interfere with the normal movement of water and nutrient through the plant.

Chemical nematicide is one of the primary means of control for plant-parasitic nematodes. However, the potential negative impact on the environment

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has led to a total ban or restricted use of most nematicides and an urgent need for safe and effective options. Application of microorganisms antagonistic to *Meloidogyne* spp, or compound produced by these microbes, could provide additional opportunity for managing the damage caused by root- knot nematode (Khan *et al*. 2005).

Many fungal and bacterial agents have been examined over a period of time for their potential as biocontrol agents (Sharma and Pandey, 2009). Direct pathogenicity of fungal biocontrol agents is one of the main mechanisms responsible for plant parasitic nematode control; however, secondary metabolites from fungi also contain compounds which are toxic to plant parasitic nematodes (Dababat and Sikora, 2007).Several attempts have been made to use *Trichoderma* species to control plant parasitic nematodes. *Trichoderma* spp. have been used as a bio control agents against plant parasitic nematodes and this fungus may also promote plant growth and have the ability to colonize root surfaces and the cortex. *Trichoderma* species led to inhibition of the nematode activity and movements *in vitro* during one week exposure. *T.viride* in combination with organic amendments was also known to produce growth hormones, which were observed to have added response in boosting the plant vigour. Some *Trichoderma* isolates were reported to enhance both plant growth and reduce root-knot nematode damage.

Only a limited work has been done on the management of nematode infecting *M.citrifolia* in India.The aim of this investigation was to isolate indigenous antagonistic fungi *Trichoderma* spp. from noni rhizhosphere and to determine their efficacy against root-knot nematode *M. incognita* infecting *M. citrifolia.*

MATERIALS AND METHODS

The antagonistic fungi *Trichoderma* species were isolated from soil samples obtained from Noni plants grown in the medicinal plants block of botanic garden, TNAU, India using the soil dilution technique. Five ml of soil suspension was placed in 15 ml molten, cooling PDA, swirled and allowed to solidify. The set up was incubated 5-7 days at 28°C. The isolates were purified by the single spore method. The fungi were identified on the basis of their morphological and reproductive characters and the pure cultures of *Trichoderma* were maintained on PDA medium and stored at - 4°C. Five isolates of *Trichoderma* were used to evaluate their efficacy against *M. incognita* under *in vitro* and pot culture conditions.

In vitro study*: Hatching and mortality test*

Approximately one egg mass and 50 second stage juveniles were suspended in syracush dish. Strains *T. viride* grown on *Trichoderma* selective medium were introduced at the centre of the chamber as a small (4 mm) disk. Each life stage of nematode was examined for infection of *Trichoderma* and the experiment was replicated 5 times, repeated two times. Control experiments were kept with the nematode *M. incognita* life stages without the antagonistic fungus. These chambers were incubated at the room temperature of 28±1°C and the interaction between the fungus and the nematodes was qualitatively monitored for one week under the microscope. Egg masses were crushed on a slide to examine possible infection of eggs by the fungus. The number of juveniles hatched out and dead was counted at 24, 48 and 72 hrs intervals. To elucidate the underlying mechanisms responsible for the antagonistic activity *viz.,* mycoparasitism and for better understanding, *Trichoderma* colonized root knot nematode eggs were observed under light microscope and electron microscope for higher resolution. Various stages of mycoparasitism *viz*., conidial attachment and hyphal penetration on nematode eggs were recorded.

Pot culture experiment

A pot culture experiment was conducted to evaluate the efficacy of *T. viride* strains.The pot mixture was filled in earthen pots of 10 kg capacity. About three months old healthy noni seedlings without root knot nematode infestation were planted in the pots @ one seedling / pot. Talc formulations of *T. viride* were applied @ 5 g/pot. Regular watering was done with tap water passing through 325 mesh sieve. Experiment was maintained at controlled conditions in CRD with three replications for each treatment. Two months after planting, the experiment was terminated and noni plants were uprooted. Observations on plant growth parameters *viz*., length and weight of shoot and root, number of leaves per plant and nematode incidence in terms of number of egg masses, eggs per egg mass, adult females, final soil nematode

population and gall index were recorded.

Field evaluation

Two field experiments were conducted during 2013 in noni garden established by Department of Medicinal and Aromatic plants in botanical garden of TNAU, Coimbatore. Field trials were aimed to assess the efficacy of *T. viride* in managing root knot nematode in Noni. The experiment was carried out in a randomized block design (RBD) with three replications with the treatment schedule as given under glasshouse experiment (3.9.3). The talc-based powder product (2.5 kg ha^{-1}) was mixed with 50 kg of sand and applied. Control plants without treatment were also maintained as control. The experiment was terminated four months after treatment and observations were made on plant growth parameters and nematode population.

Statistical analysis

The PDIs were suitably transformed into arcsine values, analyzed and presented with DMRT symbols

RESULTS AND DISCUSSION

In vitro

T. harzianum (*Hypocrealixii*) and *T. viride* strain NRRL 6418 was tested for their capacity to reduce the incidence of the root-knot nematode *M. incognita* on *L. rotundifolia.* Comprehensive studies at the laboratory revealed the nematicidal potential of selected *T.viride* and *T. harzianum* for controlling *M. incognita*. Direct interaction of *T.viride* and*T. Harzianum* with nematodes was observed *in vitro* under sterile condition. Direct parasitism of *Trichoderma* strands on separated egg/egg masses/female body was observed under the microscope (Goswami *et al*. 2006). *Trichoderma* incubated *in vitro* with the nematode gave promising results. The hyphae of *Trichoderma* penetrated and coiled the female body. Eggs were also colonized and egg masses were penetrated by fungal strands. *T. viride* and *T. harzianum* were able to colonize *M. incognita* eggs and second juveniles and female. *In vitro* studies demonstrated that both tested isolates were effective in causing nematode mortality compared with the control (Table 1and 2). Light microscopic observations of eggs revealed the attachment of fungal conidia. Therefore fungal colonized eggs were placed on the carbon coated stub of ESEM and directly introduced in to the chamber for observation for at higher magnification.

Field evaluation

Experiments were carried out to study the effect of two antagonistic fungal bioagents along with cow dung and carbofuran against root knot nematode *M. incognita* infecting *L. rotundifolia* under net house condition. Bioagents viz., *T. harzianum* (*Hypocrealixii*) and *T. viride* strain NRRL 6418 in combination with cow dung promoted plant growth, reduced number of galls/plant, female body and egg masses/root system. The *Trichoderma* sp. along with cow dung showed least nematodes reproduction factor as compared to untreated infested plants (Table 3).

In vitro efficacy of Trichoderma viride against root knot nematode eggs

Egg masses, eggs, juveniles and adult females treated with *T. viride* were observed under light and scanning electron microscopes to elucidate its mode of parasitism on root knot nematode. Various stages of mycoparasitism *viz*., conidial attachment and hyphal penetration on nematode eggs were recorded. Light microscopic observations revealed colonization of *Trichoderma* in root knot nematode eggs (Fig.1) In order to observe under high magnification and for better understanding, *Trichoderma* colonized eggs were observed under scanning electron microscope (Fig. 2). The micrographs showed attachment, complete colonization and penetration of *Trichoderma* conidia into the nematode eggs. Conidial attachment to eggs, (bar = 5 μm) , and hyphal penetration resulted in structural changes leading to shrinkage of the egg shell, (bar = 40 μm), leakage of egg contents, (bar = 4 μm) and ultimately destroying the eggs. Direct parasitic interactions between the fungus and the nematode revealed that *T. viride* was able to grow on the egg surface and penetrate the egg shell. This proves that *T. viride* is an effective egg parasite of *M. incognita* through the process of mycoparasitism.

Results showed that these *Trichoderma* species with different mechanism such as lysis of cell wall, inhibited growths of the juveniles of *M. incognita* and parasitized the eggs /female body

Table 1 : Effect of *Trichoderma viride* strains on plant growth of noni under pot culture conditions

Treatments	Shoot length (cm)	Per cent increase over control	Root length (cm)	Per cent increase over control	Shoot weight (g)	Per cent increase over control	Root weight (g)	Per cent decrease over control	No of leaves/plant	Per cent increase over control
*TvN*1(5g/pot)	97.33	24.26	25.33	58.33	87.67	30.85	87.33	7.42	31.00	47.62
TvN2 (5g/pot)	99.33	26.81	27.67	72.92	95.00	41.79	86.00	8.83	34.33	63.49
TvN3 (5g/pot)	110.33	40.86	31.67	97.92	98.33	46.77	85.33	9.54	36.67	74.60
TvN4 (5g/pot)	113.67	45.11	34.33	114.58	103.67	54.73	86.00	8.83	38.00	80.95
TvN5	104.33	33.20	30.67	91.67	97.00	44.78	86.33	8.48	32.33	53.97
Carbofuran 3G @1kg a.i./ha	121.00	54.47	38.67	141.67	111.33	66.17	80.00	15.19	42.33	101.59
Control	78.33		16.00		67.00		94.33		21.00	
CD (0.05)	2.3963		2.0602		2.7139		2.9136		2.3963	

Table 2 : Effect of *Trichoderma viride* strains against root knot nematode population in noni under pot culture conditions

Treatments	No. of egg masses/ 5g root	Per cent decrease over control	No. of adult females/5 g root	Per cent decrease over control	Final nematode population/ 250 cc soil	Per cent decrease over control	Gall index
P.f 1(5 g/pot)	21.00	37.63	25.00	44.44	352.00	53.27	3.67
T.v (5 g/pot)	21.33	36.64	23.67	47.41	322.33	57.21	3.33
Bbv 57(5 g/pot)	18.00	46.54	18.00	60.00	210.00	72.12	2.33
Bs 5 (5 g/pot)	15.33	54.46	14.67	67.41	180.33	76.06	2.33
Chitin + *P.f* 1(each 10 g/pot)	19.67	41.59	21.00	53.33	225.00	70.13	3.00
P.f 1+ *T.v*+*Bbv* 57 +*Bs* 5+Chitin	15.00	55.45	11.67	74.07	159.00	78.89	1.33
Control	33.67		45.00		753.33		5.00
CD (0.05)	1.8016		2.3169		3.5155		

of *M. incognita* and thus showed its antagonistic effects against causal agent of root knot seedlings. *T. viride* NRRL 6418 and *T. harzianum* (*Hypocrealixii*) after seven days destructed and lysis the eggs and females bodies. Identified *Trichoderma* species caused parasitize (to hyphal contact method) eggs and female bodies .*Trichoderma* had proved to infect on the eggs and adult females of the root knot nematode, *M. incognita.* Since the female adult was sedentary in its movement, the antagonistic fungus readily infected the nematode. But infection of *Trichoderma* on eggs was prudent it is best opted at the beginning of the live stage of the nematode to get controlled. Sahebani and Hadavi (2008) reported that direct parasitism of *Meloidogyne* eggs through increase in extracellur chitinase activity, which would be indicator of egg infection capability and inducing plant defense mechanisms leading to systemic resistance are two main suppression mechanisms used by *T. harzianum* against nematodes. *T. harzianum* must be able to produce extracellur chitinase and proteinase because of the proteinaceous and chitinase nature of nematode egg shell. Other extracellur protein nature has been induced by colloidal chitin which may be involved in nematode egg penetration.

In vivo

The efficacy of the potential biocontrol agent

Table 3 : Efficacy of biocontrol agents and organic amendment on fruit yield and nematode population of Noni under field conditions-Location 1 (Coimbatore)

Treatments	Fruit yield per tree (kg)	Fruit yield (Tonnes/ ha)	No. of egg masses/ 5g root*	No. of adult females/5 g root*	Final nematode population/ 250 cc soil	Gall index
*P.f*1(10 g/tree)	101.41 (12.04)	36.01 (2.04)	20.7 (40.0)	26.3 (46.32)	240.00 (44.65)	2.4
T.v(10 g/tree)	98.33 (8.64)	34.33 (2.72)	20.3 (41.16)	25.1 (48.77)	237.33 (45.27)	2.4
*Bbv*57(10 g/tree)	122.95 (35.84)	46.20 (30.91)	18.1 (47.54)	17.9 (63.47)	218.33 (49.65)	2.3
*Bs*5(10 g/tree)	125.72 (38.90)	49.63 (40.63)	15.5 (55.07)	15.4 (68.57)	176.00 (59.42)	1.3
Chitin + *P.f*1(each 10g/tree)	119.06 (31.54)	43.91 (24.42)	19.6 (43.19)	22.1 (54.90)	201.67 (53.49)	2.2
*P.f*1+ *T.v* + *Bbv*57 + *Bs*5+Chitin	139.68 (54.32)	58.81 (66.64)	14.6 (57.68)	12.3 (74.89)	154.33 (64.41)	1.2
Control	90.51	35.29	34.5	49.0	433.67	5.0
CD (0.05)	3.76	0.51	2.85	3.48	8.21	

Figures in the parentheses are per cent increase over control
*Figures in the parentheses are per cent decrease over control
Values in the column are mean of three replications
Gall Index 1-5 scale; 1= No galls, 2= 1-25%, 3=26-50%, 4= 51-75%, 5= 76-100% of the root system galled

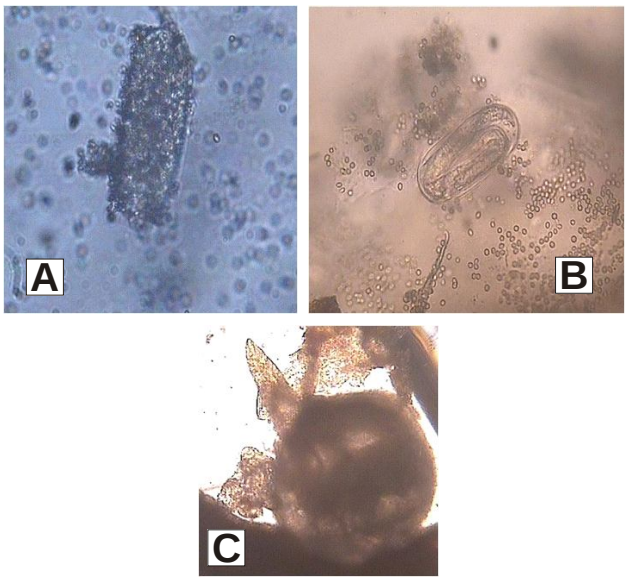

Fig. 1 : *In vitro* efficacy of *Trichoderma viride* against root knot nematode egg Light microscopic observations

A. *Trichoderma* conidia on egg, B. Juvenile inside the egg parasitized, C. Parasitized Female

of *Trichoderma* sp. in the management root knot nematode was achieved as the reduction in root gall formation and also the reduction in the number of female *M. incognita* and egg masses per root system of *L. rotundifolia*. *Trichoderma* treatment was more effective than chemical treatment in reducing the gall formation in *L. rotundifolia.* Palms treated with *Trichoderma* sp. was significantly reduced the number of galls/plant compared with standard chemical treatment (Table 1). Further application of *Trichoderma* was able to infect the eggs, adult female and reduced the number of galls/plant. Since, Carbofuran was used very frequently to control the root knots nematodes, their usage came to standstill due to the development of resistance against these inorganic chemicals. The response of application of Cabofuran was ineffective and their persistence may pose ecological problem. Therefore, biocontrol is suggested to be safer solution (Sharma and Pandey, 2009). *Trichoderma* has good scope due to its effective mode of action. In addition, the eco-friendly method of control of nematodes is prioritized among the management tactics to protect the environment from polluting with chemicals. In addition the consumers of these palms prefer organic products from any countries and as such the Green Farm Ltd. at Marawila, Sri Lanka, apply organic method of controlling insects and diseases to satisfy the requirements of their clients.

The *Trichoderma* treatment also increased the fresh weight and growth rate of the *L. rotundifolia* (Fig. 4). The plant height and root weight were sig-

nificantly improved compare to Carbofuran applications. These results are also supported by Sharma and Pandey (2009).

Combination of isolated *T. viride* strain NRRL 6418 and *T. harzianum* (*Hypocrealixii*) @ 1014 cfu ml^{-1} proved that they are capable to control *M.incognita* and showed good bio control activity at the field level (Zhang and Zhang 2009).

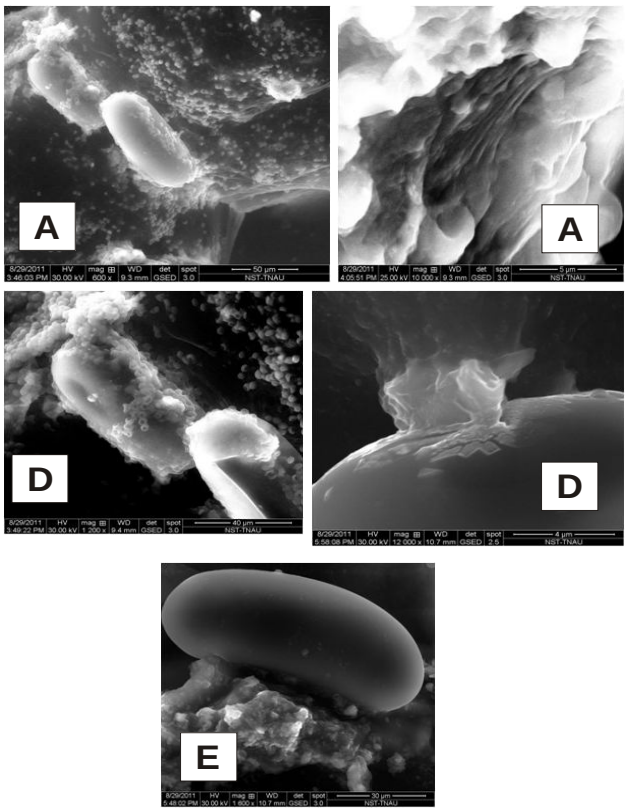

Fig. 2 : *In vitro* efficacy of *Trichoderma viride* against root knot nematode egg- A. Eggs parasitized by fungal conidia, A. *Trichoderma* conidia on egg surface (10000 X) D. Shrinkage of the egg shell (1200 X) D. Leakage of egg content (12 000 X) E. Untreated egg.

Root galling was reduced and the number of new infection had been reduced.*T. harzianum* (*Hypocrealixii*) alone also significantly reduced gall formation and improved palm growth. Both *T. viride* strain NRRL 6418 and*T. harzianum*(*Hypocrealixii*) when added together with compost + cow dung showed significant suppression of the egg masses per root system as compared to the control (Goswami *et al.* 2006). Composts, on the other hand, can serve as an ideal food base for biocontrol agents and offer an opportunity to introduce and establish specific biocontrol agents into soils, which in turn leads to sustained biological control based on the activities of microbial communities (Hoitink and Boehm, 1999). Sharma and Pandey (2009) reported that the *Trichoderma* has not only been proved to parasitize nematodes but also help in tolerence to stress conditions by enhanced root development. It participates in solubilization of inorganic nutrients.

Various mechanisms for the biocontrol activity of *Trichoderma* spp. against plant parasitic nematodes suggested were antibiosis, competition, mycoparasitism, and enzymatic hydrolysis. Under the ESEM, root knot nematode egg masses, eggs, juveniles and adult females treated with *T. viride* various stages of mycoparasitism *viz*., conidial attachment and hyphal penetration on nematode eggs were observed. The hyphal penetration resulted in structural changes in the nematode egg shell leading to shrinkage of the egg shell, leakage of egg contents and ultimately death of the eggs. Direct parasitic interactions between the fungus and the nematode revealed that *T. viride* was able to grow on the egg surface and penetrate the egg shell.

From the present study it is obvious that direct parasitic interactions between the fungus and the nematode revealed that *T. viride* is an effective egg parasite of *M. incognita*.

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