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BIO-EFFICACY OF FUNGAL BIOAGENTS AND ITS CRUDE SECONDARY METABOLITES AGAINST ROOT KNOT NEMATODE *MELOIDOGYNE INCOGNITA* [(KOFOID & WHITE, 1919) CHITWOOD, 1949] INFESTING TOMATO (*SOLANUM LYCOPERSICUM* L.)

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Prasad L., Pervez R., Gaba S. - Bio-efficacy of fungal bioagents and its crude secondary metabolites against root knot nematode *Meloidogyne incognita* [(Kofoid & White, 1919) Chitwood, 1949] infesting tomato (*Solanum lycopersicum* L.).

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crop in the world. Various obstacles are existing with this crop cultivation, among them root knot nematode, *Meloidogyne incognita* recognized as a major pathogen of tomato. The complete elimination of *M. incognita* from the soil is very difficult because of its polyphagous nature. Although, different methods are employed to manage this nematode, however, biological control has become a good alternative to the chemical nematicides. Antagonistic microorganisms are appropriate for controlling nematodes still require progressive investigation. Hence, present study on the biological activity of the different concentrations of mixer of crude secondary metabolites of the various fungal bioagents against on egg hatching, *M. incognita* (J₂) mortality *in vitro* as well as *in planta* were evaluated. Plant growth promoting activities were also recorded. Among the tested fungal bioagents *V. lecanii*, *F. chlamydosporum* and *A. niger* were found promising *in vitro* as well as *in planta* studies while *M. anisopliae* and *A. niger* was found promising to inhibit the egg hatching under *in vitro* study. It is concluded that *V. lecanii*, *F. chlamydosporum* and *A. niger* have high antagonistic/nematicidal as well as good plant growth promotion activities. These promising fungal biocontrol agents can be considering for evaluation on a larger scale in field.

KEY WORDS: Biological control, Fungi, Tomato, Root knot nematode, *M. incognita*

INTRODUCTION

Plant parasitic nematodes are a serious threat for crop production, causing an estimated US \$125 billion loss/year worldwide (CHITWOOD, 2003). In India, the estimated annual crop yield loss due to plant parasitic nematodes is 21.3%, which is equivalent to Rs. 102039.19 million or \$ 1.58 billion, and losses in 19 most common horticultural crops is 23.03% amounting Rs. 50224.98 million (KUMAR *et al.*, 2020). Root-knot nematodes, *Meloidogyne* spp. represent the most economically damaging nematode genus to crop production worldwide. The impact of these species is enhanced by their wide host ranges of more than 5000 plant species causing severe economic loss to many agricultural and horticultural crops. It has been estimated that global losses to the *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949) amount to \$78 billion (GHARABADIYAN *et al.*, 2012).

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crop in the world. Various obstacles are existing with tomato cultivation, among them root knot nematodes are recognized as a major pathogen of tomato (KAMRAN *et al.*, 2010). Many studies have been carried to assess damage of root knot nematode on toma-

to and yield loss ranged between 25 to 100% (KAUR *et al.*, 2011; EBRAHIM *et al.*, 2015).

Root-knot nematodes management includes soil fumigation, soil solarization, rotation with non-host crop and use of resistant varieties. However, due to the environmental problem caused by nematicides and the limited number of resistant varieties, more ecologically rational alternative systems like biological control and natural products are alternating attention (WILLE *et al.*, 2019; RAJKUMAR *et al.*, 2019).

Among biocontrol agents' fungi have complex strategies for capturing the nematodes by sticky branches, non-constricting and constricting rings, killing by producing toxic substances, and digesting by colonizing nematode reproductive structures (Uddin, 2019). Soil inhabiting fungi are pathogenic to soil and plant nematodes due to their antagonistic action against the nematodes more precisely *Meloidogyne* spp. (ABD-ELGAWAD, 2016; BRAND *et al.*, 2004).

In this view, present study on the nematocidal activity of the different concentrations of crude metabolites of the various fungal bioagents against *M. incognita* (J₂) egg hatching, mortality *in vitro* as well as *in planta* were evaluated. Plant growth promoting activities were also recorded.

ABBREVIATIONS:

ITCC: Indian Type Culture Collection

CFU: Colony forming unit

PDA: Potato dextrose agar

ANOVA: Analysis of variance

MATERIALS AND METHODS

SOURCE OF FUNGAL BIOAGENTS CULTURES

Six fungal cultures viz., *Metarhizium anisopliae* ((Metschn.) Sorokin) (ITCC 4034), *Nematocytus robustus* (Thorn & Barron) (ITCC 7079), *Aspergillus niger* (V Tieghem) (ITCC 4991), *Verticillium lecanii* (Zimmerman) Viegas (ITCC 7084), *Trichoderma harzianum* (Rifai) (ITCC 5593) and *Fusarium chlamydosporum* (Wollenw. & Reinking) (ITCC 8008) were obtained from ITCC, Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, India. Other cultures like *Verticillium lecanii* (MTCC 5730) was used from International Panacea Ltd., whereas *Hirsutella thompsonii* Fisher was isolated from soil. These cultures were maintained on PDA slants as per standard protocol.

SOURCE OF NEMATODE CULTURES

Meloidogyne incognita culture was maintained on tomato plant under greenhouse conditions in Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, which used as source of inoculum. Species identification was done based on juvenile measurements and examination of perineal pattern system of adult females (EISENBACK *et al.*, 1981; JEPSON, 1987). Infected tomato roots were cut into pieces about 2 cm long and placed in a conical flask with 200 ml of 0.5% Sodium hypochlorite. The tightly capped flask was shaken for 3 minutes. The shaking partially dissolved the gelatinous matrix thus freeing eggs from egg masses (HUSSEY and BARKER, 1973). The liquid suspension of eggs was poured through a 200 mesh sieve nested upon a 500 mesh sieve. Eggs collected on the 500 mesh sieve were immediately washed free of residual Sodium hypochlorite solution under a slow stream of tap water and incubated in Petri dishes at $28 \pm 2^\circ\text{C}$ until hatching. Newly hatched juveniles were collected with the help of micropipette. Emerging J₂ was collected and the aqueous nematode suspension (100 J₂ in 200 µl sterilized distilled water) was prepared by diluting the standard nematode suspension. Only freshly hatched second stage juveniles collected within 48 h were used in the study.

PREPARATION OF FUNGAL BIOAGENTS SUSPENSION

Pure cultures of all fungal bioagents were used from master culture plate. One ml of sterilized double distilled water was added onto individual fungal colony grown on master plate (9.0mm) and then scraped with spatula to create conidial suspension with different labelled stock

solution of fungal cultures used in the study. The suspension of 10 ml stock solution is diluted with 90 ml distilled water to maintain conidial load up to 2×10^6 CFU/ml. As per experimental requirement, the suspension was further diluted with sterile water.

EXTRACTION OF ROOT-KNOT NEMATODE EGGS

Meloidogyne incognita eggs used for this study were cultured on tomato plants in earthen pots. The galled roots were harvested and thoroughly washed under running water in order to remove soil particles. They were then chopped into 2-3 cm pieces and placed into a 2L conical flask. Sodium hypochlorite solution of 0.5% concentration was prepared and added into the conical flask and shaken for four minutes extracted. The content in the conical flask was poured onto a stack of sieves. The upper sieve No 1 mm was used for collecting the plant debris, followed by a 2 mm mesh for fines while the 28 mm was used to collect the eggs. The 28 mm sieve with the eggs was then quickly placed under a stream of cold water to remove residual NaOCl, and collected in a beaker. The egg suspension in the beaker was thoroughly mixed using a magnetic stirrer. The number of eggs in 1ml of suspension was counted under the stereomicroscope. The average of three counts was taken to estimate the egg population per ml of egg suspension.

EXTRACTION OF SECONDARY METABOLITES

For production of secondary metabolites, sterilized 100mL potato dextrose broth (PDB) for individual bioagents were taken in 250mL flasks and 2 discs (5mm size) taken from actively growing colony of each fungal bioagents viz., *V. lecanii* (MTCC 5730) as T₁, *T. harzianum* (ITCC 5593) as T₂, *H. thompsonii* as T₃, *M. anisopliae* (ITCC 4034) as T₄, *V. lecanii* (ITCC 7084) as T₅, *A. niger* (ITCC 4991) as T₆, *F. chlamydosporum* (ITCC 8008) as T₇ and *N. robustus* (ITCC 7079) as T₈ were inoculated into the broth. The inoculated flasks were kept for 20 d at $25 \pm 1^\circ\text{C}$ and thereafter, mycelia were removed by filtration using filter paper. The metabolites of each bioagents were extracted separately by solvent extraction. Firstly, the culture filtrate was extracted thrice with ethyl-acetate 1:1(v/v) using short-term separatory funnel. The organic phase was transferred into another clean conical flask by passing through Sodium sulphate (anhydrous) taken on a glass funnel. The organic phase collected was concentrated individually using a rotary evaporator (Heidolph, Germany). The brown coloured extract, that is crude extract, was kept in glass vials for further use (SHARMA and PRASAD, 2018).

ASSESSMENT OF BIOLOGICAL ACTIVITIES OF BIOAGENTS USING BIOASSAY TECHNIQUES

Ovicidal assay

Matured egg masses of *M. incognita* were selected and

also ensured eggs had the same embryonic stage from infected roots of tomato and 500 egg were transferred into Petri plate containing 1 ml of each tested crude secondary metabolites concentrations viz., 25%, 50% and 100% of each fungal bioagents like *V. lecanii* (MTCC 5730) as T₁, *T. harzianum* (ITCC 5593) as T₂, *H. thompsonii* as T₃, *M. anisopliae* (ITCC 4034) as T₄, *V. lecanii* (ITCC 7084) as T₅, *A. niger* (ITCC 4991) as T₆, *F. chlamydosporum* (ITCC 8008) as T₇ and *N. robustus* (ITCC 7079) as T₈. There were six replicate of each treatment along with the control (sterilized distilled water) as T₉ and experiments were conducted at 28±2°C. Total numbers of hatched juveniles from eggs were counted after five days and per cent hatching of the juveniles was calculated.

Larvicidal assay

Bioactivity of crude secondary metabolites of fungal bioagents viz., *V. lecanii* (MTCC 5730) as T₁, *T. harzianum* (ITCC 5593) as T₂, *H. thompsonii* as T₃, *M. anisopliae* (ITCC 4034) as T₄, *V. lecanii* (ITCC 7084) as T₅, *A. niger* (ITCC 4991) as T₆, *F. chlamydosporum* (ITCC 8008) as T₇ and *N. robustus* (ITCC 7079) as T₈ on the mortality of second stage juveniles of *M. incognita* was tested in Petri plate. One milliliter each of the fungal bioagents crude secondary metabolites suspension was poured into separate Petri plate. One hundred juveniles were inoculated into Petri plate containing 1 ml of each tested concentrations 25%, 50% and 100% of each fungal bioagents. All treatments were replicated six times and tested at 28±2°C along with control (sterilized distilled water) as T₉. The inactive nematodes were transferred separately into sterile distilled water and kept overnight to check whether mortality was permanent or temporary. The dead juveniles attained the straight posture and the mortality was ensured by touching the juvenile with the help of nematode picking needle. Observations were recorded on the mortality of nematodes after 24h and 48h post exposure period and per cent mortality was calculated.

EFFICACY OF FUNGAL BIOAGENTS AGAINST *M. INCOGNITA* IN PLANTA

Efficacy of seven fungal bioagents against *M. incognita* were evaluated in earthen pot under greenhouse conditions during 2017-18 and 2018-19. Earthen pot contains 1 kg sterilized potting mixture (soil: sand, 3:1) and planted tomato seedlings of cv Pusa ruby prior to root dip treatment with fungal bioagents containing 2 x 10⁶ CFU/ml individually for 2h. After the establishment of the plant, second stage of root knot nematode *M. incognita* juveniles were inoculated around the plant @ 2 J₂ g⁻¹ in the soil and watered whenever necessary. There were six replicates along with negative and positive control.

After 45 days of planting, plants were uprooted and wash properly. Observations like number of root galls and gall index calculated as per standard protocol, whereas shoot length (cm), root length (cm), shoot weight (gm) and root weight (gm) of each plant were recorded. Statistical analysis of the data was found normally distributed and did not require transformation. Mean value and completely randomized design worked out. All the data were subjected to Analysis of Variance (ANOVA). The means and standard error was calculated at 5%.

RESULTS AND DISCUSSION

BIOASSAY FOR NEMATOCIDAL ACTIVITIES:

Ovicidal assay

It was found that all the crude secondary metabolites of tested fungal bioagents were found to exhibit some level of inhibition towards root knot nematode *M. incognita* juveniles hatching from eggs. The hatching of juveniles declined with increase the concentrations of crude secondary metabolites of tested fungal bioagents. Among the tested crude secondary metabolites of fungal bioagents *M. anisopliae* (ITCC 4034) and *A. niger* (ITCC 4991) was found promising to inhibit the egg hatching, resulted only 17 and 22%, respectively egg hatched at 100% concentration, while, *V. lecanii* (ITCC 7084) was less effective towards *M. incognita* egg hatching, which shows 68% egg hatched at higher concentration, whereas hatching of juveniles from eggs was on par with control at 25% (Fig. I).

Larvicidal assay

Results showed that all the crude secondary metabolites of tested treatments were found to exhibit various level of toxicity towards juveniles of root-knot nematode, *M. incognita*. The mortality rate of juveniles increased with increase the concentrations of crude secondary metabolites of tested fungal bioagents. Data revealed that *T. harzianum* (ITCC 5593), *M. anisopliae* (ITCC 4034) and *F. chlamydosporum* (ITCC 8008) were found most pathogenic/effective which caused cent percent mortality of *M. incognita* (J₂) at higher concentrations within 24h post exposure. After 48h post exposure observation, *V. lecanii* (ITCC 5730), *T. harzianum* (ITCC 5593), *M. anisopliae* (ITCC 4034), *A. niger* (ITCC 4991), *F. chlamydosporum* (ITCC 8008) and *N. robustus* (ITCC 7079) were found most pathogenic, which caused cent percent mortality of *M. incognita* (J₂), whereas *V. lecanii* (ITCC 7084) was least pathogenic which caused only 78% after 48h at higher concentration. No mortality was found in control treatment (Fig. II).

Fig. I - Hatching of *M. incognita* (J₂) from eggs in different fungal biocontrol agents.

EFFICACY OF FUNGAL BIOAGENTS AGAINST *M. INCOGNITA* IN PLANTA

Among the tested fungal bioagents, *V. lecanii* (ITCC 7084) was found most promising followed by *F. chlamydosporum* (ITCC 8008) and *A. niger* (ITCC 4991) in comparison to other fungi. Gall formation and gall index data revealed that minimum gall formed when *V. lecanii* (ITCC 7084) followed by *F. chlamydosporum* (ITCC 8008) treated plant (7.67 and 9.0, respectively). *H. thompsonii*, *V. lecanii* (MTCC 5730) and *N. robustus* (ITCC 7079) were less effective in controlling gall formation as large number of galls (18.33, 15 and 12.67, respectively) were formed while they act as good plant growth promoter as compare to negative and positive control (Table 1).

Results shows that, *V. lecanii* (ITCC 7084) treated plants have higher plant growth promoting activity such as root length (25.40 cm), shoot length (68.67 cm) and maximum shoot weight (20.13 gm), followed by *F. chlamydosporum* (ITCC 8008) treated plants shoot length (65.17 cm), root weight (2.10 gm), shoot weight (20.23 gm) as compared to other treatments (Table 1).

Root knot nematode *M. incognita* complete elimination from the soil is very difficult because of its polyphagous nature. Although different management technologies are employed to manage this nematode. Among them, fungal bioagents are an alternative of toxic chemicals which are used to control nematodes. Besides this an additional advantage of fungal bioagents are plant growth promotion. Several studies reported that, mixing or drenching culture

supernatants of fungal bioagents in soil found to be reduced significantly the number of egg masses produced by *M. incognita* in tomato.

The present study demonstrated that crude secondary metabolites of fungal bioagents significantly causes mortality of juveniles and lower the egg hatching of *M. incognita*. However, mortality and hatching are directly related to the concentrations as well as bioagents. As mean hatching observed in different concentrations of the crude secondary metabolites of fungal bioagents showed that, there was a correspondingly hatching increased in lower concentration but all tested concentrations significantly inhibited the hatching rate as compared to control at higher concentration. Our findings agreed with previous workers that hatching inhibition was directly related to the concentration of *Beauveria bassiana* and *Metarhizium anisopliae* (ZHAO, 2013; KHOSARAVI *et al.*, 2014). The culture filtrates of *Aspergillus* sp., *T. harzianum*, *T. viride*, *Penicillium* sp. and *Fusarium* sp. efficiently control egg hatching of *M. incognita* (DEVI and BORA, 2018). The conidia and hyphae of fungus can easily be penetrated in the eggs, resulting in larval death within egg (HUSSAIN *et al.*, 2017).

Our findings indicate *V. lecanii*, *F. chlamydosporum* and *A. niger* and its crude secondary metabolites were found to be most promising, hence may be considered to be best bioagents against *M. incognita*. CAYROL *et al.* (1992) have also reported that *Verticillium* genera have ability to destroy nematode egg and considered egg parasitic fungi.

Table 1 - Antagonistic and plant growth promoting activity of fungal bioagents against root knot nematode *Meloidogyne incognita* in planta.

Treatment	Root length (cm)		Shoot length (cm)		Root weight (g)		Shoot weight (g)		Number of galls		Gall index (1-10)
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean
<i>V. lecanii</i> (ITCC 5730)	23.57	7.05	70.17	6.27	1.75	0.66	19.98	4.06	15.00	0.57	4.00
<i>H. thompsonii</i>	17.80	3.18	62.67	2.62	1.17	0.08	12.80	1.70	18.33	3.38	4.00
<i>M. anisopliae</i> (ITCC 4034)	20.07	2.72	57.57	3.82	0.93	0.09	12.73	3.10	9.33	0.67	1.67
<i>V. lecanii</i> (ITCC 7084)	25.40	1.92	68.67	2.35	1.48	0.24	20.13	1.46	7.67	0.33	1.00
<i>A. niger</i> (ITCC 4991)	24.33	5.80	52.67	8.51	1.05	0.60	13.30	3.91	9.67	0.67	1.33
<i>F. chlamydosporum</i> (ITCC 8008)	17.80	4.14	65.17	2.33	2.10	0.66	20.23	3.74	9.00	0.57	1.67
<i>N. robustus</i> (ITCC 7079)	18.23	2.62	63.67	5.67	0.83	0.22	15.13	2.63	12.67	1.20	2.67
Negative control T-8	20.23	1.35	46.00	4.91	0.83	0.16	10.73	0.63	23.67	1.20	7.33
Positive control T-9	20.53	1.92	44.83	4.53	1.18	0.40	20.30	2.10	0.00	0.00	0.00
CD @5%	N/A		14.83		N/A		N/A		3.99		0.74
CV	32.01		14.53		57.34		30.51		19.73		16.36

Fig. II - Mortality of *M. incognita* (J₂) by different fungal biocontrol agents.

Similarly, JANG *et al.* (2016) used culture filtrate of *A. niger* (F22) was highly effective as a biocontrol agent due to nematicidal component such as oxalic acid which causes mortality of *M. incognita* (J₂) and reduction in egg hatching also occurred which supports the findings in present study, which act as good plant growth promoter and effective biocontrol agent. *Fusarium* sp. and *Aspergillus* sp. are known to produce toxins and antibiotics like fusaric acid which can be considered as mechanism to reduce gall formation (DESAI *et al.*, 1972; ANKE *et al.*, 1997).

Metarhizium anisopliae also has good biocontrol potential with ingestion of spores by the larvae further conidia will germinate inside the larvae and produces hyphae and block the respiratory siphon and causes death of larva and hence reduces nematode population (KHOSRAVI *et al.*, 2014; JAHANBAZIAN *et al.*, 2015).

Previous studies suggested that the abilities of *Fusarium* species to parasitize root knot nematode eggs *in-vitro* (SUN *et al.*, 2006). Antagonistic action of *Fusarium* species has been found against root knot nematode in soil as they interact with root feeding nematodes either directly or via secondary metabolites (NITAO *et al.*, 2001). REGAIEG *et al.* (2011) have reported that promising strain of *F. chlamydosporum* which have an ovicidal effect on root knot nematode. All these mechanism has contributed our research study to test the effect of *F. chlamydosporum* against *M. incognita* and have an effect on reducing gall formation and enhanced plant growth promotion. However, BHAGWATI *et al.* (2007) reported that simultaneous inoculation of nematode and *F. chlamydosporum* show reduction in plant growth parameters and favoring the host more vulnerable for fungal infection. Further attack by nematode modifies the rhizosphere favoring the fungal growth.

Verticillium lecanii emerged as plant growth promoter and biocontrol agent in our studies were also be supported by previous studies have found to be act as in improving growth of plants and also reduces J₂ population in soil or egg masses (MEYER *et al.*, 1999). They also used a formulation (mycotal) contains fungus *V. lecanii* against *M. incognita* in tomato crops and whiteflies and they found good antagonistic against *M. incognita* and also showed a strong action on thrips and spider.

It is concluded that *V. lecanii*, *F. chlamydosporum* and *A. niger* have high antagonistic as well as good plant growth promotion activities. These promising fungal biocontrol agents can be considering for evaluation on a larger scale in field study.

ACKNOWLEDGEMENTS

Authors express their gratitude to the Director, and Joint Director (Research), ICAR- Indian Agricultural Research

Institute, New Delhi for provided all the facilities for this study. Thanks also extended to the Head, Division of Plant Pathology and Head, Division of Nematology, ICAR-IARI, New Delhi, India.

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