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Effect of cell free extract of fungal isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* on egg hatching on *Meloidogyne incognita*

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Abstract
The study was conducted to find out the effect of cell free extracts of isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* on mortality and inhibition of egg hatching of *Meloidogyne incognita in vitro.* Mortality of *Meloidogyne incognita* was directly proportional to the cell free extracts of all the tested fungi and the duration of exposure. Maximum mortality of larvae was observed in higher concentration of *Trichoderma viride* followed *by Metarhizium anisopliae* and *Fusarium verticillioides.* Likewise different dilution of culture filtrates significantly inhibited the hatching of *M. incognita eggs.* Larval emergence was inversely proportional to filtrate concentrations. Significantly lower hatching was observed with higher concentration of CFE.

Keywords: root knot nematode *Trichoderma viride* followed by *Metarhizium anisopliae* and *Fusarium verticillioides*

Introduction
Among plant parasitic nematodes *Meloidogyne incognita* is the most damaging species attacking horticultural and field crops, causing a projected yield loss of 12.3 per cent ($157 billion dollars) worldwide and of which $40.3 million is reported from India (Singh *et al*., 2015) [18]. Introduction of antagonists in the soil microenvironment has resulted in an efficient method for biological control of nematodes (Akhman *et al*., 2002) [3] and gaining importance. In nature, fungi continuously destroy nematodes in virtually all soils because of their constant association with nematodes in the rhizosphere. The fungal antagonism consists of a great variety of organisms which vary considerably in their biology and taxonomy and play a major role in recycling the carbon, nitrogen and other important elements from the rather substantial biomass of nematodes. *Trichoderma harzianum* was able to penetrate nematode eggs and significantly decrease the hatching of *Meloidogyne javanica* eggs. The tomato plants inoculated with the fungi showed an increase in the activity of resistance related enzymes *viz*, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase (Sahebani and Hadavi, 2008) [13]. Narayana *et al.* (2017) [11] reported that *Purpureocillium lilacinum* $1x10^7$ cfu @ 30g/plant was equally effective in increasing the yield of cardamom and reducing the population of *Meloidogyne javanica* in soil and roots. Varkey *et al.* (2017) [20] reported that a consortium of rizhobacteria and *Piriformospora indica* suppressed the population of *Meloidogyne incognita* in soil and roots of tomato.
Hence attempts have to be initiated for the isolation of native strains of antagonistic fungi that can be developed as an effective bio-agent against root knot nematodes. Information pertaining to different indigenous species of antagonistic fungi effective against root knot nematodes in Kerala is meager. Hence the present study was undertaken to isolate indigenous fungal antagonists from vegetable growing areas of Thiruvananthapuram district and to evaluate the ovicidal and larvicidal effect of cell free extracts of fungal isolates against *Meloidogyne incognita* under laboratory conditions.

Materials and Methods
A survey was conducted at six Taluks of Thiruvananthapuram district during 2017- 18 for the isolation of indigenous fungi. 20 soil and root samples were took from the rhizosphere of vegetable crops like bhindi, tomato, chilly, cucumber and cowpea grown in each taluk by

random sampling. Plants showing different disease symptoms such as yellowing, necrosis, withering, galling etc. were selected for sampling. These plants were collected and brought back to the laboratory for the isolation of egg masses and females of root knot nematodes. The soil adhered to roots was also collected carefully in polythene bag and labelled for the isolation of soil fungus. The soil samples (weighing 10g each) were collected using sterile spatula and kept in a clean and dry polythene bag under sterile conditions. Fungus from soil samples were isolated by serial dilution technique. These isolates were sub-cultured on Potato Dextrose Agar [PDA] media to get pure culture of the fungus and the fungus was streaked onto PDA slants for further analysis and storage.

Meloidogyne spp. was identified by observing perineal pattern of adult females as described by Taylor and Netscher (1974) [19] later modified and described by Hartman and Sasser (1985) [5]. *M. incognita* population was obtained from naturally infected tomato plant and was multiplied and maintained on susceptible tomato plant variety, Vellayani Vijay, grown in grow bags filled with sterilized loamy soils. The egg masses from infected plants were extracted and placed on culture plates containing distilled water and kept for 48-72 hours in dark at 23 °C to allow hatching. 5mL of freshly hatched juveniles were inoculated in grow bag. Inoculated plants were maintained throughout the experiment for the assortment of egg masses and second stage juveniles as and when required.

Inoculated tomato plants were uprooted and roots are thoroughly washed for egg masses. Small spherical honey dew like brown colored egg mass adhered to the root surface were picked up carefully using forceps. These are collected in sterile distilled water and surface sterilized in 0.1 per cent sodium hypochlorite for two minutes and 95 per cent ethanol for one minute, followed by three continuous washings with sterile water (Singh and Siddique, 2010) [17].

Egg masses were collected in sterile distilled water after surface sterilization and incubated at 28±2 °C in BOD for 2 days for juvenile emergence (J2) using Modified Baermann's funnel technique (Schindler, 1961) [16]. The bottom of Petri plates (10 cm diameter) were poured with sterile water and above that a concave wire mesh covered with double layered tissue paper was placed carefully without breaking the tissue paper. The egg masses were then spread uniformly on the tissue paper. The edges of the tissue paper spreading outside the wire mesh were bend back to keep away from the trickling of water drops from the edges as it might transmit nematodes. Petri plates were then completely filled with water and maintained a level 5 mm over the wire mesh. This plate was incubated at room temperature. After 24 to 48 hours the wire mesh along with filter paper was removed and the extracted nematodes in the Petri plate were collected and counted under a stereo zoom microscope using hand tally counter.

Five mm diameter mycelium disc was cut out from the fungus grown in PDA plates for 7 days using a sterile cork borer and used for inoculating 100ml of PDB broth (pH 7) in 250 ml conical flask and incubated at 28±2 °C for 10 days at 100 rpm in an incubator shaker. Fungal mycelium was separated by centrifugation at 8000 rpm for 10 minutes. CFE was collected aseptically and filter sterilized using Bacteriological Filter.

Different concentration of cell free extracts of each isolates *viz*., S, S/2, S/3 and S/4 were prepared as follows: S - 100% cell free extract (5 mL cfe), S/2- 50% cell free extract + 50% sterile water (2.5 mL cfe+2.5 mL sterile water), S/3- 33.3% cell free extract + 66.7% sterile water (1.67 mL cfe+3.33 mL sterile water) and S/4- 25% cell free extract + 75% sterile water (1.25 mL cfe+3.75 mL sterile water)

Effect of cell free extracts of selected isolates on mortality of *M. incognita* was studied in four different concentrations (100, 50, 33.3 and 25%) at 24, 48 and 72 hours after treatment (HAE). Hundred freshly hatched second stage juveniles of *M. incognita* were suspended in 5mL of each concentration of cell free extracts in sterile vials. All vials were kept in, BOD incubator at a temperature of 30 °C. Larval mortality was estimated by counting the number of dead juveniles at 24, 48 and 72 hours after treatment. Of these isolates, most effective three isolates were screened for ovicidal effects against *M. incognita in vitro*. Sterile water and plain broth were maintained as control.

Results and Discussion

Cell free extracts (CFE) of isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* at different concentrations (100, 50, 33.3 and 25%) showed significantly superior mortality of juveniles of M. *incognita* at 24, 48 and 72 hours after exposure (HAE) (Table 1). At 100% concentration, CFE extracts of all isolates of these fungi recorded the highest mortality and were significantly higher than all other concentrations of *M. Incognita* juveniles from 24 to 72 HAE. At 100% concentration, the mortality percent of fungal isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* were 17.93%, 59.00% and 46.50% at 24 HAE, 46.88%,74.75% and 52.50% at 48 HAE, and 85.05%,76.50% and 62.50% at 72 HAE respectively. While at 25% concentration CFE extracts of all fungal isolates (*Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides*) recorded the lowest mortality and were significantly lower than all other concentrations of *M. Incognita* juveniles from 24 to 72 HAE. At 25% concentration, the mortality percent of fungal isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* were 12.13%, 17.25% and 17.00% at 24 HAE, 21.25%, 19.50% and 20.00% at 48 HAE, and 34.50%, 34.50% and 24.25% at 72 HAE respectively. It is seen that in this experiment the mortality of *M. incognita* juveniles increased with concentration and time of exposure. Higher mortality (85.05 to 62.50 per cent) was observed in higher concentration of isolates (100%) while it was lesser (34.5 to 24.25 per cent) in lower concentration (25%) at 72 HAE. Similar trend was observed in 24 and 48 HAE also. CFE of isolates at 100% concentration recorded 17.93 to 59.00 percentage mortality of juveniles at 24 hours after exposure. This observation clearly highlights the presence of nematicidal compounds in the cell free extract of indigenous fungal isolates. In this study, the fungal colonies selected for screening showed colony characters similar to *Trichoderma* and *Purpureocillium.* Acharya *et al*. (1988) [18] reported that field application of *T.viride* resulted in good control of root knot nematodes. Sankaranarayanan *et al*., (1997) [15] have reported that *Trichoderma* and *Gliocladium* could be used as fungal biocontrol agents against many plant pathogens and plant parasitic nematodes.

Reddy *et al.*, (I996) [12] and Sankaranarayanan and Sundarababu (1997) [14] observed increased population of nematode trapping fungus which led to increased plant growth when the fungi *T. viride* was applied along with the organic amendment. Khan and Saxena (1997) [8] also reported reduced *M. incognita* damage in plants when filtrates of *A. niger*, *P. lilacinum* and *T. viride* were used. The free-living soil fungi

Trichoderma spp. are potential nematode bio-control agents on many food, vegetable and cash crops (Dababat and Sikora, 2007; Affokpon *et al*., 2011) [4, 2]. Lal and Rana (2013) [9] found that *T. harzianum* was the most effective fungus in reducing nematode growth followed by *T. viride*, *Gliocladium virens* and *Aspergillus ochraceous*.

CFE of isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* reported significantly higher inhibition of egg hatching of *M. incognita* at concentrations of 100, 50, 33.3 and 25%, three to eight days after exposure in comparison with sterile water and plain broth. CFE of isolate 10 at 100% concentration recorded minimum hatching of *M. incognita* eggs (14.00 to 23.00 per cent) at three to eight days after treatment while at lower concentrations (33.3 and 25%) the inhibition of egg hatching of *M. incognita* ranged from 16.25 to 22.00% and 17.25% to 27.25% respectively, three to eight days after treatment. CFE of isolate of *Trichoderma viride* at 100% concentration recorded significantly lower hatching of *M. incognita* eggs compared to 25% concentration on 4th, 5th, 6th, 7th and 8th day after treatment.

As the concentration of CFE increased the mortality also increased. Among different concentrations of CFE of isolates of *Trichoderma viride, Metarhizium anisopliae* and *Fusarium verticillioides*, the *highest* egg hatching was observed in lowest concentration (25%) which ranged from 17.25 to 27.25 per cent from three to eight days after treatment while at highest concentration (100%) it was 14.00 to 19.75 per cent.

Cell free extracts of isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* at 100, 50, 33.3 and 25% concentration were tested for egg hatch inhibition of *M.incognita* (Table 2). On third day after exposure CFE of these three isolates exhibited 14.00 to 28.23 per cent hatching of eggs while it was 19.75 to 30.50 in 8th day after exposure. Egg hatching percent at highest concentration (100%) ranged from 19.75 to 27.25 on 8th day after exposure while at lowest concentration of 25%, it ranged from 27.25 to 30.50 against cent per cent in control (sterile water). This finding clearly highlights the inhibitory effect of cell free extract of the isolated indigenous fungus on *M. incognita* eggs. Jones *et al.*, (1983) [7] reported that *V. chlamydosporium* was capable of preventing egg hatching of *M. arenaria* and to colonize eggs by hyphal penetration. Jatala *et al*., (1985) [6] also reported that *P. lilacinus* caused substantial egg deformation in *M. incognita* and these deformed eggs never matured or hatched. Fungal penetration of eggs might be enabled by the high production of extracellular cuticle degrading protease (Yang *et al*., 2007) [21]. Monjil *et al*. (2017) [10] also found that fungus attacked eggs of *Meloidogyne spp*. and inhibit the nematode hatching from the egg masses and were more susceptible to penetration and killing by fungus. From this study it is clearly evident that the fungal isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* have inhibitory effect on hatching *M. incognita* eggs and mortality of its juveniles.

Table 1: Effect of cell free extracts of Isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* on mortality of *M. incognita in vitro*

Concentrations	24 HAE*	48 HAE	72 HAE	24 HAE*	48 HAE	72 HAE	24 HAE*	48 HAE	72 HAE
100%	17.93	46.88	85.05	59	74.75	76.5	46.5	52.5	62.5
	(25.02) a	(43.19) a	(67.60) a	(50.18) a	(59.83) a	(61.00) a	(42.99) a	(46.43) a	(52.24) a
50%	16.5	37.5	68.25	47.25	52.5	68.25	39.5	42.75	47
	(23.95) ab	(37.72) b	(55.72) b	(43.42) b	(46.43) b	(55.70) b	(38.94) b	(40.83) b	(43.28) b
33.30%	14.5	27.25	45.25	32.25	36	45.25	27.75	30.75	32.75
	(22.36) by	(31.45) c	(42.26) c	(34.60) c	(36.86) c	(42.27) c	(31.79) c	(33.68) c	(34.91) c
25%	12.13	21.25	34.5	17.25	19.5	34.5	17	20	24.25
	(20.36) c	(27.34) c	(35.93) d	(24.54) d	(26.21) d	(35.97) d	(24.39) d	(26.57) d	(29.50) d
Sterile water	0.5	0.5	0.5	0.75	1	1.25	0.5	1.25	1.75
	(2.87) d	(2.87) d	(2.87) e	(4.97) e	(5.74) e	(6.42) e	(4.05) e	(6.42) e	(7.60) e
Plain broth	0.25	0.75	1	0.25	0.5	1	0.5	1	2
	(1.43) d	(3.47) d	(4.06) e	(2.87) e	(4.05) e	(5.74) e	(4.05) e	(5.74) e	(8.13) e
CD (0.05)	-2.965	-4.561	-5.419	-4.694	-5.843	-3.976	-3.824	-3.352	-2.336

Figures in the parenthesis are Angular transformed values
*Hours After Exposure

Table 2: Effect of cell free extracts of fungal isolates of *Trichoderma viride* and *Metarhizium anisopliae* and *Fusarium verticillioides* on egg hatching of *M. incognita* under *in vitro* conditions

Isolates	Concentration	Percentage of egg hatching (in days after exposure)					
		3D*	4D	5D	6D	7D	8D
Trichoderma viride	100%	h 14.00(21.95)	i 15.25(22.97)	f 16.50(23.95)	g 18.50(25.46)	h 25.28(18.25)	g 19.75(26.37)
	50%	g h 15.00(22.76)	i 15.75(23.37)	f 16.75(24.15)	g 18.75(25.65)	h 24.78(17.50)	fg 22.00(27.95)
	33.3%	f g h 16.25(23.75)	h 18.00(25.07)	ef 18.75(25.65)	f g 20.50(26.91)	g 26.37(19.75)	fg 23.00(28.63)
	25%	f g 17.25 (24.52)	g 19.00(25.87)	de 19.75(26.37)	e f 21.75(27.79)	e 28.30(22.50)	cde 27.25(31.44)
Metarhizium anisopliae	100%	e f 18.00(25.07)	g 18.75(25.64)	de 20.00(26.55)	e f 22.00(27.96)	f g 27.26(21.00)	ef 24.00(29.32)
	50%	e f 18.50(25.46)	f g 19.75(26.37)	de 20.25(26.72)	e f 22.2(28.12)	e f 27.76(21.75)	e f 24.00(29.32)
	33.3%	e f 19.13(25.89)	e f g 20.50(26.89)	d 21.50(27.61)	de 23.50(28.98)	e 28.29(22.50)	d ef 24.50(29.61)

	25%	de 20.50(26.91)	e f 21.50(27.61)	d 21.75(27.79)	de 23.75(29.15)	e 28.47(22.75)	d ef 24.75(29.80)
Fusarium verticillioides	100%	de 20.88(27.17)	e f g 20.75(27.08)	d 21.75(27.78)	de 23.75(29.15)	d 26.37(25.00)	cde 27.25(31.45)
	50%	d 22.50(28.27)	c d 24.00(29.29)	c 26.00(30.62	c 28.00(31.91)	d 31.93(28.00)	cd 28.25(32.09)
	33.3%	d 23.75(29.14)	c 24.75(29.79)	c 26.00(30.63)	c 28.00(31.92)	c 31.44(27.25)	c 30.25(33.35)
	25%	c 28.23(32.08)	d e 22.38(28.22)	c 23.75(29.15)	cd 25.75(30.48)	c 28.30(25.25)	c 30.50(33.51)
Sterile water		a 76.59(61.54)	a 84.03(66.63)	a 93.62(73.55)	a 100.00(89.88)	a 100.00(89.88)	a 100.00(89.88)
Plainbroth		b 72.31(55.87)	b 81.32(57.90)	b 89.63(65.70)	b 92.05(74.39)	b 95.43(76.79)	b 98.01(84.21)
CD (0.05)		(2.274)	(1.479)	(1.905)	(1.753)	(1.404)	(2.708)

Figures in the parenthesis are Angular transformed values
*Days

Conclusion

Trichoderma viride was found to be efficient in preventing egg hatching of *Meloidogyne incognita* from three to eight days following treatment using cell free extracts of isolates of *Trichoderma viride, Metarhizium anisopliae*, and *Fusarium verticillioides*. It may be concluded that a 100 percent concentration of *Trichoderma viride* was effective in increasing the mortality of *M. incognita* juveniles 24 hours, 48 hours, and 72 hours after treatment.

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