

**NEMATO-TOXIC POTENTIAL OF *TRICHODERMA HARZIANUM* AND
T. VIRIDE EXTRA-CELLULAR METABOLITES AND *ANNONA GLABRA*
CRUDE EXTRACTION ON *MELOIDOGYNE INCOGNITA* INFESTATION**

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Received 20th December 2020 / Accepted 18th March 2021

ABSTRACT

Root-knot nematode, *Meloidogyne incognita* (Kofoid and White) is one of the most destructive plant parasitic nematode species in almost all agriculture and horticulture crops in Sri Lanka. Biologically sound controlling measures against this nematode species has been attempted from long past with ambiguous efficacy. This paper highlights the nemato-toxic potential of crude leaf extract of *Annona glabra*, and extracellular metabolites of *Trichoderma harzianum* and *T. viride* on root-knot nematode species, *Meloidogyne incognita* infesting in spinach plants under semi-field conditions. Results revealed that nematode infested plants treated with *T. harzianum* extract showed a significantly higher plant growth together with reduced root galling compared to that of *T. viride* and *A. glabra* crude extract. Both *T. harzianum* and *T. viride* treatments significantly increased the root growth of the nematode infested plants compared to that of *A. glabra* crude extract. *Annona glabra* crude leaf extract at the rate of 125 g/L and fungus mycelium of *T. viride* and *T. harzianum* at the rate of 140 mg/L and 100 mg/L

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DOI: <http://doi.org/10.4038/josuk.v14i0.8028>



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respectively resulted the highest nemato-toxic potential against *M. incognita*. This study concludes positive effect of antagonists tested against *M. incognita* and suggests that activity of lytic enzymes of *T. harzianum* and *T. viride* has enhanced the nemato-toxic effect.

Keywords: biological control, lytic enzymes, root galls, soil amendments

INTRODUCTION

Meloidogyne incognita (Kofoid and White) Chitwood is a sedentary endoparasitic nematode species affecting many agricultural and horticultural crops (Sharon *et al.*, 2001). The second stage juveniles of this nematode penetrate the epidermal cells closer to the root tip of the host plant. Once invaded, they locate the conductive tissues of the roots and commence feeding. Physiological changes due to the nematode feeding cause enlargement of the feeding cells and disrupt the functioning of conductive tissues. Formation of root galls is the characteristic symptom of root-knot nematode infestation while the nematode damage leads to a retarded plant growth, wilting during dry weather and reduction of crop (Taylor and Sasser, 1978).

Synthetic nematicides play a major role in controlling plant parasitic nematodes. However, considerable negative impacts have been reported over their use. Therefore, as an alternative approach, several plant species and antagonistic fungi with strong nematicidal compounds have been assessed against root knot nematodes (Meyer *et al.*, 2000; Khalil *et al.*, 2012). It has been reported that crude leaf extract of *Annona glabra* (Family Annonaceae) contains Acetogenin, a high molecular weight compound having cytotoxic, anti-tumor, anti-parasitic, anti-fungal, anti-spasmodic, repellent, and insecticidal activities (Dang *et al.*, 2011; Moghadamtousi *et al.*, 2015; Alka *et al.*, 2017). In addition, previous studies also reported larvicidal effects *A. glabra* crude extract against mosquito species (Amarasinghe and Ranasinghe, 2017; Amarasinghe *et al.*,

2020). Antagonistic fungus of the genus *Trichoderma* is a soil borne, spore forming Ascomycetes. *Trichoderma* spp. are ubiquitous colonizers of cellulose materials and therefore, found wherever decaying plant material is available (Kubicek *et al.*, 2008). It has the ability to colonize root surfaces and the cortex of higher plants (Sharon *et al.*, 2001). Biological control ability of *Trichoderma* spp. contain multiple factors; they are rich in extra-cellular metabolites (Prasun *et al.*, 2012; Mironenka, *et al.*, 2020) and produce peptaibols, polyketides and terpenes (Kumar *et al.*, 2014). *Trichoderma harzianum* exhibits extracellular enzymatic activities that include lipolytic, amylolytic, pectinolytic, chitinolytic and cellulolytic activities whereas *T. viride* displays lipolytic, proteolytic, amylolytic and cellulolytic activities (Emma and Simeon, 2008). *Trichoderma harzianum* and *T. viride* have been experimentally used to reduce the effect of root-knot nematode, *Meloidogyne incognita* on different crop plants (Dababat *et al.*, 2006; Jegathambigai *et al.*, 2008; Siddiqui and Akhtar, 2009; Degenkolb, 2016, Khan *et al.*, 2020). *Trichoderma* spp. penetrate and gain nutrients from other soil fungi and are known to be antagonistic to other phyto-pathogenic microorganisms, hence indirectly improve the plant growth (Emma and Simeon, 2008; Sing *et al.*, 2012). Present study was conducted to determine the nemato-toxic activity of crude leaf extract of *Annona glabra*, an alien plant species in Sri Lanka, and two commonly available *Trichoderma* species namely *T. harzianum* and *T. viride* on root knot nematode, *Meloidogyne incognita* infesting spinach, *Basella alba* L (F. Basellaceae).

MATERIALS AND METHODS

Preparation of crude extract of *Annona glabra* plant leaves

Field collected (N 06° 58.904. E 079° 54.281), fresh mature leaves of *A. glabra* were washed well with running tap water, rinsed with deionized water and air dried for ten days until they become brittle. Dried leaves were crushed until become a powder using

an electric blender. Pulverized leaves, were subjected to soxhlet extraction using ethanol as the solvent. The thimble of the apparatus was filled with the powdered leaf sample while ethanol was added to the round bottom flask of the apparatus. Extraction was carried out for 3½ hours maintaining the temperature at 78 °C. The resulted solution with extracted compounds were subjected to rotary evaporation at 40 °C until become a semi solid. The resultant crude extract was air dried for several hours until become a solid state and stored at 6 °C. Crude extract, 250 mg, was dissolved in 10 mL of deionized water and used as the stock solution (250 mg/L). Concentrations of 70, 125 and 170 mg/L of the crude were prepared by diluting the stock 30%, 50%, and 70% respectively for experimental use.

Preparation of liquid cultures of *Trichoderma viride* and *T. harzianum*

Laboratory maintained cultures of *Trichoderma viride* and *T. harzianum* were used for the study. A loop-full of the culture (5 mm diameter) from each species was inoculated on Potato Dextrose Agar (PDA) plates (5 x 5 x 1.5 cm) (n = 6) under aseptic condition (Amarasinghe & Madurusinghe 2012). Culture plates were maintained at room temperature, 28 ± 2°C, for six days and identified using morphological and reproductive characteristics (Watanabe, 2002; Anonymous, 2006). Pure cultures were maintained on PDA medium and stored in the incubator at 10 °C. Fungal mycelium, of *T. viride* and *T. harzianum*, 20 mg each, were cut separately using an autoclaved cork-borer and was inoculated separately in fresh coconut water autoclaved at 15 psi (103 kPa) at 121 °C for 20 minutes, aseptically (Emerson and Mikunthan, 2015). Cultures were incubated for 30 days with occasional shaking at room temperature, 28 ± 2 °C. The liquid cultures were filtered through a muslin cloth and the mycelial mass was discarded. The culture filtrates were used as the fungal stock solutions (200 mg/L). Concentrations, 60, 100, and 140 mg/L (30%, 50%, and 70%) were prepared by adding deionized water to *Trichoderma* stock solution and used for experimentations.

Potting of Spinach, *Basella alba* plants and inoculation

Transparent polythene bags measuring 9 cm diameter and 15cm height were filled with 250 g of pre examined nematode free soil and served as a pot. Three sets each of 40 pots were maintained for the experiments. Four week old *Basella alba* plants with six opened leaves were transplanted singly in each pot. Plants were given recommended NPK fertilizer at the planting and, watering done daily. Mature adult females of *Meloidogyne incognita* with egg masses were collected from field infested spinach plants and transferred individually into the rhizosphere of all the potted plants. These pots were kept in a screen house in semi field conditions for two weeks prior to the treatments.

Bio assay with *A. glabra* crude extract and extracts of *Trichoderma*

Annona glabra crude extract, 10 mL each from each concentration (30%, 50% and 70%) was applied into the rhizosphere of randomly selected ten potted plants each, previously inoculated with nematodes. Untreated control pots (n=10) received 10 mL water (4 T x 10 R). This was repeated for *T. viride* and *T. harzianum*. Pots were arranged in a randomized block design. Pots arrangement was rotated weekly to avoid the edge effect. Shoot height (cm), stem diameter (cm) at the level between first and the second leaf and total number of leaves were recorded and watered every other day. After six weeks, plants were carefully uprooted and root system was washed in running tap water; root length (cm) and galling in each root system were recorded.

Data analysis

The data analysis was performed using MINITAB 14 version. One-way ANOVA was performed to test whether there was a significant difference among the concentrations of treatments at 95% confidence interval. Anderson-Darling test was performed to determine the normality of the observed data. Tukey's pairwise comparison

tests were carried out to determine whether there was a significant difference between test plants and controls.

RESULTS

Post treatment measurements, namely stem height, stem diameter, number of leaves and root length of treated and control plants infected with nematodes are given in Table 1. This shows that there is a significant difference in the number of leaves, stem height, and root length between treated and respective control plants ($P = 0.001$, $F = 19.04$; $P = 0.074$, $F = 3.4$; $P=0.001$ $F=16.40$ respectively) (Table 1).

Table 1: Post treatment measurements (Mean \pm SD) of the treated plants at 30%, 50% and 70% of fungal and herbal extracts compared to control plants

Treatment	Post treatment measurement	30%	50%	70%	Control
<i>A. glabra</i>	No. of leaves	8.66 \pm 0.00 ^b	11 \pm 2.00 ^{ab}	13.66 \pm 1.52 ^a	9.33 \pm 1.15 ^b
	Stem height	35.56 \pm 3.98 ^a	39.73 \pm 2.89 ^a	46.2 \pm 2.95 ^a	30.6 \pm 3.10 ^b
	Stem diameter	6.7 \pm 0.832 ^a	5.9 \pm 0.16 ^a	6.33 \pm 0.47 ^a	4.25 \pm 0.34 ^b
	Root length	11 \pm 0.70 ^{ab}	7 \pm 1.78 ^c	8.26 \pm 1.38 ^{bc}	14.1 \pm 1.30 ^a
<i>T. harzianum</i>	No. of leaves	11.33 \pm 0.57 ^a	14 \pm 2.00 ^a	14 \pm 1.00 ^a	7.66 \pm 0.57 ^b
	Stem height	36.83 \pm 3.38 ^c	46.56 \pm 1.45 ^b	59.33 \pm 4.60 ^a	32.06 \pm 2.94 ^c
	Stem diameter	5.66 \pm 0.34 ^a	5.62 \pm 0.84 ^a	6.44 \pm 0.52 ^a	5.33 \pm 0.36 ^b
	Root length	11.06 \pm 0.72 ^a	9.96 \pm 3.40 ^a	7.2 \pm 1.08 ^a	11.8 \pm 0.30 ^a
<i>T. viride</i>	No. of leaves	8.66 \pm 0.57 ^{ab}	8.66 \pm 0.57 ^{ab}	10 \pm 1.73 ^a	7 \pm 0.00 ^b
	Stem height	32.2 \pm 2.69 ^b	36.5 \pm 1.68 ^{ab}	42.2 \pm 3.24 ^a	32.5 \pm 1.87 ^b
	Stem diameter	5.86 \pm 0.44 ^a	6.47 \pm 0.15 ^a	6.24 \pm 0.51 ^a	4.31 \pm 0.48 ^b
	Root length	8.53 \pm 1.19 ^a	7.13 \pm 1.60 ^a	10.8 \pm 3.80 ^a	10.46 \pm 0.40 ^a

Mean values of the rows in a table described in results section with different superscript letters are significantly different from each other (One-way ANOVA, Tukey's pairwise test, $P \leq 0.05$)

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Results revealed that the stem height, root length and number of leaves in plants received 70% concentration of the treatments are significantly different from that of 30% and 50% concentrations. However, there was no any significant difference on mean stem circumference among three concentrations of the treatments.

Table 2 shows the root galls in treated plants and control plants. It shows that the root galls in treated plants using *A. glabra* has reduced significantly lower level while it is reached to zero in fungal extract. Plates 1-3 shows the gall formation in highest concentration of treatments, T1, T2, and T3 compared to control.

Table 2: Root galls (Mean \pm SD) caused by *Meloidogyne incognita* in treated plants with different concentrations compared to control plants

Treatment	Mean (\pm SD) number of galls			
	30%	50%	70%	Control
<i>A. glabra</i> crude	6.66 \pm 6.11	0	0	24.66 \pm 10.50
<i>T. harzianum</i>	0	0	0	12.33 \pm 4.16
<i>T. viride</i>	0	0	0	19.66 \pm 2.52 ^b

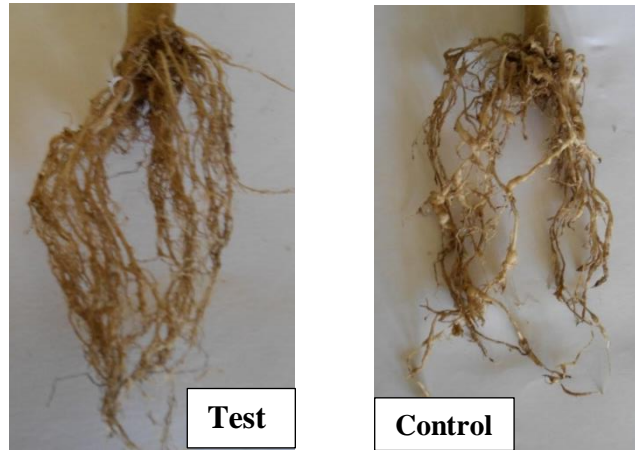


Plate 1: Root galls caused by *Meloidogyne incognita* on infested plants treated with *Annona glabra* extract compared to control

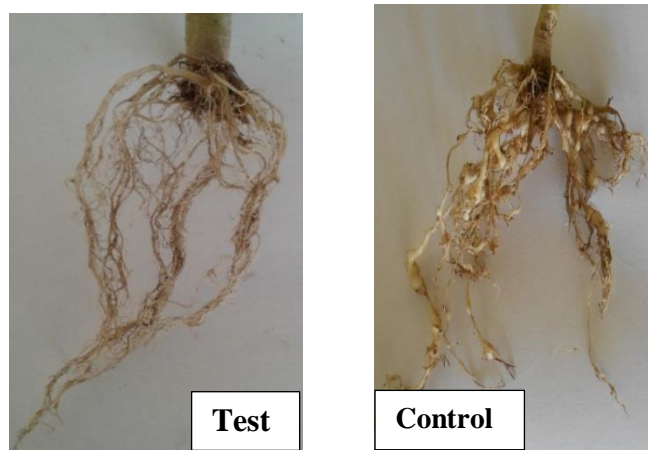


Plate 2: Root galls caused by *Meloidogyne incognita* on infested plants treated with *Trichoderma harzianum* compared to control plants

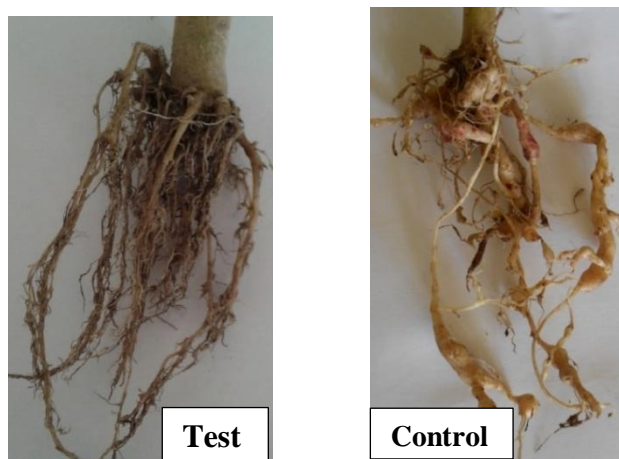


Plate 3: Root galls caused by *Meloidogyne incognita* on infested plants treated with *Trichoderma viride* compared to control plants

DISCUSSION

Meloidogyne incognita is one of the widely distributed and injurious among other root knot nematode species infesting agriculture and horticulture crops in Sri Lanka (Ekanayake and Toida, 1997). Even though synthetic nematicides to control this nematode species have been recommended, there are some negative effects of the use of them. Approaches to use biological methods to overcome the infestation of *Meloidogyne* spp. have been reported by many authors worldwide (Gupta and Sharma, 1993; Amarasinghe and Kariapperuma, 2007; Wiratno, *et al.*, 2009; Khali *et al.*, 2012; Khan *et al.*, 2019). Further, Oka *et al.*, (2000) suggested that the essential oils and their main components of aromatic plants cause immobilization of root knot nematodes, *Meloidogyne javanica* and inhibit their egg hatching, hence, serve as nematicides. Mani & Chitra, 1989 and Dang *et al.*, 2011 reported that petroleum ether extracts of *Annona squamosa* leaves and methanol extracts of *A. squamosa* seeds respectively, have nematicidal activity against *M. incognita*. The suppressive effects of some phytochemical compounds on nematode population has been documented in several studies (Chitwood, 2002; Desmedt, *et al.*, 2020). Such compounds may further develop to produce synthetic herbal nematicidal derivatives. Records on the use of *Annona glabra* extracts for their larvicidal activity against dengue vector mosquito species in the recent past (Amarasinghe and Ranasinghe, 2017). Therefore, present study was conducted for more economical and environmentally feasible, low cost method to control the root knot nematode, *Meloidogyne incognita*.

Meloidogyne incognita infested spinach plants treated with *A. glabra* crude extract indicated an antagonist effect of the extract against the nematodes *in situ*. Among the concentrations of *A. glabra* plant extract, 170 mg/L was more effective than the lower concentrations tested in this study. When the juveniles of root-knot nematode,

Meloidogyne incognita established their infestation, it adversely affected the shoot height and number of leaves of the host plant. This has been explained by Siddiqui and Akhtar, 2009 and Abd-Elgawad & Kabeil, 2010 showing that annihilation of water uptake interrupts the photosynthetic activities of the plant. Number of root galls directly reflect the damage that the nematodes cause to the host plant. Secondary metabolites that produce by antagonists potentially encounter toxic substances with relatively non-specific effects on a wide range of molecular targets. The targets are range from proteins such as receptors, enzymes, ion-channels and structural proteins, nucleic acids, bio membranes and other cellular components (Rattan, 2010). Rattan (2010) reviewed the mechanism of action of plant secondary metabolites on insect body and documented several physiological disruptions, such as inhibition of acetylcholine-esterase by essential oils, GABA-gated chlorine channel, sodium and potassium ion exchange disruption, and inhibition of cellular respiration. A secondary metabolite can cause the blockage of calcium channel nerve cell membrane action, mitotic poisoning, disruption of molecular events of morphogenesis and alteration in the behavior and memory of cholinergic system (essential oils), hormonal balance disruption etc. The most important activity is the inhibition of acetylcholine-esterase activity (AChE). These toxic compounds may enhance the toxicity of other toxic compounds (Rattan, 2010). However, the secondary metabolites are promising agents of nematicidal activity. There are several studies proven that the secondary metabolites act on root knot nematodes. It is reported that the two fungal isotes selected to test in this study, *T. harzianum* and *T. viride*, secrete the volatile toxic compounds (Degenkolb and Vilcinskas, 2016). Meyer *et al.* (2000) suggested that extracellular factors of *Trichoderma* species inhibit the egg hatch and second-stage juvenile (J2) mobility of *M. incognita*. Kerry, 1989, reported that some fungal isolates have improved the plant growth as well. *T. harzianum* and *T. viride* significantly reduced

the number of root galls compared to that of organic fertilizer (Jegathabigai, *et al.*, 2008). In the present study the effect of extracellular metabolites of two species of *Trichoderma* on nematode infestation was found to be more comparably effective. Therefore, use of 100 mg/L concentration of the fungal over 140 mg/L could be used against the *Meloidogyne incognita* nematode infestation.

CONCLUSIONS

Application of *Trichoderma viride* and *T. harzianum* extra-cellular metabolites enhanced the shoot growth of root knot nematode infested spinach plants and reduced the nematode infestation. Concentrations of 125 g/L of *Annona glabra* crude leaf extract, and 100 mg/L of *T. harzianum* and 140 mg/L of *T. viride* fungal extracts were the most suitable to act as nemato-toxic on *M. incognita*.

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