RESEARCH ARTICLE

Harnessing biocontrol potential of *Trichoderma harzianum* for control of *Meloidogyne incognita* in tomato

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ABSTRACT: The aim of the study was to evaluate the biocontrol potential of *Trichoderma harzianum* against *Meloidogyne incognita* and decipher mechanisms of induced systemic resistance and disease suppression in tomato grown in net house conditions. The fungal biocontrol agent *T. harzianum* UBSTH-501 was evaluated against *M. incognita* on dual plate under *in vitro* conditions and *in planta* under nethouse conditions. The results of *in vitro* parasitism on dual plates showed that *T. harzianum* causing infection on the eggs and juveniles of root-knot nematode, whereas, *in planta* assay showed that plants treated with talc based bioformulation *T. harzianum* UBSTH-501 exhibited manifold increase in the accumulation of total chlorophyll and enzymes, viz. chitinase, phenylalanine ammonia lyase (PAL) and peroxidase which is known to confer systemic resistance in tomato against *M. incognita* resulting into decreased nematode population and disease severity. Results revealed that *T. harzianum* UBSTH-501 modulated phenylpropanoid pathways led to enhanced accumulation of defence related mediator molecules and enzymes in tomato resulted in disease suppression to a significant extents.

Keywords: Chitinase, disease suppression, induced systemic resistance, PAL, peroxidase

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop grown in India after potato, and ranks first in terms of area and production. It is one of the largely grown vegetable crops throughout the world for consumption in various forms (Singh *et al.*, 2013a). In the protected and open field cultivation, several pests and pathogens attack to the crops, resulting severe yield losses. Among the category of difficult pests, plant parasitic nematodes (PPNs) are possibly most notorious ones (Walia and Bajaj, 2003). PPNs are capable of inciting diseases in a wide range of crop species, vegetables in particular, causing yield losses to considerable extents mainly in sub-tropical and tropical agro-climatic conditions (Sikora and Fernandez, 2005). Among various nematode species, southern root-knot nematodes (SRKN) *Meloidogyne incognita*, a sedentary endoparasite, is one of the most important plant parasitic nematodes causing root-knot disease in a wide range of vegetable crops of economic importance (Sahebani and Hadavi, 2008). Historically, management of nematode-induced crop damage has been achieved through the utilization of resistant cultivars, crop rotation with non-host crop plants and other agronomic practices, or through toxic chemical pesticides (Latha *et al.*, 2000; Pandey, 2011). Most of the chemical nematicides were

found to be ineffective over a period of time and development of resistance in the nematodes takes place (Chitwood, 2002). Also, most of the PPNs reside within plant roots and therefore, most often the location of the target nematode is fairly distant from the site of application of the chemical. Moreover, chemical control of nematodes is also not desirable and they may affect the agro-ecosystem and have detrimental effects on numerous beneficial flora and fauna native to agricultural soils (Walia and Bajaj, 2003; Sharma *et al.*, 2014). Further, development of nematode resistant cultivars is another important approach conferring sustainability to the crop production but periodical break-down of resistance in cultivars bred for the purpose has remained a great concern (Singh *et al.*, 2013a, b). Demand for alternatives to chemical control of PPN has become ever increasing due to rising concerns about environmental safety.

Trichoderma harzianum, avirulent plant symbiont, has been known and widely tested as potential biological control agents against a number of soilborne plant pathogens comprising of several species of fungi and bacteria (Harman *et al.*, 2004; Sharma *et al.*, 2014). The parasitism of PPNs especially SRKN by *T. harzianum* is a complex process and very few reports are available as compared to that by other soilborne fungal bioagents.

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However, the genus *Trichoderma* also has potential to control plant-parasitic nematodes (Sahebani and Hadavi, 2008; Yang *et al.*, 2010). Simultaneously, several reports have indicated that *T. harzianum* induced systemic resistance in the plant challenged with fungal and bacterial pathogens (Harman *et al.*, 2004; Singh *et al.*, 2016a, b). Few reports indicate the application of *T. harzianum* in the control of PPN, *M. incognita* in tomato is available so far. The biological relevance of *T. harzianum* in ISR signalling in Tomato–*M. incognita* interaction need to be explored thoroughly. Keeping in view the importance of *T. harzianum* and the SRKN in tomato, the present study was carried out with the objectives to evaluate the potential of *T. harzianum* against *M. incognita*, and also to decipher the mechanisms of induced systemic resistance in *M. incognita* challenged tomato plants treated with *T. harzianum*.

MATERIALS AND METHODS

Strains and culture conditions

The fungal biocontrol agent *Trichoderma harzianum* UBSTH-501 was taken from Plant-Microbe Interaction and Rhizosphere Biology Lab, ICAR-National Bureau of Agriculturally Important Microorganisms, Kushmaur, India. The culture was maintained on Potato dextrose agar (PDA) (HiMedia Pvt. Ltd.) at 25±1°C by sub-culturing at 15 days interval.

Extraction of egg, juveniles and adult nematodes from root galls

Nematode egg mass, juveniles and adults were extracted from infected roots and galls employing the methods (Singh *et al.*, 2013a) with slight modifications. Briefly, *Meloidogyne incognita* infected tomato plants were uprooted gently from infested farmers' field of Village Medhia in Mirzapur District of India (25°08'20.80N 82°51'55.44E, Elevation: 79m). Single egg mass was used to establish a population on susceptible tomato cultivar (cv. HS-101) for experiments. Plants grown in sterilized soil mixture were inoculated with single egg mass under controlled nethouse conditions. After 45 days of inoculation, infected parts of root and galls were separated from plants and gently washed under running tap water. Infected root parts and galls were cut with a sterilized sharp razor blade and placed on moist tissue paper mounted on cavity blocks containing 50 ppm streptomycin aqueous solution in sterilized distilled water. The egg masses were separated from root tissue, kept on moist tissue paper under aseptic conditions for further experiments. Further, few cavity blocks containing sliced gall tissues and egg masses were incubated for one week at 25±1°C. The second stage juveniles (J_2) came out from the egg mass and gall tissue in the water. These J_2 were further used for *in vitro* and *in planta* assay under nethouse conditions. The number of J_2 , adult males and females was measured with the help of nematode counting disc (HiMedia, India).

In vitro T. harzianum-M. incognita interaction

In vitro parasitism of eggs, J_2 , and adults were observed for seven days on PDA (1:10) at 25±2°C. Eggs, J_2 , and adult males (100 in each plate) were inoculated into Petridishes containing *T. harzianum* UBSTH-501 (48 h old) grown on PDA medium (1:10, pH 6.5). The percentages of parasitized eggs, J_2 and adult males were recorded after seven days of incubation from 10 microscopic fields (1.6 mm²) at 10 × magnification from the centre, middle and periphery of the fungal colony. The experiments were repeated thrice in five replications to assess the consistency of the results.

Plant material and growth conditions

The experiments were carried out with tomato cultivar HS-101, susceptible to *M. incognita* grown in a sterile potting mixture (sand, vermi-compost and soil in 1:1:2 ratio w/w) under nethouse conditions in 2014-15. Tomato seeds (cv. HS-101) were surface sterilized with 1% sodium hypochlorite (5 min) and sown in pots containing sterile potting mixture. Thirty days old seedlings (four leaf stage) were taken for further *in planta* assay. The experiments were conducted during October to December (October 15-December 15) with relative humidity 80-85% under 11/-13 h light/dark photoperiod.

Effect of *T. harzianum* on plant growth and *M. incognita* infection

The tomato seedlings (four-leaf stage) were uprooted from nursery, treated with *T. harzianum* UBSTH-501 formulation (Green Fungicide: Talc based bioformulation of *T. harzianum*) at 10⁶ colony forming unit/g by root dipping (10 min) and were transplanted in pots (20 × 20 cm²) containing sterile potting mixture (3 kg/pot). Gum acacia (HiMedia, India, 0.1%) was used as adhesive. Two seedlings were planted in each pot. After seven days of transplanting, seedlings were inoculated with 3000 J_2 per individual seedling at subsurface near tap root system in the pot. Tomato seedling inoculated with nematode without fungal bioagent served as positive control, whereas, uninoculated plants (neither pathogen nor fungal bioagent) served as negative control. Plants treated with carbofuran 3G at 2 kg a.i. ha⁻¹ (Walia and Bajaj, 2003) were taken as chemical control. Experiments were repeated twice in ten replications at 15 days interval under nethouse conditions and pool analysis was done.

Sampling and analysis

Plant growth promotion and disease development

Five plants were sampled from each treatment after 30 and 45 days of pathogen inoculation (DPI). Shoot and root biomass were recorded at 30 and 45 DPI, whereas, number of galls per plant, egg mass per plants, eggs per individual egg mass and J_2 per plants were evaluated after 45 DPI as per the method described by Bridge *et al.* (1981).

Estimation of total chlorophyll and defence related enzymes

Plant leaves were sampled from each treatment and total chlorophyll content was measured as per the methods described by Sadasivam and Manickam (1996), whereas, chitinase, phenylalanine ammonia lyase (PAL) and peroxidase activity were measured spectrophotometrically as per the procedures described by Thimmaiah (2012) after 30 DPI.

Statistical analysis

In vitro laboratory experiment were conducted in the layout of completely randomized design, whereas, nethouse experiments were setup in randomized block design. Laboratory experiments were repeated thrice with five replications. However, nethouse experiments were repeated twice with ten replications to assess the consistency in the results and the data were subjected to pool analysis and Duncan's Multiple Range Test (DMRT) using statistical package for Social Sciences Version 16.0 (SPSS 16.0) programme. The experimental data were compared with DMRT at $P < 0.05$.

RESULTS AND DISCUSSION

Plant parasitic nematodes especially root-knot nematodes (RKNs) are one of the most important and notorious pathogens causing severe economic losses in a number of crops. Many researchers supported non-chemical methods for controlling soil-borne plant pathogens including PPNs. Several nematode trapping fungi were found effective in controlling root-knot nematodes in tomato and wheat (Singh *et al.*, 2013a, b). The present study revealed the impact of *T. harzianum* on parasitism and killing of nematode eggs and juveniles and elicitation of chitinase, PAL and peroxidase in plant system and plant growth promotion under pathogenic stress conditions in tomato. The results of this investigation clearly showed the efficacy of *T. harzianum* UBSTH-501 in controlling SRKN and promoting plant growth directly or indirectly.

In vitro parasitism of eggs and juveniles

Trichoderma species are well known biocontrol agent and has potential to colonize and spread in the root, soil

and foliar environments and capable of suppressing phytopathogens (Sahebani and Hadavi, 2008; Singh *et al.*, 2016a, b). The results of *in vitro* parasitism on dual culture indicated that *T. harzianum* caused infection to the eggs and juveniles of root-knot nematode. Result showed that 69.32% eggs were parasitized by *T. harzianum* UBSTH-501, whereas, 56.25% adult males and 81.66% J_2 were infected and paralysed by the bioagent tested. Based on these results, *T. harzianum* UBSTH-501 appeared to be more aggressive to infect and parasitize J_2 as compared to eggs and adult males under *in vitro* laboratory conditions.

In vitro results showed that *T. harzianum* UBSTH-501 parasitized and killed the eggs and juveniles of the SRKN on dual plate. Similar observations were also reported by many researchers (Szabó *et al.*, 2012; Zhang *et al.*, 2014). It is well known that nematode egg shell and juvenile's epidermis are made up of chitin and its derivatives. Reports indicated that *Trichoderma* spp. utilize chitinolytic enzymes to disintegrate and degrade the pathogen's cell walls (Sahebani and Hadavi, 2008; Singh *et al.*, 2016a, b) and therefore *T. harzianum* is thought to be a rich source of chitinolytic enzymes which might degrade the eggshell during parasitism of eggs and juveniles (Szabó *et al.*, 2012; Sharma *et al.*, 2014).

Plant growth promotion and disease development

Direct effects of *T. harzianum* are associated with reduction in nematode population by parasitizing and killing them in rhizosphere and plant systems (Szabó *et al.*, 2012). Indirect mechanisms include modulation of host physio-biochemical pathways that trigger defence cascades inside the plant leading to suppression of penetration, invasion and development of relationship with plant system resulting into reduction in the rate of disease development that eventually translates into better plant growth. Results from the nethouse experiments showed significant effects of *T. harzianum* UBSTH-501 colonization on number of galls per root system, number of egg mass per root system, number of eggs/individual egg mass, and number of juveniles (J_2) per root system after 45 DPI under nethouse conditions (Table 1). Result revealed that significant reduction in the root gall development in the plant roots treated with *T. harzianum* UBSTH-501 as compared to *M. incognita*

Table 1. Effect of *T. harzianum* UBSTH-501 colonization on number of gall per root system, number of egg mass per root system, number of eggs/individual egg mass, and number of juveniles (J_2) per root system after 45 days of transplanting under nethouse conditions

Treatments	Parameters			
	No. of galls/ root system	No. of egg mass/ root system	No. of eggs/ individual egg mass	No. of J_2 / root system
<i>M. incognita</i>	71.26 ± 4.45 ^a	234.97 ± 5.56 ^a	339.25 ± 11.50 ^a	1898.46 ± 12.44 ^a
<i>M. incognita</i> + <i>T. harzianum</i> UBSTH-501	48.60 ± 3.29 ^b	176.49 ± 9.33 ^b	186.92 ± 5.50 ^b	1248.26 ± 13.75 ^b
<i>M. incognita</i> + Carbofuran 3G	21.96 ± 3.12 ^c	101.32 ± 6.33 ^c	126.23 ± 5.23 ^c	742.32 ± 9.25 ^c
Control	00 ± 00 ^d	00 ± 00 ^d	00 ± 00 ^d	00 ± 00 ^d

Data are mean ± SEM (n=5). Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test

Table 2. Effect of *Trichoderma harzianum* UBSTH-501 colonization on biomass production after 45 days of transplanting under nethouse conditions

Treatments	Plant biomass on fresh weight basis			
	Shoot biomass (g)		Root biomass (g)	
	30 days	45 days	30 days	45 days
<i>M. incognita</i>	15.45 ± 1.45 ^c	20.12 ± 2.12 ^c	8.96 ± 0.50 ^a	10.28 ± 1.33 ^a
<i>M. incognita</i> + <i>T. harzianum</i> UBSTH-501	25.97 ± 1.20 ^b	32.46 ± 2.35 ^b	6.12 ± 0.25 ^b	8.45 ± 0.75 ^b
<i>M. incognita</i> + Carbofuran 3G	29.91 ± 2.25 ^a	40.32 ± 2.66 ^a	5.05 ± 0.45 ^c	7.12 ± 0.66 ^c
Control	31.39 ± 2.33 ^a	42.56 ± 2.50 ^a	4.92 ± 0.33 ^c	6.67 ± 0.33 ^c

Data are mean ± SEM (n=5). Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test

Table 3. Effects of *T. harzianum* UBSTH-501 inoculation on activities and accumulation of total chlorophyll (mg g⁻¹ fresh wt.), chitinase [μ mole Glc NAc (mg prot)⁻¹ h⁻¹], phenylalanine ammonia lyase (mM cinnamic acid h⁻¹ g⁻¹ fresh wt.) and peroxidase activity (unit mg⁻¹ min⁻¹ fresh wt.) induced in tomato plants pre-challenged with *M. incognita* after 30 days of pathogen inoculation under nethouse conditions

Treatments	Total chlorophyll and defense related enzymes in plant leaves			
	Total chlorophyll	Chitinase	PAL	Peroxidase
<i>M. incognita</i>	50.14 ± 2.13 ^d	32.66 ± 1.15 ^b	35.65 ± 1.95 ^b	5.32 ± 0.50 ^b
<i>M. incognita</i> + <i>T. harzianum</i> UBSTH-501	69.32 ± 2.25 ^c	42.76 ± 1.33 ^a	54.32 ± 2.55 ^a	8.23 ± 0.75 ^a
<i>M. incognita</i> + Carbofuran 3G	86.25 ± 3.02 ^b	26.92 ± 1.25 ^c	16.32 ± 1.01 ^c	4.91 ± 0.54 ^b
Control	96.32 ± 2.50 ^a	14.6 ± 1.66 ^d	9.26 ± 0.75 ^d	2.67 ± 0.25 ^c

Data are mean ± SEM (n=5). Data with different letters show significant difference in column data in randomized block design test at $p < 0.05$ under Duncan's multiple range test

(alone) treated plants. The minimum gall per plant was recorded in the plants treated with carbofuran 3G. Similar trend was observed in case of number of egg mass per root system, number of eggs/individual egg mass, and number of juveniles (J_2) per root system after 45 DPI. In the present study, *T. harzianum* strain was found to parasitize the eggs and J_2 in the rhizosphere. Applications of *T. harzianum* UBSTH-501 restricted the secondary infection. This is in agreement with the findings of several other workers (Szabó *et al.*, 2012).

T. harzianum stimulated plant growth by producing plant growth hormones and other secondary metabolites that led to increase in root biomass through formation of adventitious roots and root hairs. *T. harzianum* mediated increased root density led to higher uptake and translocation of water and other mineral nutrients. Significant differences ($p < 0.05$) between treatments in nethouse experiments were also noted regarding accumulation of fresh biomass (Shoot and root). Results showed that plants treated with *T. harzianum* UBSTH-501 were found to have faster growth from day one to the last day of the study (Table 2). Results revealed that inoculation of tomato roots with *T. harzianum* UBSTH-501 significantly increased fresh weight of shoot as compared to *M. incognita*, carbofuran treated and control plants after 30 and 45 DPI, respectively. However, maximum root biomass was recorded in the plants inoculated only with *M. incognita* followed by *T. harzianum* UBSTH-501 inoculated and carbofuran treated plants, whereas, minimum biomass was recorded in control plants (Table 2). These results are in agreement with the

findings of several workers (Harman *et al.*, 2004; Singh *et al.*, 2016a, b).

Estimation of total chlorophyll and defence related enzymes

Results showed that inoculation of tomato roots with *T. harzianum* UBSTH-501 significantly increased total chlorophyll content, and chitinase activity as compared to the plants inoculated with the nematode (alone) and other treatments (Table 3). Maximum chlorophyll content was recorded in control plants (neither pathogen nor bioagent) followed by *T. harzianum* UBSTH-501 inoculated and carbofuran treated plants pre-challenged with *M. incognita* after 30 DPI. However, minimum chlorophyll content was recorded in *M. incognita* (alone) treated plants. The chitinase activity proved highest in plants treated with *T. harzianum* UBSTH-501 followed by *M. incognita* (alone) treated, carbofuran treated and control plants after 30 DPI. Similar pattern was recorded for PAL and peroxidase activity in the plants treated with *T. harzianum* UBSTH-501 compared to *M. incognita* treated plants after 30 days of pathogen inoculation (Table 3). Present findings clearly indicate that inoculation of *T. harzianum* UBSTH-501 induced various defence related activities like chitinase, PAL and peroxidase activity (Sahebani and Hadavi, 2008; Sharma *et al.*, 2014). *T. harzianum* reprogramming the mechanisms and cascades in tomato via phenylpropanoid pathways which further leads to lignin synthesis and accumulation of phytoalexins and other metabolites toxic to nematodes.

This may impart resistance in tomato plants against the invasion caused by *M. incognita*.

In brief, the present study describes mechanisms involved in the *T. harzianum* mediated disease control in *M. incognita*-tomato pathosystem. The application of *T. harzianum* revealed well-coordinated mode of action during nematode parasitism and expression of defence related mediator molecules/enzymes ultimately lead to reduction in the rate of disease development and improved plant growth under biotic stress condition.

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