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Effect of carrier media on population fluctuation of *Trichoderma harzianum* (MTCC5179) in black pepper (*Piper nigrum* L.) rhizosphere and their interaction with soil microflora and fauna

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ABSTRACT: ***Trichoderma harzianum*** **is used as a biocontrol agent to manage** ***Phytophthora*** **foot rot of black pepper (*Piper nigrum* L.) in India. Several organic materials such as sorghum, neem cake, decomposed coir pith and farmyard manure are used both as multiplication and carrier media for *T. harzianum*. The effect of these carrier media on the population build up of *T. harzianum* in black pepper rhizosphere and their effect on microflora and fauna was studied. The organic materials increased the population of introduced *T. harzianum* and also increased populations of fungi, bacteria, microarthropods and nematodes. In case of sorghum, there was a sudden spurt in the population of saprophytic nematodes and mycophagous mites within 15 days followed by a succession of predatory mites and nematodes. The unspent carbohydrate and mycelial form of *T.harzianum* in sorghum perhaps helped in the population build up of soil microarthropods and nematodes, which in turn affect the biocontrol efficiency of the introduced organism.**

KEY WORDS: Biocontrol, black pepper rhizosphere, carrier media, mycophagous mites, mycophagous nematodes, *Trichoderma harzianum*

INTRODUCTION

Black pepper (*Piper nigrum* L.) is one of the major spice crops of India; the mature dried berries are the black pepper of commerce. *Phytophthora* foot rot caused by *Phytophthora capsici* and slow decline due to feeder root damage by plant parasitic nematodes, *Meloidogyne incognita* and *Radopholus similis*, either alone or in combination with *P. capsici*, are a major threat to black pepper cultivation (Anandaraj, 2000; Ramana and Eapen, 2000). To reduce the use of hazardous chemicals against diseases, ecologically safer biological control is preferred as an important crop protection strategy in many crops (Cook and Baker, 1983).

Trichoderma spp. are free living fungi that are highly interactive in root, soil and foliar environments and used as biocontrol agents (Harman *et al.*, 2004). Their wide range of applications is due to the various antagonistic mechanisms found in different *Trichoderma* isolates, enabling them to function as potent biocontrol agents on many crops against a range of pathogens and in several ecological situations. Antagonistic fungi like *T. harzianum* (MTCC5179), *Paecilomyces lilacinus* and *Pochonia chlamydosporia* are reported to bring about

suppression of pathogenic fungi and nematodes affecting black pepper. *T. harzianum* is used to control *P. capsici* in black pepper cultivation (Anandaraj, 2000; Ramana and Eapen, 2000). There are several reports that *T. harzianum* isolates stimulate plant growth even in the apparent absence of pathogens and induce resistance (Harman *et al.*, 2004).

Biocontrol agents are formulated in the form of powder using talc as carrier medium by commercial firms. Several agricultural wastes / by-products such as tapioca rind and farmyard manure were found to be suitable media for mass culturing of *Trichoderma* (Kousalya and Jayarajan, 1990). *Trichoderma* spp. are also mass multiplied on inexpensive carrier media and applied in the field with promising results (Anandaraj and Sarma, 1997; Prakash *et al.*, 1999; Saju *et al.*, 2002).

When the organic wastes are added to the soil, several biotic and abiotic changes take place. The soil contains a rich and varied soil microflora and fauna including nematodes and microarthropods (Acarina and Collembola). Interaction among fungi, bacteria and microartropods are central to many processes in litter from decomposition to the functioning of rhizosphere (Ananthakrishnan, 1996, Curl *et al.*, 1988). The several hundred families of mites represented in soil include predatory species as well as detritus feeders and fungivores whereas Collembola are largely fungal feeders. Predation on nematodes has been observed in both groups (Curl and Truelove, 1986). In perennial crops such as black pepper, it is reported that continuous application of *T.harzianum* has not resulted in corresponding increase in soil population levels of *T. harzianum* (IISR2001). The utilization of added substrates by *T. harzianum*, other microflora and microfauna is poorly understood. The present study was, therefore, undertaken to investigate the population dynamics of *T.harzianum* in relation to fungi, bacteria, microarthropods and nematodes after introducing *T. harzianum* grown on different organic substrates.

MATERIALS AND METHODS

Mass multiplication of *T. harzianum*

The culture of *T.harzianum* (MTCC5179) was obtained from the Repository of Biocontrol Agents at Indian Institute of Spices Research (IISR), Calicut, India. The inoculum was prepared by growing *T. harzianum* in petri plates containing potato dextrose agar (PDA-pH adjusted to 6.0) and incubated at room temperature (28-30°C) for 48h. From this, a 5mm diameter disc was cut using a cork borer, inoculated to 50ml PDA medium in a 250ml conical flask, and incubated at room temperature for 96h. Conidial suspension of *T. harzianum* was prepared by adding 50ml sterile distilled water into the conical flask and the mycelial mat scraped out aseptically using an inoculation loop and filtered into a sterilized bottle through two layers of sterile cheesecloth. This procedure was repeated three times to extract maximum conidia from the medium. The volume of this spore suspension was made up to 300ml and the population was calculated in terms of colony forming units (CFUs) at 10^8 dilutions by plating on *Trichodema* selective medium (TSM) (Elad and Chet, 1983).

Different carrier media (250g) like sorghum, neem cake, decomposed one-year-old farmyard manure, and coir pith compost, talc and vermiculite were taken and moistened with distilled water. The moisture content of each carrier medium was estimated. These were taken in polypropylene bags and sterilized at 121°C for 30 minutes. Using a sterile syringe, 5ml spore suspension of *T.harzianum* containing 10^8 spores ml^{-1} was transferred aseptically to each of the carrier media, mixed thoroughly and incubated at 26°C for 15 days. *T. harzianum* multiplied on sorghum (10g) was added to 250g of talc and vermiculite with sorghum residue since they are inert materials and the population of *T.harzianum* in each carrier medium was estimated in TSM after 5 days of incubation.

Pot culture experiment

T. harzianum with different carrier media was delivered into the rhizosphere of two-year-old rooted black pepper laterals grown in 30cm earthern

pots. There were seven treatments as follows. T_1 - neem cake, T_2 – Sorghum, T_3 – Farmyard manure (FYM), T_4 – one-year-old coir pith compost, T_5 – talc, T_6 – vermiculite, T_7 – Control. For each treatment, four individual pots were maintained as replications. As per the treatment, 10g of 15-days old inoculum of *T. harzianum* grown on various carrier media was mixed thoroughly with 100g of respective sterile carrier medium, then applied to each pot by raking surface soil and mixed well and the pots were watered.

Enumeration of microflora

Natural populations of bacteria, fungi and *T.harzianum* were monitored from the rhizosphere of black pepper by collecting the soil adhering to feeder roots and serial dilution (1g of rhizosphere soil was mixed aseptically with 9ml sterile distilled water and serially diluted upto 10^{-6} dilution in 9ml sterile distilled water) followed by pour plating in different media, namely, nutrient agar, rose bengal agar (RBA) and TSM, respectively. Population was monitored at three intervals – before application of *T.harzianum*, 15 days and 30 days after treatment with *T.harzianum*.

Enumeration of microfauna

The populations of mites, Collembola, beetles, larvae of insects and nematodes (including saprophytes, predators and parasites) were monitored before application of *T. harzianum*, 15 days and 30 days after treatment with *T. harzianum*.

Enumeration of nematodes

Centrifugal floatation technique (Jenkins, 1964) was used for nematode isolation. The soil sample (100cc) was mixed with 600 ml water, then stirred for 20 seconds, and allowed to settle for 60 seconds. The supernatant was decanted through a 0.15mm mesh sieve, which was placed over a 38μm-mesh sieve at 35-40° angle during the decanting process to minimize the chance of small nematodes passing directly through the pores of the sieve. Using a wash bottle, the 0.15mm-mesh sieve was rinsed while still over 38-μm-mesh sieve. The debris and nematodes from the 38-μm-mesh sieve were washed into a 150ml beaker and centrifuged at 420g for 5 minutes. Then the supernatant was decanted as nematodes were in the soil pellet at the bottom of the tubes. The nematodes were separated from debris by centrifuging the soil pellet on sucrose solution (45.4%) cushion at 420g for 30 sec. The nematodes were suspended in sucrose solution while soil particles settled. The sucrose-nematode suspension was carefully decanted onto a 25-μm-mesh sieve and rinsed into a 150ml beaker. This was used for counting the total nematodes as predators, saprophytes and parasites under a stereomicroscope.

Enumeration of microarthropods

For the enumeration of microarthropods, the method developed by Berlese (1905) was adopted. The funnel, supporting mesh and sample container, was fabricated locally using tin sheets. It involves a gentle application of heat to drive the fauna from the sample into a funnel and then into a container placed under it. An ordinary electric light bulb (40w) was placed above the sample as heat source. The sample-collecting vessel was placed on gauze below and care was taken to leave a free area of gauze between samples and funnel to allow air to circulate thus avoiding condensation. The collecting vessels contained 30ml of 70% alcohol. The soil was added into the sample-collecting vessel and kept for 48h. The collecting fluid in the vessels was then used for estimating the population.

Data Analysis

Data on the population of total mites, Collembola, beetles, insect larvae and nematodes were counted as total number per 100cc of soil. The population of *Trichoderma*, bacteria and other fungi were estimated as number of colony forming units (CFUs) per gram of soil. The Statistical software MSTATC was used for analysis of variance (ANOVA) and the means were separated using Duncan's multiple range test (DMRT) at 5% level.

RESULTS AND DISCUSSION

Mass multiplication of *T. harzianum* in various carrier media

Population of *T. harzianum* in the conidial suspension was $84x10^8$ CFUsml^{-1}. *T. harzianum* inoculated on various media grew within 10 days and colonized the entire carrier media, except in talc and vermiculite by forming a greenish coloration. Among the carrier media, maximum number of CFUs was observed in sorghum ($18.4x10^8$), followed by neem cake ($16x10^8$), coir compost ($78x10^7$), farmyard manure ($36x10^7$), vermiculite ($12x10^7$) and talc ($36x10^6$).

Microflora

In the present study, introduction of *T. harzianum* along with organic and inert carrier media in black pepper soil resulted in an immediate increase in the population of *T.harzianum* in all the treatments within 15 days. However, *T. harzianum* populations declined sharply in treatments where neem cake, FYM, talc, vermiculite and coir pith compost were used as carrier media. The only exception was with sorghum, which retained higher population of *T. harzianum* after 30 days. At the same time, there was no significant increase or decrease in the native population of *T.harzianum* in the control (Table 1). Hence, it is evident that the addition *T. harzianum* with carrier media helped to increase the population of *T. harzianum*.

After introducing *T. harzianum* grown on different substrates, the population of other fungi like *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Mucor* sp. and *Rhizopus* sp. also increased after 15 days. The maximum CFUs were observed in sorghum followed by neem cake. Population in treatments with talc, vermiculite and coir compost also significantly increased compared to control. However, the total population of other fungi was significantly less than *T.harzianum* in all the treatments. No reduction in population of total fungi was observed after 30 days (Table 1).

After introducing *T. harzianum* into black pepper rhizosphere along with various substrates, the population of soil bacteria also increased after 15 days. The maximum number of CFUs was noticed in treatment with sorghum followed by neem cake and coir compost. The increase in population was maintained even after 30 days of treatment (Table 1). Chung and Hoitink (1990) reported that these types of organic and inert materials affect the soil microbes, bacterial/fungal populations. Therefore, after application of *T. harzianum* along with the above organic and inert carrier media into the soil, the resultant interactions between microflora and fauna are very important since they affect the population dynamics of *T. harzianum* and consequently, the consistency of control obtained.

Microfauna

The carrier media also affected the microfauna of the rhizosphere. Populations of mites, Collembola, beetles, larvae of insects and saprophytic, parasitic, predacious nematodes were monitored from each treatment. Population of saprophytic nematodes increased in the treatment with sorghum after 15 days and declined after 30 days. However, it recorded highest population at both the sampling intervals (Table 2). There was also an increase in predatory nematode population after 30 days and the increase was significant in case of sorghum (Fig. 1). On the other hand, parasitic nematodes declined to zero after 30 days in all the treatments, suggesting that fungal biocontrol agents enhance general activity of saprophytic and predatory nematodes and reduce parasitic nematodes. Sudden increase in mycophagous nematodes and other microbivorous nematodes has been observed in potato fields, consequent to incorporation of organic food sources (Hoffman and Jacob, 1989). The sudden increase in non-parasitic nematodes in this experiment may be due to unspent starch residues in sorghum and also due to the mycelial phase of *Trichoderma* present in sorghum. *Trichoderma* level in the soil has shown a declining trend 15 days after the treatment, probably due to mycophagy by large number of nematodes. Bae and Knudsen (2001) reported that fungivorous nematodes such as *Aphelenchoides* spp. and *Aphelenchus* spp. were a significant biotic constraint on the activity of *T. harzianum* in the field. The longevity of free-living nematodes in soil varies with temperature and nutrition (Gems, 2000). The decline in nematode population after 30 days may be due to the reduction in *Trichoderma* mycelium.

Table 1. Population fluctuation of microflora after introducing *Trichoderma harzianum* with various carrier media

Treatment	*T.harzianum*-Colony forming units*			Other fungi- Colony forming units*			Bacteria- Colony forming units*		
	Before application	15 DAT	30 DAT	Before application	15 DAT	30 DAT	Before application	15 DAT	30 DAT
Neem cake	$2x10^3$a	$80.7x10^4$b	$62.7x10^3$b	$12x10^3$a	$63.2x10^4$b	$83.0x10^3$b	$55x10^5$c	$65.5x10^6$ab	$59.7x\ 0^6$b
Sorghum	$1x10^3$b	$82.0x10^6$a	$60.7x10^6$a	$10x10^3$a	$87.2x10^5$a	$79.7x10^5$a	$34x10^5$d	$77.0x10^6$a	$79.7x10^6$a
FYM	$1x10^3$ b	$27.5x10^4$cd	$5.5x10^3$cd	$5x10^3$b	$16.2x10^3$de	$16.7x10^3$de	$61x10^5$b	$46.2x10^6$b	$37.5x10^6$c
Coir pith compost	$2x10^3$a	$40.7x10^4$c	$10.5x10^3$c	$5x10^3$b	$58.0x10^3$c	$40.5x10^3$c	$51x10^5$c	$64.5x10^6$a	$64.2x10^6$ab
Talc	$1x10^3$b	$22.2x10^4$d	$6.2x10^3$cd	$6x10^3$b	$19.7x10^3$d	$23.5x10^3$d	$50x10^5$c	$28.5x10^6$c	$33.2x10^6$c
Vermiculite	$1x10^3$b	$28.0x10^4$cd	$5.7x10^3$cd	$9x10^3$a	$38.0x10^3$c	$31.2x10^3$c	$80x10^5$a	$16.7x10^6$d	$34.2x10^6$c
Control	$2x10^3$a	$3.2x10^3$e	$4.0x10^3$e	$4x10^3$c	$12.5x10^3$e	$14.7x10^3$c	$50x10^5$c	$57.5x10^5$e	$56.7x10^5$d

* Mean of 4 replicates; DAT = Days of treatment; Values followed by the same letter(s) in a column do not differ significantly at 5% level by DMRT

Table 2. Population fluctuation of microfauna after introducing *Trichoderma harzianum* with various carrier media

Treatments	Population of saprophytic nematodes*			Population of mites*		
	Before application	15 DAT	30 DAT	Before application	15 DAT	30 DAT
Neem cake	9.5^{bc}	14.5^{b}	18.0^{abc}	17.0^{b}	52.25^{b}	71.5^{c}
Sorghum	10.5^{bc}	2337.0^{a}	24.5^{a}	19.0^{b}	19830.0^{a}	471.8^{a}
FYM	5.0^{c}	8.5^{b}	4.7^{cd}	33.5^{b}	75.0^{b}	46.0^{c}
Coir pith compost	81.0^{a}	8.75^{b}	4.25^{d}	32.5^{b}	327.0^{b}	175.5^{b}
Talc	12.5^{bc}	5.0^{b}	7.0^{cd}	32.0^{b}	34.5^{b}	2.25^{c}
Vermiculite	18.0^{bc}	10.25^{b}	9.0^{bcd}	30.5^{b}	149.0^{b}	30.0^{c}
Control	44.5^{ab}	11.0^{b}	22.0^{ab}	97.0^{a}	73.0^{b}	73.75^{c}

* Mean of 4 replicates; DAT = Days of treatment; Values followed by the same letter(s) in a column do not differ significantly at 5% level by DMRT

The population structure of mites in various treatments showed that there was a numerical increase in all the treatments except for talc after 15 days. In treatment with sorghum, there was a sudden increase in mite population (Table 2). In many of the field applications of *T. harzianum* with sorghum as carrier media, it has been observed that there was no appreciable increase in *T. harzianum* population despite continuous application (IISR 2001) and the population declined sharply over a period of time. Hyphal growth rate is the central adaptation of fungi that determines their biology. It is not certain whether this decline is associated with the increase in the number of mites and consequent predation of mycelia. Mycophagous mites have been reported to selectively feed on the mycelium and carry conidia on their body or on mycangiums, which disperse the spores. Although the identity of these mites needs confirmation, the spurt in the increase of population in logarithmic proportion within 15 days indicates that the unspent carbohydrates present in sorghum medium besides the mycelium of *T.harzianum* must have supported such an increase in population. Substrate quality is the major factor, which determines the rate of litter decomposition, diversity of the microbes associated with litter, composition of mycoflora, and microarthropods such as mites, Collembola and thrips. Various fungal species affect the reproductive success of oribatids indicating the nutritional quality of the fungal material, which may affect the overall density and structure of mite community. Some oribatid mites show fungal feeding preferences, selective feeding tends to alter the composition of microbial community by allowing the less palatable species to colonise more rapidly. Different species of fungi such as *Trichoderma, Phoma, Penicillium and Paecilomyces* have been noticed in the midgut of mites (Ananthakrishnan, 1996). There was also a succession of predatory mites and predatory nematodes within 30 days of imposition of treatment and a sudden decrease in mycophagous mites and saprophytic nematodes. In general, mites have a short generation time at proper temperature and nutrition. The pattern of egg laying frequency varies among species and ranges between a few hours. For instance, at the moderate temperature of 25°C the nematode-predatory pachylaelapid, *Zygoseius furcifer* develops from newly laid egg to adult female in

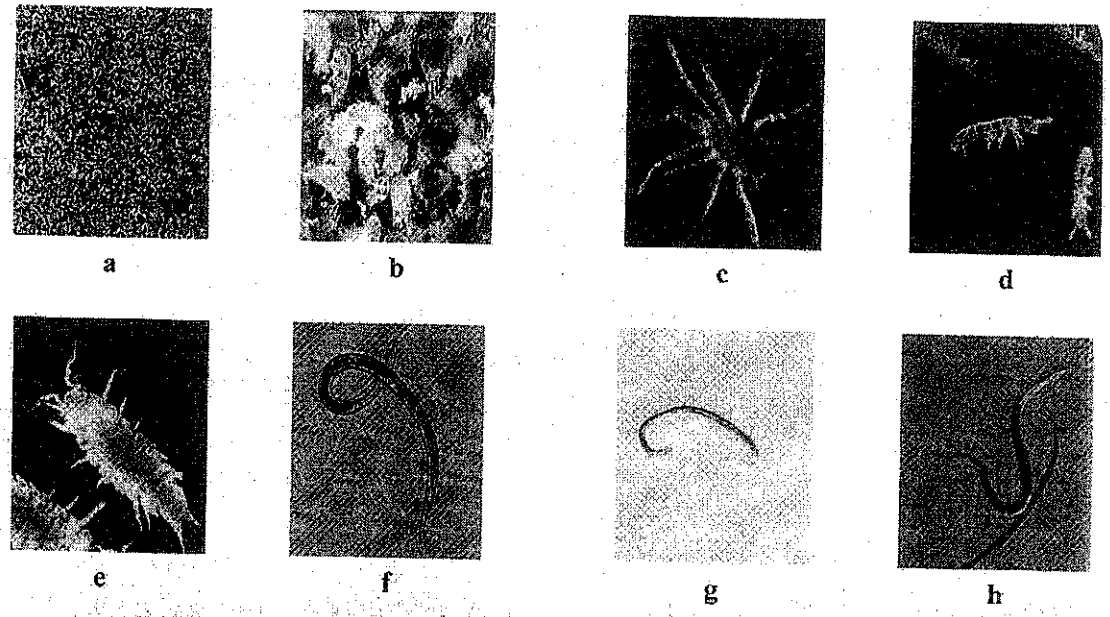

Fig. 2. Population of microfauna in rhizosphere soil of black pepper after application of *Trichoderma harzianum* with various carrier media (a. population density of mites in 100cc of soil treated with sorghum; b. enlarged view *of T. harzianum* growth; c. predatory mite; d. collembolan; e. crustacean larvae; f. plant parasitic nematode, *Radopholus similis*; g. saprophytic nematode; h. predatory nematode)

108±4 hours. On the other hand, minimum developmental time of *Macrochelis* species is 34-51 h at 30°C (Walter and Proctor 1999). Hence, the logarithmic increase of mite population in the treatment with sorghum may be due to the availability of nutrition in the form of *Trichoderma* mycelium and unspent starch. There was no significant difference in the population of Collembola, larvae of insects and beetles.

The influence of soil environment on the biological control of *Phytophthora* foot rot of black pepper with *T.harzianum* is poorly understood. This study has indicated the complexities of population of various groups of organisms in soil in response to addition of various organic matters. A thorough knowledge of soil ecology would be essential for application of biocontrol as a viable option for the management of soil borne diseases. The introduced biocontrol agent faces the twin challenges of establishing itself in the rhizosphere of black pepper and survival in the new ecological niche with increase in microfauna when there is organic base and fungal mycelium. It is prudent to use a formulation where the unspent organic matter is minimum and the fungus is present in the conidial or chlamydospore form, as these spores have better ability to tide over adverse conditions.

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