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Effect of culture filtrates of *Pseudomonas striata*, *Trichoderma harzianum*, *T. viride* and *Aspergillus awamori* on egg hatch of *Meloidogyne javanica*

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Abstract. The root-knot nematode, *Meloidogyne javanica*, is an important parasite of field crops in the tropics. To develop eco-friendly management options for this nematode, culture filtrates of *Pseudomonas striata* (strain 303), *Aspergillus awamori*, *Trichoderma harzianum* OPT NAB and *T. viride* were screened for their effects on egg-hatch in laboratory experiments. Five egg masses of *M. javanica* approximately the same size and colour were transferred in 5 ml of culture filtrates in 2.5-cm dia. Petri dishes and incubated at 25°C. Filtrates of non-inoculated nutrient broth, potato dextrose broth and distilled water were used as negative controls. The number of hatched second-stage juveniles in 5 replications for each treatment was determined after 7 and 14 days. Results suggest that culture filtrates of *P. striata* strain 303 was the most effective in reducing egg hatch of *M. javanica* by up to 95% compared with controls, whereas culture filtrates from *A. awamori*, *T. harzianum* and *T. viride* reduced egg hatch by 13 to 69%. The inhibitory effect on egg hatch of the organisms tested was reversible, except of *P. striata* strain 303, by transferring the eggs to distilled water. The results indicated that culture filtrate of *P. striata* 303 is lethal for *M. javanica* eggs and may be considered in a nematode management strategy.

Keywords. Biocontrol, egg-hatch, *Meloidogyne javanica*, *Pseudomonas striata*, Root-knot nematode, *Trichoderma* spp.

INTRODUCTION

Root-knot nematode, *Meloidogyne javanica*, is one of the most common and important plant parasites in tropical and subtropical regions of the world (Taylor & Sasser, 1978; Sasser *et al.*, 1983). Its infection causes root-knots (galls) in many crops and impedes normal flow of nutrients and water to the above ground parts of the plants. The infection starts with root penetration by second-stage juveniles (J_2). In India, *M. javanica* is a major agricultural pest causing crop losses ranging between 27 to 90% (Ali, 1997). At present, control of root-knot nematodes relies mainly on crop rotations and chemical pesticides; however, the latter are toxic compounds and their use is detrimental to the environment. Several options are currently being assessed around the world to identify and develop ecologically sustainable management options for controlling nematode damage to plants.

Application of microorganisms antagonistic to *Meloidogyne* spp., or compounds produced by these microbes, could provide additional opportunities for managing the damage caused by root-knot nematodes. Research in this area has resulted in commercial biocontrol preparations reported to act against root-knot nematodes (Stirling, 1991; Fravel, 2000). During the last decade, research on nematode management was focused on proposing strategies for inhibition of egg hatch (Westcott & Kluepfel, 1993), degradation of the hatching factor (Oostendorp & Sikora, 1989) or production of metabolites toxic to juvenile nematodes (Meadows *et al.*, 1989). Number of fungi isolated from nematodes, soil and plants were proved to produce substances that inhibit hatching of eggs or

kill nematodes (Nitao *et al.*, 1999). Some of these fungi produced toxic metabolites in culture filtrates (Khan & Saxena, 1997; Siddiqi *et al.*, 2000).

The objective of this study was to evaluate the effects of culture filtrates of two species of *Trichoderma* and one species each of *Aspergillus* and *Pseudomonas* on the egg hatch of *M. javanica*. These microorganisms were used as amendments to add value to compost prepared from rice-straw in another study (Rupela *et al.*, 1998). *Pseudomonas striata* strain 303 and *Aspergillus awamori* were selected for solubilizing rock-phosphate, *T. harzianum* OPT NAB for rapid degradation of rice-straw, and *T. viride* for suppressing soil-borne disease of crop plants such as Fusarium wilt. An abstract of this paper has been published (Sharma *et al.*, 1998).

MATERIALS AND METHODS

Egg masses of *M. javanica*

Nematode egg masses were collected from *M. javanica* cultures maintained on susceptible chickpea (*Cicer arietinum* L.) cv. K 850 plants at ICRISAT, Patancheru, Andhra Pradesh, India. Roots of the infected plants were washed under tap water and egg masses were carefully extracted with forceps under a binocular microscope. The egg masses were surface disinfected in streptomycin sulphate (0.1%) for 45 min (Sawhney & Webster, 1975) followed by rinsing several times with sterilized distilled water before use in experiments.

Bacterial and fungal filtrates

Pseudomonas striata strain 303 was obtained from the National Bio-fertilizer Development Corporation (NBDC), Gaziabad, Uttar Pradesh, India. The fungi used in this study were *A. awamori* obtained from Dr. K. K. Kapoor at Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India, *T. harzianum* OPT NAB obtained from Dr. V. C. Cuevas, University of Los Baños, Philippines and *T. viride* from Nagarjuna Agricultural Research and Development Institute, Secunderabad, Andhra Pradesh, India. The bacterium was grown in nutrient broth (Difco Laboratories) at 28°C on a rotary shaker (150-rpm) for 24 h. The fungi were grown on potato dextrose broth prepared as per Johnston & Booth (1983) and incubated as static culture at 28°C for 72-96 h. Broth cultures were centrifuged at 10,000 rpm for 15 min., supernatent collected and passed through a 0.45µm Millipore filter and the resulting culture filtrates were stored at 13°C until used, generally within a week. Three concentrations, 20, 50 and 100%, of bacterial and fungal filtrates were made with sterilized distilled water.

Effect on egg hatch

Five ml of culture filtrates of *P. striata* strain 303, *A. awamori*, *T. harzianum*, and *T. viride* were transferred into 2.5-cm dia Petri dishes. Distilled water and the different media (pre-sterilized) were used as controls. It was also an important control to determine if any of the bacterial and fungal filtrates would stimulate hatch. Five randomly picked *M. javanica* egg masses of approximately the same size and colour were transferred to the Petri dishes, which were then sealed with parafilm and incubated at 25°C. Five replicates (Petri dishes) for each concentration were used. The Petri dishes were observed periodically for emergence of J_2 from egg masses. The number of J_2 emerged after 7 days in each replication were counted under a binocular microscope. The unhatched egg masses were transferred in to new Petri dishes containing five ml of fresh filtrates. The Petri dishes were again sealed and incubated for seven more days. At the end of seven days of incubation, the number of emerged J_2 was counted and percentage of cumulative hatch was calculated. The unhatched eggs were counted after dissolving the egg masses of each treatment in sodium hypochlorite (0.5%, 1 min.).

To determine the inhibitory effects and viability of eggs treated with culture filtrates after 14 days, eggs from the different treatments were washed thoroughly with distilled water and transferred to 2.5-cm dia. Petri dishes containing 5 ml of distilled water. The Petri dishes were then sealed with parafilm and incubated at 25°C. After three days, J_2 emergence was counted and percentage egg hatch calculated.

Analysis of variance was used to compare the mean % egg hatch in the different culture filtrates based on the arcsine-transformed data. This analysis was followed by the Duncan's Multiple Range Test to establish the differences between the treatments (SAS Institute, Cary, NC, USA).

RESULTS

Culture filtrates of *P. striata* strain 303, *A. awamori*, *T. harzianum* and *T. viride* significantly ($P < 0.001$) reduced the egg hatch of *M. javanica*. The duration of exposure and concentration of culture filtrate had a direct effect on reduction of egg hatch. Among the tested microorganisms, the culture filtrate of *P. striata* strain 303 was toxic to the nematode eggs as eggs in the treated egg masses did not hatch even after transfer to distilled water (Tables 1 and 2). Analysis of cumulative hatch revealed that the reduction in hatching at all concentration of *P. striata* strain 303 was significantly ($P = 0.05$) different from other treatments.

The culture filtrates of fungal species reduced the egg hatch (Table 1) as compared with controls. The eggs, however, were not killed by the culture filtrates as they hatched readily when transferred into distilled water (Table 2).

Computing the percent inhibition over controls, the highest inhibition (95%) was observed in *P. striata* strain 303 at 100% concentration and the lowest from the same filtrate (85%) at 20% concentration (Table 2). A high proportion of these remaining eggs appeared normal in

Table 1. Effect of different concentrations of culture filtrates of microorganisms on hatching of second-stage juveniles from egg masses of *Meloidogyne javanica*.

Microorganisms	% concentration	% Juveniles hatched in filtrates*		
		week 1	week 2	Cumulative hatch**
Aspergillus awamori	20	63.0 ± 2.0 [b***]	24.3 ± 2.2 [bcd]	87.2 ± 1.5 [b]
	50	45.4 ± 4.0 [c]	21.0 ± 2.3 [de]	66.3 ± 3.5 [d]
	100	31.3 ± 3.0 [de]	17.4 ± 1.8 [def]	48.7 ± 2.2 [f]
Pseudomonas striata	20	10.0 ± 1.2 [fg]	4.8 ± 0.8 [g]	14.8 ± 1.7 [h]
(strain 303)	50	6.5 ± 1.2 [g]	3.3 ± 0.8 [g]	9.8 ± 1.7 [hi]
	100	3.5 ± 0.7 g	1.2 ± 0.3 [g]	4.7 ± 0.9 [i]
Trichoderma harzianum	20	55.8 ± 4.6 [b]	28.3 ± 4.0 [bc]	84.4 ± 2.1 [b]
	50	38.6 ± 4.7 [cd]	20.5 ± 3.8 [de]	59.0 ± 2.5 [e]
	100	18.9 ± 3.9 [f]	12.4 ± 1.3 [f]	31.2 ± 3.5 [g]
T. viride	20	41.7 ± 2.7 [c]	36.8 ± 3.0 [a]	78.5 ± 2.8 [c]
	50	29.0 ± 2.6 [e]	31.3 ± 3.0 [ab]	60.3 ± 3.4 [e]
	100	18.0 ± 2.0 [f]	15.6 ± 1.8 [ef]	33.6 ± 2.9 [g]
Controls				
Filtrates (NB, 100%)	-	77.2 ± 1.9 [a]	22.7 ± 1.8 [cde]	99.9 [a]
Filtrates (PDB, 100%)	-	71.3 ± 3.0 [a]	28.7 ± 3.6 [bc]	100 [a]
Distilled water	-	77.3 ± 2.3 [a]	22.6 ± 2.3 [cde]	99.9 [a]

NB = nutrient broth; PDB = potato dextrose broth (culture medium); con. = concentration
* = The culture filtrates were replaced by fresh ones after one week.
** = Cumulative percentage hatch = egg hatch in week 1 + week 2.
*** = Values are means of 15 replicates, with 5 egg masses per replicate. Means followed by the same letter (s) in columns are not significantly different at $P = 0.05$ according to Duncan's Multiple Range Test.

shape. The general pattern of eggs hatch reduction was similar when the experiment was repeated. Hatch in distilled water and culture media were 100%, and majority of the eggs in distilled water and filtrates of culture media hatched after 14 days of incubation. There was no significant difference found in control treatments.

DISCUSSION

Culture filtrate of *P. striata* strain 303 significantly inhibited egg hatch of *M. javanica* within a short period of exposure. This is the first report on inhibitory effect of culture filtrate of *P. striata* strain 303 against *M. javanica*. Although, bacterial suspensions of other *Pseudomonas* spp. have been reported antagonistic against *M. incognita* (Becker *et al.*, 1988). All the fungi included in these tests inhibited egg hatch (by 4 to 8%) but this activity was markedly lower from the proportion of egg hatch inhibited by *P. striata* strain 303 (85 to 95%). Our experiments did not demonstrate the mechanism by which *P. striata* strain 303 inhibiting hatch of *M. javanica* eggs. Possible mechanisms of inhibition of *M. javanica* eggs include production of toxic metabolites in culture broth of bacteria (Samaliev *et al.*, 2000). Previous research (Prasad *et al.*, 1972) indicates that the *Bacillus thuringiensis* appears to produce a number of exotoxins that kill eggs of *Meloidogyne* spp. In our tests,

Table 2. Inhibitory effects of microorganisms on hatching of second-stage juveniles from eggs of *Meloidogyne javanica*

Microorganisms	% concentration	% inhibition* over control	Hatching** resumed in water	Total*** % hatch
Aspergillus awamori	20	12.7 ± 1.5 ^{h****}	8.4 ± 1.2 ^{e}	95.6 ± 0.7 ^{bc}
	50	33.6 ± 3.5 ^{f}	29.9 ± 3.2 ^{c}	96.3 ± 0.5 ^{bc}
	100	51.3 ± 2.2 ^{e}	47.4 ± 2.1 ^{b}	96.2 ± 0.5 ^{bc}
Pseudomonas striata	20	85.2 ±1.7 ^{c}	0 ^{f}	14.8 ± 1.7 ^{e}
(strain 303)	50	90.2 ± 1.7 ^{b}	0 ^{f}	9.8 ± 1.7 ^{ef}
	100	95.3 ± 0.8 ^{a}	0 ^{f}	4.7 ± 0.9 ^{f}
Trichoderma harzianum	20	15.6 ± 2.1 ^{gh}	11.9 ± 1.8 ^{de}	96.4 ± 0.6 ^{b}
	50	41.1 ± 2.5 ^{f}	36.7 ± 2.7 ^{c}	95.8 ± 0.7 ^{bc}
	100	68.7 ± 3.5 ^{d}	62.7 ± 3.6 ^{a}	93.8 ± 1.2 ^{cd}
T. viride	20	21.6 ± 2.8 ^{g}	15.6 ± 2.9 ^{d}	94.1 ± 0.9 ^{cd}
	50	39.8 ± 3.4 ^{f}	32.9 ± 3.4 ^{c}	93.0 ± 0.9 ^{d}
	100	66.3 ± 2.9 ^{d}	58.3 ± 3.4 ^{a}	91.9 ± 1.6 ^{d}
Controls				
Filtrates (NB, 100%)	-	0 ^{i}	0 ^{f}	100 ^{a}
Filtrates (PDB, 100%)	-	0 ^{i}	0 ^{f}	100 ^{a}
Distilled water	-	0 ^{i}	0 ^{f}	100 ^{a}

NB = nutrient broth; PDB = potato dextrose broth (culture medium).
* = Percent inhibition over control = 100 × (hatch in control – hatch in filtrates) / hatch in control.
** = After 14 days hatching in culture filtrates, the eggs were transferred in distilled water.
*** = Total % hatch = 100 × (egg hatch in culture filtrates and water) / total eggs
**** = Values are means of 15 replicates, with eggs from 5 egg masses per replicate. Means followed by the same letter (s) in columns are not significantly different at $P = 0.05$ according to Duncan's Multiple Range Test.

no evidence of eggshell rupture or change in the shape of eggs was observed. However, change in permeability could occur without dramatic changes in egg shape, as has been documented for exotoxins produced by *B. thuringiensis* (Bone *et al.*, 1987). This potential mechanism of action requires further study for *P. striata* strain 303.

Our results showed that *A. awamori*, *T. harzianum* and *T. viride* in culture filtrates containing their metabolites reduced egg hatch of *M. javanica* (range 12.7 to 66.3%) as the concentration of filtrates was increased from 20 to 100%, although, hatching resumed when the eggs were transferred in to distilled water. The non-toxic activity of culture filtrates of fungi in this study, however, does not agree with the previous reports, in which culture filtrates of these fungi reduced egg hatching of *M. javanica* (Khan & Saxena, 1997; Siddiqi *et al.*, 2001). There could be many possible reasons for this, including differences in fungal isolates, nematode species, culture media, and experimental conditions. Studies by Cayrol *et al.* (1989) and Roberts & Lumsden (1990) demonstrated that culture medium, aeration and pH affected production of inhibitory compounds with some microorganisms. Cayrol *et al.* (1989) reported that higher toxin production occurred at lower pH. However, further study is needed to determine whether pH has an effect on toxin production and toxic activity of *A. awamori*, *T. harzianum* and *T. viride*.

In conclusion, our results highlight that *P. striata* strain 303 produced some factor (s) that reduce egg hatch of *M.*

javanica. If the factor (s) prove to be unique compounds, they may be utilized in a number of ways in nematode management programs. Further work to elucidate the active factors produced by *P. striata* strain 303, including its characterization, should improve our understanding in order to provide explanations for these results.

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