CULTURE FILTRATE OF PLEUROTUS OSTREATUS ISOLATE Poa3 EFFECT ON EGG MASS HATCHING AND JUVENILE 2 OF *Meloidogyne incognita* AND ITS POTENTIAL FOR BIOLOGICAL CONTROL

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ABSTRACT

A possible alternative to chemical nematicides is the use of biological control agents for the management of root - knot nematode. This research was conducted from 2006 to 2008. One *Pleurotus ostreatus* isolated from Nakhon Pathom locations in Thailand, was evaluated for their *in vitro* antagonistic effects against egg masses and the juveniles 2 (J2) stage of root knot nematode, *Meloidogyne incognita*. Toxic droplets water agar tests demonstrated that *P. ostreatus* isolate Poa3 gave significantly higher colonized egg masses and parasitized hatching J2. Filtrates of the tested Poa3 grown in 1% malt extract broth were toxic to the infected egg masses and J2. The results showed that the culture filtrate of Poa3 reduced egg hatching and paralyzed juveniles of *M. incognita* after inoculation. The egg masses on tomato roots, the density of J2 and the number of galls were reduced in screen house bioassays of tomato plants parasitized by *M. incognita*.

Key words: nematode management, egg hatching and paralysis.

INTRODUCTION

In Thailand, the most destructive group of plant parasitic nematodes (PPN) is root-knot nematodes (RKN) or *Meloidogyne* spp., which exist in soils fromthe temperate and tropical regions. The most prevalent root-knot nematode in the northern part of Thailand is *Meloidogyne hapla* (*Mh*), whereas *M. incognita* (*Mi*) and *M. javanica* (*Mj*) are found in the central part of the country. The *Mi* is the most widespread, attacking more than 2,000 plant species including the families of Solanaceae, Cucurbitaceae and many others. They cause severe yield losses, approximately 78 billion US dollars worldwide annually (Sun et al., 2006; Caillaud et al., 2008). Nematicides are widely used for pest control, nevertheless current effective nematicides, such as carbofuran and others will be prohibited in the future because of their negative environmental impact. In addition, the safer newly introduced chemical pesticides are not 100 percent effective. Moreover, various problems were found in plants grown in greenhouses after continue application of the pesticides. This effect may consequently cause environment pollution, resulting in reduced biodiversity and impacts on food safety.

Currently, biological control of plant parasitic nematodes is one of the alternative measures receiving much attention around the world. The existing management procedures for nematode control can be enhanced by biological control strategies (Siddiqui and Shahid, 2003). Mushrooms have been reported for the ability of controlling root-knot nematodes. The fungi can directly infect some nematodes using their mycelia and produce nematotoxin (Khun-in et al., 2005). The mushroom *Pleurotus ostreatus* is considered to be a successful biological control agent against nematode. It immobilizes the second stage juveniles of *M. incognita* by parasitized oyster mushroom mycelia (Khun-in et al., 2005 and Heydari et al, 2006). Barron and Thorn (1987) reported that nematode which touched such droplets, showed an instant response in the form of the head region shrinking, hyphae infected the body orifices and homed.

Previously, the *P. ostreatus* isolates Poa3 could directly parasitize the infective juvenile 2 of *M. incognita* (Khun-in et al., 2005**)** (Fig 1). However, another stage of the nematode is the egg mass which is the critical stage for producing abundant infective juvenile 2. It has been reported that the nematode produce the secondary metabolite (Sun et al., 2006 and Ruanpanun et al., 2010), resulting in a deformed egg (Khun-in et al. 2005**).** *P. ostreatus* isolate Poa3 had the ability of antibiosis mechanism against the egg mass and juvenile 2 of *M. incognita*. Therefore, the objectives of this study were to investigate the effects of culture filtrate of *P. ostreatus* isolate Poa3 on egg hatching and juvenile 2 of *M. incognita* and to examine its potential for controlling *M. incognita*.

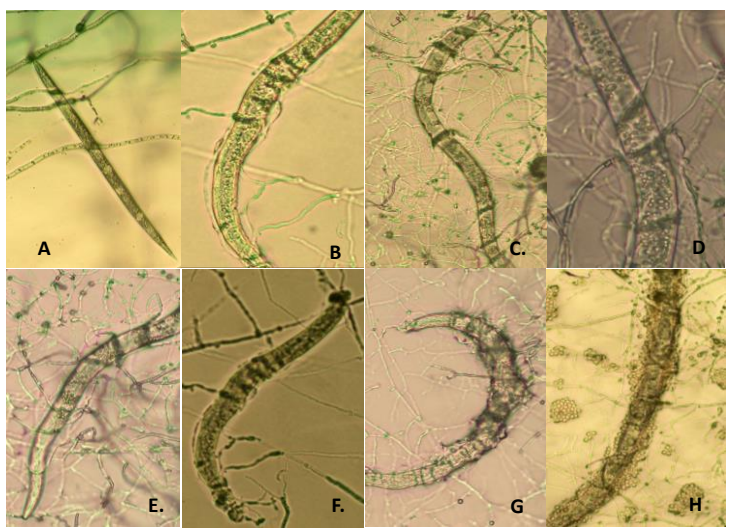

Fig. 1. Nematode trapping structures of *P. ostreatus* isolate Poa3 on water agar culture (A-G). Hysis of *M. incognita* J2 after infection and colonization by hyphae of *P. ostreatus* isolate Poa3.

MATERIALS AND METHODS

Nematode collection

Infective juvenile 2 (J2) of *M. incognita* were collected from Petchaburi Province (12º41'49.5"N99º54'15.1"E). The nematode was identified using the perennial pattern as described by Taylor and Netscher (1974). *M. incognita* was inoculated on tomato seedlings in a greenhouse from a single egg mass for 45 days. Just one egg mass was collected from infested roots and surface-

disinfected with 0.1% sodium hypochlorite (NaOCl) solution. For the J2 preparation, eggs in water were incubated at room temperature (RT) for 48 hr or more until eggs hatched, the J2 were carefully removed and immediately used for experiments (McClure et al., 1973).

P. ostreatus isolate Poa3 cultures

The fungal isolation of Poa3 was provided by Associate Professor Dr. Prapaporn Tangkijchote. Sampling sites were in Nakhon Pathom Province (lat.14º01'54.5"N and long 99º58'11.6"E) in 2003. Fruit body morphogenesis and amplified fragment length polymorphism (AFLP) technique (Anna et al., 2012) was used to identify genus and specie of the fungus. The fungus were cultured onto potato dextrose agar (PDA) and incubated at 25°C for 7 days. The hyphae were transferred to PDA for testing *in vitro* for antagonistic activity against *M. incognita*.

Parasitism and antibiosis effects of _P. ostreatus_ isolate Poa3 on nematode egg mass

The active hyphae of isolate Poa3 grown on PDA were used to test for its parasitism effect on nematode egg mass and its antibiosis effect on nematode egg hatching. One plug of the fungal isolate Poa3 was placed at the center of water agar (WA) plates and cultured for 7 days at room temperature. The fungi approximately radiated 3-3.5 cm from the agar plug. Four egg masses of *M. incognita* were placed far from the edge of the fungal colony about 1 cm. The plate was incubated at room temperature and periodically observed for number of infected nematode egg mass and number of eggs hatched by using a compound microscope at 6, 12, 24, 48, 72 and 96 h after application. The eggs, juveniles and dead juveniles were then counted and egg hatching rate and juvenile mortality were calculated according to the following formulas: egg hatching rate = 100 x juveniles/(eggs + juveniles) and juvenile mortality = 100 x dead juvenile/total juvenile (Sun et al., 2006). All experiments were conducted in 7 replications.

P. ostreatus isolate Poa3 culture filtrates

The fungal isolate Poa3 was cultured in Erlenmeyer flasks (500 mL) containing 250 ml of potato dextrose broth (PDB) + 1% of yeast extract. Each flask was inoculated with a 1 cm agar block derived from 5-day-old fungal colonies grown on PDA. The fungi were cultured for one week on a rotary shaker, at a low speed of forward and backward motion at room temperature (25-28ºC). The filtrate was first passed through a layer of filter paper (Whatman Cat. No: 1001110) and then filtered through a 0.45 µm membrane filter. The filtrate was used at concentrations of the original preparation and diluted to 25/100, 50/100 (Heydari et al., 2006).

Effect of _P. ostreatus_ isolate Poa3 culture filtrate on _M. incognita_ juvenile mortality

Two hundred J2 of nematode were added into each well of a 24-well plate containing 1 ml of each concentration of isolate Poa3 filtrates mentioned above. The same amount of avermectin and a sterile water was used as positive and negative control, respectively. Five replicates were tested per treatment. Each well was used as a replicate. Viability of the J2 was determined after 12, 24, 48, 72, and 96 h of incubation. The infective juveniles and dead juveniles were then counted and juvenile mortality rates were calculated according to the following formula: Juvenile mortality = 100 x dead juveniles/total juvenile (Sun et al., 2006).

Juveniles that did not move even after touching the tail were considered to be dead. After the incubation period, the fungal culture filtrate was replaced with tap water and mortality of the egg mass and infective juveniles were checked after 12-120 h. All experiment were conducted in 7 replications.

Effect of *P. ostreatus* isolate Poa3 culture filtrate on *M. incognita* egg mass

One sterilized egg mass of *M. incognita* was mixed with 1 ml Poa3 filtrate of each different dilutions, 25/100, 50/100 and 100 and dropped in 24 wells tissue culture plate. Egg mass in a sterile distilled water and 10µg ml^{-1} abamectin were served as negative and positive control, respectively. Each treatment contained five replication. The plate was incubated at room temperature (25-30°c) and the number of egg hatching was checked at 6, 12, 24, 48, 72 and 96 h post inoculation, respectively. The percentage of hatched eggs were calculated according to the procedures described by Heydari et al., 2006. Eggs that did not hatch and juveniles that did not move were considered to be dead. The culture filtrate was replaced with a tap water after incubating for 12-96 h and the mortality of the egg mass and juveniles were checked. All experiments were conducted in 7 replications.

Relationship between inoculum density and plant growth after applying the *P.* ostreatus isolate Poa3 culture filtrates

The experimental design was a completely randomized design with 12 treatments, 7 replications (tomato seedlings). The number of galls and total egg masses were recorded, and recoded to galling index which based on a scale of 0 = no galls; 1 = 1 to 5 galls; 2 = 6 to 20 small galls; 3 = more than 20 galls homogeneously distributed in the root system; 4 = reduce and deform root system with some large galls; and 5 = completely deformed root system with few but large galls (Di et al., 1979).

The treatment were the treated hatched eggs and J2 from each plate after 6, 12, 24, 48, 72, 96 and 120 h post inoculated and untreated hatched and J2 for the control. All treatments were inoculated on 10-day-old tomato seedlings in a pot for 45 days under screen house conditions.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using Tukey's range test (p<0.05) T-test was used to rank the pathogenicity level of Poa3 with *M. incognita* egg and juveniles. All experimental data were analyzed by SPSS program version 18.0 (2007 SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Parasitism and antibiosis effects of *P. ostreatus* isolate Poa3 on nematode egg mass

Results showed that egg mass of *M. incognita* was aggressively attacked by isolate Poa3 as time went by. The fungus infected the eggs using its hyphal tips to penetrate and colonize the eggs, and then digested the embryos within eggs during the early stages of infection (Fig. 2). This also revealed the ability of the fungus to destroy J2 of nematode inside egg masses, as well as after hatching (Table 1). Our results were similar to that of other fungal egg-parasites report by Gortari and Hours (2008) who demonstrated that the parasite caused death of the nematode by halting the embryo development, resulting in the reduction of host population growth.

Table 1. Percentage of mycelial infectivity and egg mass hatching after *M. incognita* were infected by hyphae of *P. ostreatus* isolate Poa3.

Treatment	Period (h)	Percent infection	
		Mycelia infectivity	Egg mass hatching
Control	12	0 ±0.0^{d}	3.1±1.6^{h}
	24	0±0.0^{d}	27.7±2.9^{e}
	48	0±0.0^{d}	60.0±6.2^{d}
	72	0±0.0^{d}	80.1±5.6^{c}
	96	0±0.0^{d}	95.8±4.1^{b}
	120	0±0.0^{d}	135.1±5.2^{a}
P. ostreatus	12	14.2±13.4^{d}	1.14±1.1^{h}
	24	42.8±12.2^{c}	14.7±2.6^{g}
	48	75.0±14.4^{b}	21.5±2.4^{ef}
	72	100.0±0^{a}	16.0±2.4^{fg}
	96	100.0±0^{a}	14.7±1.8^{g}
	120	100.0±0^{a}	12.5±2.4^{g}

Note: Means followed by different superscript letter in each column are significantly ($p < 0.05$) different from each other.

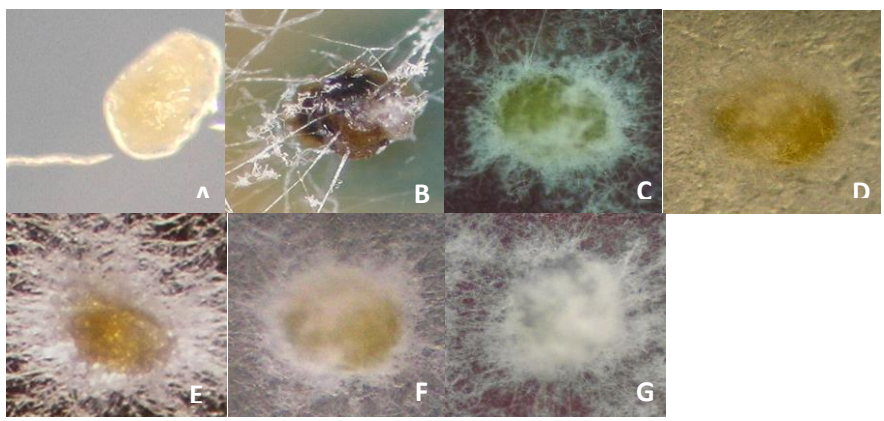

Fig 2. Egg mass of *Meloidogyne incognita* parasitized by *Pleurotus ostreatus* isolate Poa3 after egg mass inoculation with the fungus 12, 24, 48, 72 and 96 hours (panel B-G, respectively). Panel A represents only egg mass as a control treatment without fungal inoculation.

Effect of *P. ostreatus* isolate Poa3 culture filtrate on *M. incognita* juvenile 2

The effect of culture filtrates of the Poa3 in potato dextrose broth; paralyzed J2 after an exposure period of 48 h and all J2 after 72h. The dilute 10^{-2} of culture filtrates isolate Poa3 at 100% was tested for effect on J2 of *M. incognita*. The mortality showed significance compared with the control except for 50/100 and 75/100 dilutions. At 72 and 96 h after filtrates exposure, the effect of

avermectin on percentage mortality significantly higher than the effects of the culture filtrates, except at 10 mg/ml, (Table 2). The isolate Poa3 was capable of producing tiny appendages on the vegetative hyphae which secreted droplets of a potent toxin as described by Thorn and Barron (1984), Barron and Thorn (1987) and Peterson (1993). When nematodes came into contact with this culture filtrate, they suddenly and dramatically became immobilized in a few minutes (Tadaaki Satou et al. 2008).

Table 2. Effect of Poa3 culture filtrates on Juvenile 2 mortality at various exposure time.

Treatment	Dilution	Juvenile mortality (%)					
		6 h	**12 h**	**24 h**	**48 h**	**72 h**	**96 h**
Control (water)	0	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}
Avermectin	10 ppm	1.4 ± 1.1^{e}	4.8 ± 1.1^{e}	25.8 ± 1.6^{d}	35.8 ± 2.5^{c}	43.8 ± 2.3^{b}	56.6 ± 2.5^{a}
	50 ppm	6.8 ± 1.9^{d}	10.2 ± 1.1^{d}	35.2 ± 1.9^{c}	37.2 ± 2.9^{c}	50.4 ± 1.9^{b}	65.4 ± 2.1^{a}
	100ppm	10.6 ± 1.9^{e}	15.0 ± 1.8^{d}	42.0 ± 2.2^{c}	42.6 ± 2.1^{c}	58.0 ± 1.6^{b}	70.2 ± 2.8^{a}
Poa3	25/100	0 ± 0.0^{d}	0.6 ± 0.5^{d}	4.0 ± 1.0^{c}	6.8 ± 1.3^{c}	17.0 ± 2.0^{b}	25.2 ± 2.9^{a}
	50/100	0 ± 0.0^{d}	1.4 ± 0.5^{d}	12.8 ± 1.9^{d}	14.8 ± 1.8^{c}	25.8 ± 1.9^{b}	33.4 ± 2.7^{a}
	75/100	0.6 ± 0.5^{e}	3.0 ± 1.0^{e}	17.8 ± 2.2^{d}	24.6 ± 2.3^{c}	37.2 ± 2.9^{b}	44.8 ± 2.8^{a}
	100%	1.0 ± 0.0^{f}	5.4 ± 1.9^{e}	24.0 ± 1.6^{d}	36.0 ± 2.7^{c}	46.4 ± 2.4^{b}	55.2 ± 2.6^{a}

Note: Means followed by different superscript letter in the same row are significantly ($p < 0.05$) different.

Effect of *P. ostreatus* isolate Poa3 culture filtrate on *M. incognita* egg mass

The hatchability test (Table 3) demonstrated that culture filtrates of Poa3 strongly suppressed hatching of *M. incognita* egg masses. The percentage reduction ranged from 0.4 to 43.5%. The different concentrations of the culture filtrate exhibited significant differences compared to the control; only the 25/100 dilution had no significant difference when compared to the avermectin.

Table 3. Effect of *P. ostreatus* isolate Poa3 cultures filtrate on *M. incognita* egg hatching at various exposure times.

Treatment	**Dilution**	**Egg hatched (%)**					
		6 h	**12 h**	**24 h**	**48 h**	**72 h**	**96 h**
Control	0	0 ± 0.0^{f}	10.7 ± 1.8^{e}	20.1 ± 3.2^{d}	40.5 ± 2.6^{c}	71.0 ± 2.4^{b}	107.0 ± 5.8^{a}
Avermectin	10 ppm	0 ± 0.0^{f}	4.1 ± 1.3^{e}	12.8 ± 2.5^{d}	29.1 ± 2.0^{c}	42.7 ± 3.6^{b}	52.1 ± 3.0^{a}
Poa3	25	0 ± 0.0^{f}	5.0 ± 1.9^{e}	16.8 ± 2.4^{d}	34.2 ± 3.7^{c}	56.4 ± 2.9^{b}	70.0 ± 2.6^{a}
	50	0 ± 0.0^{e}	2.1 ± 0.7^{e}	9.4 ± 1.7^{d}	25.7 ± 3.7^{c}	34.5 ± 2.7^{b}	43.5 ± 3.7^{a}
	75	0 ± 0.0^{e}	1.0 ± 0.8^{de}	4.2 ± 1.5^{d}	12.5 ± 1.1^{c}	22.1 ± 3.6^{b}	32.1 ± 2.7^{a}
	100	0 ± 0.0^{d}	0.4 ± 0.5^{d}	1.1 ± 0.4^{d}	5.2 ± 1.8^{c}	11.0 ± 2.6^{b}	23.4 ± 2.1^{a}

Note: Means followed by different superscript letter in row are significantly ($p < 0.05$) different from each other.

The variables evaluated included egg parasitism, egg hatch rate variations could be related to other mechanisms like antibiosis, competition and predation besides parasitism (Cayrol 1983; Kwork et al., 1992; Zaki 1994). Previous findings showed that culture filtrate from the same Poa3 isolated induced juvenile mortality and inhibited *M. incognita* egg hatching. A compound microscopy study of treated egg masses showed severe alterations caused by the filtrate of isolate Poa3 on *M. incognita* egg masses suggesting that enzyme and toxin-based strategies were part of the Poa3 nematocidal behavior (Regaieg et al.2010).

Relationship between inoculum density and plant growth after applying the *Pleurotus* culture filtrates

The efficacy of the root-knot nematode control agents were tested under greenhouse conditions to evaluate the most antagonistic activity of Poa3. The culture filtrates of Poa3 reduced the numbers of root galls index after exposure for periods of 72 to 120 h when compared with other periods and the control (inoculated) at 45 days after inoculation with *M. incognita*, (Table 4).

The number of *M. incognita* egg masses on roots were significantly (P\0.05) affected by Poa3 (Table 3). The application of Poa3 reduced the J2 density as well as the number of galls by 0.1gall/plant/ period of 120 h, when compared to the control. Finally, the galling inside roots clearly demonstrated that Poa3 filtrate inhibited the hatching of nematode egg masses. Comparison of Poa3 to others which are good candidates for practical exploitation in bio-control, such as *Paecilomyces lilacinus* (Kiewnick and Sikora, 2006) and *Trichoderma* spp. (Thienhirun et al. 1997), Poa3 shows similar or higher levels of nematode control.

Table 4. Average numbers of galls and egg masses of *M. incognita* in tomato seedlings roots after were infected by hyphae of *P. ostreatus* isolate Poa3

Treatment	Period (h)	Index of investigation	
		Number of galls	Egg mass in roots
Control	12	98.1±6.6^{a}	21.0±2.2^{a}
	24	97.8±9.9^{a}	21.8±3.9^{a}
	48	103.8±5.2^{a}	20.4±2.3^{a}
	72	97.4±5.9^{a}	19.5±1.4^{a}
	96	98.8±9.0^{a}	18.2±2.8^{a}
	120	99.8±4.9^{a}	20.0±3.0^{a}
P. ostreatus	12	48.4±3.5^{b}	3.7±0.8^{b}
	24	51.4±3.3^{b}	3.7±0.8^{b}
	48	28.2±3.9^{c}	1.5±1.0^{b}
	72	0.7±0.8^{d}	0±0.0^{b}
	96	0.2±0.5^{d}	0±0.0^{b}
	120	0.1±0.4^{d}	0±0.0^{b}
Control (healthy)	-	0±0.0^{d}	0±0.0^{b}

Note: Means followed by different superscript letter in each column are significantly ($p < 0.05$) different from each other.

CONCLUSIONS

The culture filtrate from *P. ostreatus* isolate Poa3 shows antagonistic effect on both egg hatching and juvenile 2 of *M. incognita*. Results from greenhouse experiments demonstrate its potential for biological control of root-knot nematode. Field experiments should be investigated in the future to test the efficacy of the fungal filtrate for controlling the disease caused by *M. incognita*. In addition, the biologically active substance(s) in fungal filtrate of isolate Poa3 affecting both nematode egg hatching and juvenile 2 should be further investigated.

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