

## Research Article

## The effects of *Arthrobotrys oligospora* and *Arthrobotrys conoides* culture filtrates on second stage juvenile mortality and egg hatching of *Meloidogyne incognita* and *Meloidogyne javanica*

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**Abstract:** Culture filtrates (CF) of two species of the nematophagous fungi, *Arthrobotrys oligospora* and *Arthrobotrys conoides* at three concentrations (25%, 50% and 100%) of stock, were tested on the mortality of second stage juveniles (J2) and egg hatching rate of *Meloidogyne incognita* and *Meloidogyne javanica*. Results showed that the percent juvenile mortality was directly proportional to concentration of the filtrates. Egg hatching rate of these nematodes was inversely affected by increasing concentrations. Also CFs had various impacts on the mortality of J2 and egg hatching rate. In case of *M. incognita* maximum J2 mortality (28.98%) occurred after 24 hours of exposure to *A. conoides* filtrate at concentration of 100%. The minimum toxicity (12.5% J2 mortality) was recorded for *A. oligospora* at 25% filtrate concentration. At the same time, the highest rate of J2 mortality of *M. javanica* (19.18%) belonged to the 100% concentration of *A. conoides*, while minimum toxicity belonged to 25% concentration of *A. oligospora* causing 9.09% mortality. Maximum egg hatching rate for *M. incognita* (30.75%) belonged to control and minimum hatching rate (1.25%) belonged to 100% concentration of *A. conoides*. The highest hatching rate of *M. javanica* (36.25%) belonged to control and minimum hatching rate (1.25%) occurred at 100% concentration of *A. conoides*.

**Keywords:** *Arthrobotrys oligospora*; *A. conoides*; culture filtrate; mortality; egg hatching rate, *Meloidogyne incognita*; *M. javanica*

### Introduction

Antagonistic fungi are continuously attracting great attention as potential alternatives to chemical control of root-knot nematodes (Kalele *et al.*, 2010) which are the most important group of plant parasitic nematodes, causing great economic losses (Sasser and Freckman, 1987;

Moens *et al.*, 2009). These nematodes are polyphagous, have a highly specialized and complex feeding relationship with their hosts and are very difficult to control (Hussey and Janssen, 2002). Several fungi such as nematophagous fungi directly parasitize nematodes or secrete nematotoxic compounds and enzymes that affect nematode viability (Nitao *et al.*, 1999). There are many reports regarding the production of anti-nematode compounds by these fungi (Cayrol *et al.*, 1989; Anke *et al.*, 1995; Hallmann and Sikora, 1996; Anke and Sterner, 1997; Chen *et al.*, 2000; Meyer *et al.*, 2000; 2004). The study of fungal

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culture filtrates as sources of nematicidal metabolites and antagonistic compounds has continuously increased worldwide (Ciancio, 1995; Liu *et al.*, 2008; Lopez-Llorca *et al.*, 2008). Secondary metabolites from *Fusarium oxysporum*, were toxic to *M. incognita*, and toxins from various *Fusarium* spp. have been demonstrated to reduce nematode viability (Hallmann and Sikora, 1996; Ciancio, 1995; Anke and Sterner, 1997; Nitao *et al.*, 2001). The wood-rotting basidiomycetes such as *Pleurotus* species especially *P. ostreatus* produce the nematotoxin *trans*-2-decenedioic acid (Kwok *et al.*, 1992). Also extracts of *P. ostreatus* owing to anti-nematode compounds could paralyze 90% of *Heterodera schachtii* J2 (Palizi *et al.*, 2009). Acetic acid is an active component of culture filtrates of *Paecilomyces lilacinus* and *Trichoderma longibrachiatum* and linoleic acid produced by nematophagous fungi *Arthrobotrys conoides* and *A. oligospora* was identified as a nematotoxic compound (Djian *et al.*, 1991; Anke *et al.*, 1995).

Also *A. oligospora*, when grown in liquid culture, produced two extracellular proteases that hydrolyze the chromogenic substrate Azocoll (Tunlid *et al.*, 1994). Also extracellular serine protease Ac1 with a molecular mass of 35 kDa, was purified from *A. conoides*. Ac1 can degrade a broad range of substrates including casein, gelatin, bovine serum albumin, collagen and nematode cuticle (Yang *et al.*, 2007).

*Pochonia chlamydosporia* secretes proteases, one of which hydrolyzes proteins in egg shell of *M. incognita* (Segers *et al.*, 1994; 1999; Meyer *et al.*, 2004). Nematicidal activity of *P. chlamydosporia* culture was primarily attributed to phomalactone, which acts against *M. incognita* J2 and inhibits hatching of eggs (Khambay *et al.*, 2000). Many soil borne fungi such as nematode trapping or predacious fungi inhibit the hatching of eggs and cause mortality of root-knot nematodes (Hallmann and Sikora, 1996; Anke and Sterner, 1997; Khan, 1999; Sharma, 1999; Wang *et al.*, 1999; Costa *et al.*, 2000; Randhawa *et al.*, 2001; Nitao *et al.*, 2001).

This study was performed to estimate the antagonistic activity of CFs of nematophagous fungi *A. oligospora* and *A. conoides* on second stage juvenile mortality and egg hatching rate of *M. incognita* and *M. javanica*

## Materials and Methods

### Nematode inoculum preparation

Egg masses of *Meloidogyne incognita* and *M. javanica*, collected from Mazandaran Province, were separately cultured and maintained on tomato seedling in pots in greenhouse condition for six weeks. Infected tomato roots were washed with tap water to remove adhering soil then roots bearing egg masses were surface sterilized in 200 ml of 0.5 percent sodium hypochlorite for four minutes. They were then poured onto 350 and 500 mesh sieves and washed with sterile distilled water for 15 minutes. The collected suspension of larvae and egg masses were poured on a paper tissue spread on the bottom of a plastic sieve placed in a tray containing water. The second stage larvae were collected every 24 hours and kept in refrigerator (Hussy and Barker, 1973). The larvae were surface sterilized by streptomycin solution 4000 ppm before inoculation (Pourjam *et al.*, 1999).

### Antagonistic fungi

Fungal species used in this study were *Arthrobotrys oligospora* (IRAN678C), isolated from Noor area of Mazandaran Province provided by Iranian Research Institute of Plant Protection, Tehran. *A. conoides* (CBS575.91) provided by CBS-KNAW Fungal Biodiversity Centre. These isolates were grown and maintained on Corn Meal Agar medium (CMA).

### Fungal filtrates preparation

The fungi were cultured in autoclaved 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth. Cultures were shaken at 100 rpm for 14 days. Distilled water was used as control. Filtrates were sterilized using filter membrane (0.2µm) and were used at 25, 50 and 100%.

**Effect of filtrates on nematode mortality**

The experiment was carried out in 1.5 ml tubes filled with 1 ml of fungal filtrate and then nematode suspension was added (ca.100 nematodes/tube). After 24 hours, 200 µl of suspension was transferred into new 1.5 ml tube and then the nematodes were washed three times with tap water, followed by centrifugation for three minutes at 1000 rpm, and then incubated in 1 ml water for 24 hours at 22 °C. The mortality rate was calculated by counting the numbers of nematodes that were inactive.

**Effect of filtrates on egg hatching**

10 µl of egg suspension were placed into sterile 1.5 ml tubes (ca.50 eggs/tube) to which 1 ml fungal culture filtrates was added. The tubes were incubated at 25 °C. The numbers of J2 at the end of two weeks of incubation time were counted using a stereo microscope, and the percentages of hatched eggs were calculated as follows: number of hatched eggs/number of eggs originally placed in the tubes × 100.

**Data analysis**

All analyses were done using SPSS (Statistical Package for Social Science) statistical program version 18. The factorial experiment was conducted in Completely Randomized Design with four replications. Data was analyzed statistically by analysis of variance, the comparison of means was done at 1% ( $p \leq 0.01$ ) and 5% ( $p \leq 0.05$ ) levels of significance according to the Duncan's multiple range test (DMRT).

**Results****Effect of culture filtrates on J2 mortality**

Results showed that there were significant differences in nematicidal activity of *A. oligospora* and *A. conoides* filtrates and at different concentrations.

**Effect of filtrates on mortality of J2 of *M. incognita***

Results indicated that there were significant differences at 1 percent ( $p \leq 0.01$ ) level between filtrates of different concentrations. Juvenile

mortality in the filtrates of fungus was found to be directly proportional to concentration of filtrates and the duration of exposure. After 24 hours exposure to culture filtrates, the maximum mortality rate of J2 (28.98%) occurred at 100% concentration of *A. conoides*. The minimum death rate (12.50%) belonged to *A. oligospora* with a concentration of 25% (Table 1).

**Effect of filtrates on mortality of J2 of *M. javanica***

The findings indicated that there were significant differences at the 1 percent ( $p \leq 0.01$ ) level between the filtrates as well as their concentrations. A progressive increase in the concentrations of the filtrates resulted in an increase in the mortality of juveniles. Maximum mortality (19.18%) occurred after 24 hours exposure to highest concentration (100%) of *A. conoides* filtrate. The minimum mortality (9.09%) belonged to *A. oligospora* with a concentration of 25% (Table 1). Results indicated that CF from *A. conoides* caused greater mortality than that from *A. oligospora*. The results also showed that second stage juveniles of *M. incognita* compared with *M. javanica* were more sensitive to secondary metabolites of these fungi.

**Effect of culture filtrates on egg hatch rate**

Results showed that there were significant differences in ovicidal activities of *A. oligospora* and *A. conoides* filtrates and their concentrations.

**Effect of filtrates on *M. incognita* egg hatch**

The results indicated that there were significant differences ( $p \leq 0.05$ ) between different culture filtrates and their concentrations. Also egg hatch rate of *M. incognita* was correlated inversely with concentrations of filtrates i.e. the hatching rate decreased with increasing concentrations. After two weeks maximum egg hatch rate belonged to control (30.75%) and minimum (1.25%) to eggs treated with a 100% concentration of *A. conoides* culture filtrate. Meanwhile, the lowest hatching rate (6.25%) occurred in presence of 100% CF of *A. oligospora* (Table 2).

**Table 1** Mean percent mortality ( $\pm$  SE) of *M. incognita* and *M. javanica* J2, in stock dilutions of fungal culture filtrates after 24 hours.

Fungal species	Concentration (%)	J2 mortality (%) <sup>1</sup>	
		<i>M. incognita</i>	<i>M. javanica</i>
<i>Arthrobotrys conoides</i>	0 (Control)	0 $\pm$ 0.00h	0 $\pm$ 0.00h
	25	16.83 $\pm$ 0.24cd	15.50 $\pm$ 0.28de
	50	19.42 $\pm$ 1.31b	17.48 $\pm$ 0.40c
	100	28.98 $\pm$ 1.13a	19.18 $\pm$ 0.28a
<i>Arthrobotrys oligospora</i>	0 (Control)	0 $\pm$ 0.00h	0 $\pm$ 0.00h
	25	12.50 $\pm$ 0.28f	9.09 $\pm$ 0.33g
	50	15.25 $\pm$ 0.25de	14.93 $\pm$ 0.38e
	100	19.62 $\pm$ 0.97b	16.14 $\pm$ 0.45cde

<sup>1</sup> Means followed by different letters in each column are significantly different from each other (DMRT,  $P \leq 0.01$ ).

**Table 2** Mean percentage of eggs hatching rate ( $\pm$  SE) of *M. incognita* and *M. javanica* in fungal culture filtrates after two weeks.

Fungal species	Concentration (%)	Eggs hatching (%) <sup>1</sup>	
		<i>M. incognita</i>	<i>M. javanica</i>
<i>Arthrobotrys conoides</i>	0 (Control)	30.75 $\pm$ 0.75b	36.25 $\pm$ 1.25a
	25	14.00 $\pm$ 0.00ef	20.00 $\pm$ 0.00d
	50	10.00 $\pm$ 0.00g	12.00 $\pm$ 0.00fg
	100	1.25 $\pm$ 1.25i	1.25 $\pm$ 1.25i
<i>Arthrobotrys oligospora</i>	0 (Control)	30.75 $\pm$ 0.75b	36.25 $\pm$ 1.25a
	25	16.00 $\pm$ 0.00e	26.00 $\pm$ 0.00c
	50	12.00 $\pm$ 0.00fg	15.00 $\pm$ 0.00e
	100	6.25 $\pm$ 1.25h	2.50 $\pm$ 2.50i

<sup>1</sup> Means with different letters in a column differ significantly from each other (DMRT,  $P \leq 0.05$ ).

#### Effect of filtrates on *M. javanica* egg hatch

Incubation of the surface sterilized eggs for two weeks in water (control) resulted in a 36.25% hatching rate. The findings indicated that there were significant differences ( $p \leq 0.05$ ) between culture filtrates and their different concentrations. Also egg hatch rate of *M. javanica* was inversely correlated with concentrations of filtrates i.e. the hatch rate decreased with increasing concentration. Minimum hatch rate for 100% concentration of *A. conoides* culture filtrate was the same as that for *M. incognita*. Meanwhile, the lowest hatching rate caused by *A. oligospora* was 2.5% at 100% concentration of filtrate (Table 2).

#### Discussion

Wide range of activities has been attributed to the culture filtrates of fungal isolates. Many fungi are known to produce nematicidal or nemastatic compounds (Anke *et al.*, 1995; Hallmann and Sikora, 1996; Anke and Sterner, 1997; Chen *et al.*, 2000; Meyer *et al.*, 2000; Kopcke *et al.*, 2001). Adverse effects of culture filtrates of several fungi on hatching and mortality of root-knot nematodes have been reported (Cayrol *et al.*, 1989; Saifullah, 1996; Zaki, 1999). In this study, culture filtrate of two fungal species *Arthrobotrys conoides* and *A. oligospora* showed significant nematicidal activity by killing second stage juveniles and inhibition of egg hatch in

*Meloidogyne incognita* and *M. javanica*. This study confirmed the lethal effect of *Arthrobotrys* exudates on J2. Results showed that CF from *A. conoides* compared with that of *A. oligospora* was more lethal to J2 and also egg hatching rate was less in presence of *A. conoides* filtrate compared with *A. oligospora*. This can be assigned to Ac1 protein produced in *A. conoides*, compared with serine protease produced by *A. oligospora*, of which the former can destroy broad spectrum of substrates (Yang et al., 2007). It was also observed that J2 and eggs of *M. incognita* compared with *M. javanica* were more sensitive to CF of both fungi. Different concentrations of filtrates illustrated that mortality rates are directly proportional to concentration and that exposure time is also important in mortality rates. Other studies have showed similar results; for instance the culture filtrates of *Verticillium leptobactrum* inhibited egg hatching and were lethal to *M. incognita* J2 (Hajer et al., 2010). Also the antagonistic effect of *V. chlamydosporium* against *M. javanica* may be attributed to the production of certain enzymes (Webb et al., 1972; Segers et al., 1994). There is an increasing willingness to use nematophagous fungi or their products as biological agents for control of plant and animal parasitic nematodes. There are some data indicating that hydrolytic enzymes including proteases can be used to control plant parasitic nematodes (Kerry, 1990; Miller and Sands, 1977). Among the nematophagous fungi, *Arthrobotrys* spp. are outstanding nematode predators, but little research has been conducted on the effects of these fungi against nematodes.

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## تأثیر عصاره‌های *Arthrobotrys conoides* و *Arthrobotrys oligospora* روی میزان مرگومیر لاروهای سن دو و نرخ تفریخ تخم نماتدهای *Meloidogyne javanica* و *Meloidogyne incognita*

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**چکیده:** عصاره‌های دو گونه قارچ نماتدخوار *Arthrobotrys conoides* و *Arthrobotrys oligospora* در سه رقت (۱ ×)، (۰/۵۰ ×) و (۰/۲۵ ×) از عصاره پایه روی میزان مرگومیر لاروهای سن دو و نرخ تفریخ تخم نماتدهای *Meloidogyne incognita* و *Meloidogyne javanica* مورد ارزیابی قرار گرفتند. نتایج نشان داد که درصد مرگومیر لاروها با غلظت عصاره‌ها نسبت مستقیم داشت. اما نرخ تفریخ تخم با افزایش غلظت نسبت معکوس داشت. هم‌چنین نتایج نشان داد که عصاره‌های این دو قارچ اثرات مختلفی روی میزان مرگومیر لاروهای سن دو و نیز نرخ تفریخ تخم نماتدهای فوق داشتند. درخصوص لاروهای سن دو *M. incognita* پس از ۲۴ ساعت بیش‌ترین میزان مرگومیر مربوط به عصاره پایه (۱ ×) گونه *A. conoides* بود که باعث نابودی ۲۸/۹۸ درصد لاروها گردید. هم‌چنین کم‌ترین میزان مرگومیر مربوط به رقت (۰/۲۵ ×) گونه *A. oligospora* با ۱۲/۵ درصد بود. این در حالی است که بیش‌ترین میزان مرگومیر لاروهای *M. javanica* مربوط به عصاره (۱ ×) گونه *A. conoides* با ۱۹/۱۸ درصد و کم‌ترین میزان مرگومیر مربوط به رقت (۰/۲۵ ×) گونه *A. oligospora* با ۹/۰۹ درصد بود. پس از گذشت دو هفته بیش‌ترین نرخ تفریخ تخم‌های گونه *M. incognita* مربوط به شاهد به‌میزان ۳۰/۷۵ درصد و کم‌ترین نرخ تفریخ مربوط به عصاره پایه (۱ ×) گونه *A. conoides* به‌میزان ۱/۲۵ درصد بود. درخصوص گونه *M. javanica* بیش‌ترین نرخ تفریخ تخم مربوط به شاهد به‌میزان ۳۶/۲۵ و کم‌ترین نرخ تفریخ مربوط به عصاره پایه (۱ ×) گونه *A. conoides* به‌میزان ۱/۲۵ درصد بود.

**واژگان کلیدی:** *Arthrobotrys oligospora*، *A. conoides*، عصاره قارچی، مرگومیر، نرخ تفریخ تخم، *Meloidogyne incognita* و *M. javanica*