BIOCONTROL POTENTIAL OF *METARHIZIUM ANISOPLIAE* (HYPOCREALES: CLAVICIPITACEAE), ISOLATED FROM SUPPRESSIVE SOILS OF THE BOYER-AHMAD REGION, IRAN, AGAINST J2S OF *HETERODERA AVENAE*

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Abstract: In order to study the nematode parasitic fungi, eighty soil samples were collected from different regions of Boyer-Ahmad, Iran. Extracted nematodes were examined for fungal parasitism. The naturally parasitized nematodes were surface sterilized and cultured on lactic acid containing media, then incubated at 25°C for 7–10 days. *Metarhizium anisopliae* was isolated from five soil samples. An *in vitro* test of the potential antagonistic efficiency of *M. anisopliae* against J2s of *Heterodera avenae* was evaluated. Nematodes were exposed to concentrations of 10^3, 10^4, 10^5, 10^6, and 10^7 conidia per ml. Results showed that the isolated *M. anisopliae* is highly pathogenic to them. The number of parasitized J2s varied between 14.9% and 47.1% for 10^3 and 10^7 conidia per mL, respectively. To assess the optimum temperature of the isolate, cultured fungi were incubated at 20, 25, 27, 30, 35, 37, and 38°C. The optimum temperature for growth was 27–35°C and no growth was observed at 38°C. This is the first report of a natural occurrence as well as the biocontrol potential of *M. anisopliae* on nematodes in Iran.

Key words: biological control, entomophagous fungi, integrated nematode management, nematophagous fungi

INTRODUCTION

Plant parasitic nematodes are one of the major factors limiting the productivity of many agricultural crops grown for profit (Luc *et al.* 2005). The majority of the synthetic chemical nematicides are being taken off the market because of their hazardous effect on human beings and animals (Ghazalbash and Abdollahi 2011). Also unreliable results from crop rotation systems have necessitated the search for sustainable, effective, and environmentally acceptable nematode management options (Sikora and Fernandez 2005).

Some soil inhabiting fungi are pathogenic to some pests of plants, including insects and nematodes. *Metarhizium anisopliae*, the agent of green muscardine disease of insects, is the most important entomopathogenic fungus (Driver *et al.* 2000). This is a facultative parasite which can affect a group of insects and is a well-studied species for microbial control of insect pests (Liu *et al.* 2007; Hoe *et al.* 2009). This fungus produces some cyclic peptides, destruxins (Hsiao and Ko 2001) which may play a role in its pathogenicity (Kershaw *et al.* 1999).

Large numbers of organisms including fungi, bacteria, viruses, insects, mites and some invertebrates have been found to invade or prey on the nematodes (Stirling 1991). Among these organisms, fungi are great potential candidates for biocontrol of nematodes (Dijksterhuis *et al.* 1994). Fungi have a significant association with nematodes in rhizosphere and thus, they can constantly destroy nematodes in nearly all soils in different geographical areas (Siddiqui and Mahmood 1996). Although more than 70 genera and 160 species of fungi have been associated with nematodes, only a few of them are known as nematophagous fungi (Duddington 1994). Fungi can directly parasitize nematodes (Holland *et al.* 1999; Olivares-Bernabeu and Lopez-LIorca 2002; Chen and Chen 2003; Fatemy *et al.* 2005) or secrete nematicidal metabolites and enzymes that affect nematode viability (Cayrol *et al.*1989; Nitao *et al.* 1999; Chen *et al.* 2000). These active compounds have the potential for being applied as novel nematicides (Meyer *et al.* 2004). Biological control of sugarcane nematodes using *Penicillium oxalicum* and *M. anisopliae* has been studied by Zorilla (2001). He has reported the significant inhibitory effect of *M. anisopliae* on the studied nematode population. Inhibition of the population growth of *Rotylenchus reniformis* by *M. anisopliae* has also been reported (Tribhuvaneshwar *et al.* 2008).

During a study on the nematode parasitic fungi, soil samples were collected from different regions of Boyer-Ahmad, Iran. In the course of the investigation, some naturally parasitized nematodes were observed (Fig. 1). The main objective of this study was to investigate the biocontrol potential of the naturally occurring fungus, *M. anisopliae*, on J2s of *H. avenae*. The fungus *M. anisopliae* is found in the suppressive soils of the Boyer-Ahmad region of Iran.

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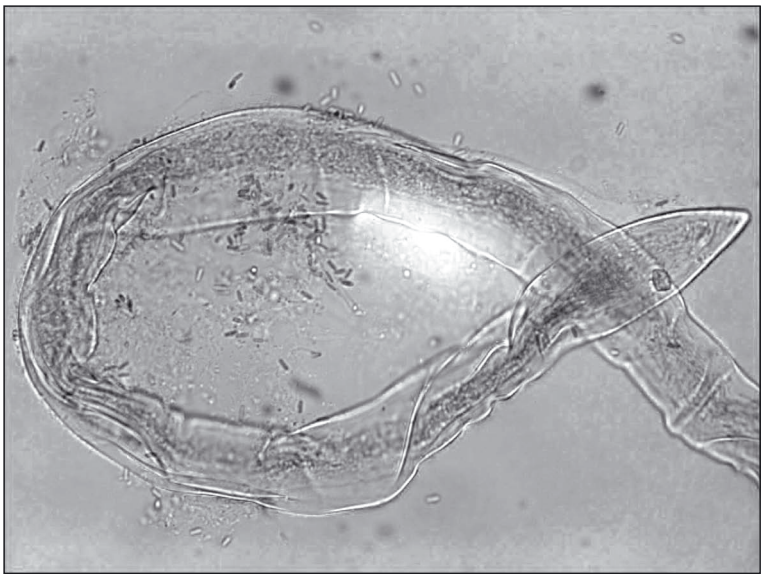

Fig. 1. A naturally parasitized nematode, isolated from suppressive soils of the Boyer-Ahmad region

MATERIALS AND METHODS

Isolation of the fungus

Eighty soil samples were collected from the Boyer-Ahmad region of Iran, during the summer months of 2010. The samples were washed and the nematodes were extracted by a centrifugal flotation technique according to the modified method of de Grisse (1969). Nematode suspension was then examined using a stereo microscope, and the parasitized nematodes were handpicked to isolate the nematophagous fungi. Nematodes were surface-sterilized for 2 minutes in 0.5% (w/v) sodium hypochlorite and washed five times in sterile water. The parasitized nematodes were then transferred to petri dishes containing 1.5% water agar (WA) medium supplemented with 0.1% chloramphenicol solution and left for two days (Sikora *et al.* 1990).

After the incubation period, individual nematodes were aseptically transferred onto new WA plates and incubated for four days to allow fungal growth. To obtain pure fungal cultures, each fungal colony was aseptically transferred to new WA plates using a sterile platinum loop and again incubated for four days. Single fungal colonies were transferred and cultured on Potato Dextrose Agar medium (PDA) in 9 cm-diameter Petri-dishes for five days at 25°C (Dackman 1990). In order to suppress bacterial growth, the media was acidified to a pH of 3.5 by adding 1 ml of Lactic Acid 10% to each 100 ml of sterilized medium, at 50°C. The mycelial plugs of pure culture were transferred to PDA and CMA media.

Identification of the fungus

The identification of isolated fungi was performed by macroscopic and microscopic morphometry of conidia and conidiophores using a compound microscope (Olympus Cx31) at 100X, 400X and 1000X magnifications. These measurements were compared with dichotomous keys (Barnett and Hunter 1987; Samson *et al.* 1988; Humber 1997) for the identification of fungi genera. Cultures also have been sent to prof. E.M. Goltapeh, Department of Plant Pathology, Tarbiat Modares University, Tehran, Iran, for identity confirmation.

Determination of optimal temperature

To quantify the effects of temperature on the growth rate of the isolates, the radial growth of the isolated fungi was measured on PDA plates at various temperatures of 20, 25, 27, 30, 35, 37, and 38°C, after 48 and 96 hours, as described by Kerry (1990). Five mm mycelial plugs from one week old PDA cultures were transferred to fresh PDA plates. The experimental design was a randomized block with four replications for each temperature.

Pathogenicity assays

In the pathogenicity test, fungi were grown on PDA and incubated at 25°C for 7 days. Aerial conidia were harvested by dislodging them from a culture and suspending in 2 ml sterile distilled water with 0.01% Tween 80 (Merck, Germany). The spore suspension was stirred and filtered through gauze (mesh: 500 μm) to remove mycelium particles. The spore concentration in the resulting suspension was determined using a haemocytometer (Neubauer, Germany). A concentration of 10^9 conidia/ml was standardized and suspensions containing 10^3, 10^4, 10^5, 10^6, and 10^7 conidia/ml were obtained by serial dilution. For inoculation purposes, the second stage juveniles of *H. avenae* were obtained from mature cysts. Cysts were placed in fresh water at 37°C and the J2s were allowed to emerge. The healthy J2s were hand-picked. Nematodes were surface sterilized with 0.5% sodium hypochlorite (NaOCl) for 2 minutes and rinsed five times with sterilized double distilled water. They were then transferred aseptically to 9 cm diameter glass petri dishes containing 10 ml of conidial suspension. Petri dishes with distilled water served as the controls. There were four replicates for each concentration with 100 nematodes added to all plates. The petri dishes were kept at 25°C in the dark for 7 days (Liu *et al.* 2003). After the incubation period, the parasitized nematodes were counted.

RESULTS AND DISCUSSION

Identification of the fungus

Based on the observation of colony morphology as well as the size and shape of conidia, the isolated fungus was identified as *M. anisopliae*. Colonies of this fungus grow rather slowly on PDA and sometimes are a pale luteous to citrine in the centre with yellow pigment diffusing into the medium. Conidia color may differ in colony size and condition (Latch 1964). After 10 days of incubation, the culture produces a white mycelial margin with clumps of more or less verticillate branching conidiophores. These branching conidiophores become colored with the development of the spores. The colors vary from olivaceous buff to cream color to dark green (Fig. 2). This is akin to the observations of Bridge *et al.* (1993). They described the colonies of *M. anisopliae* as yellowish green or olivaceous green, sometimes even as pink buff colonies. The conidiophore and phialide dimensions were 4–13.5x1.4–2.6 μm and 6.3–13.5x1.8–3.6 μm, respectively. Conidial chains were round, columnar phialides in a dense parallel arrangement, and conidia were cylindrical to oval measuring 2.5–3.5x2.5–7 μm (Fig. 3, Table 1).

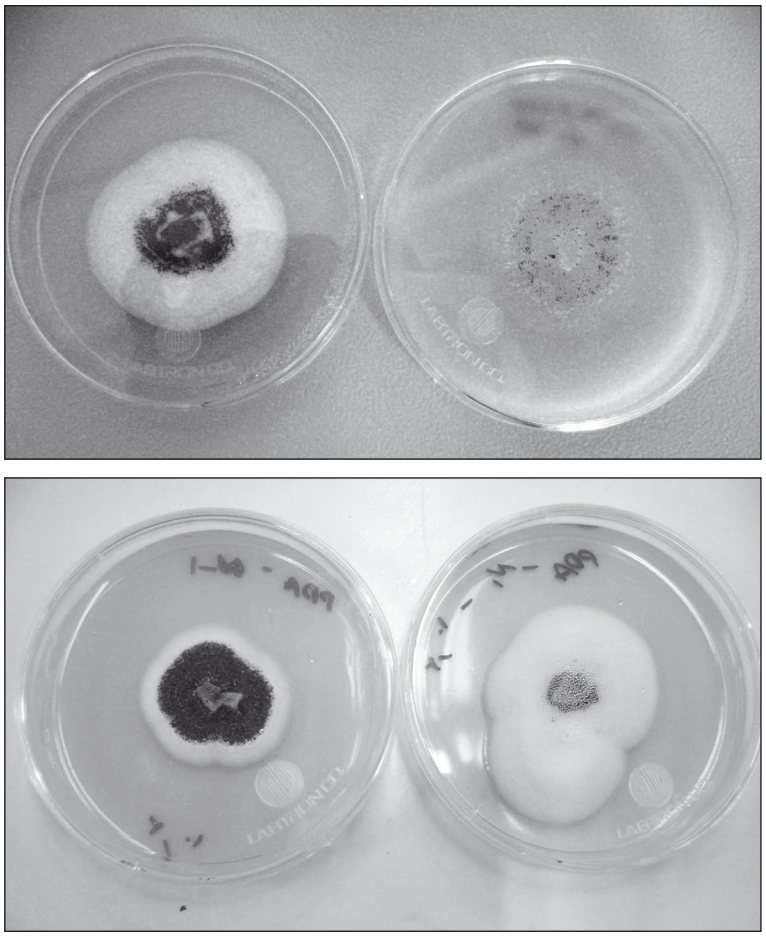

Fig. 2. Ten-days-old culture of *M. anisopliae* on CMA (up) and PDA (down)

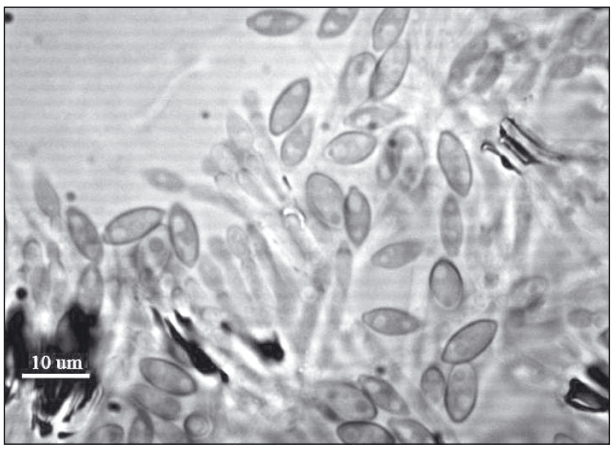

Fig. 3. Conidia and phialides of M. anisopliae, isolated from soil nematodes in Boyer-Ahmad

Table 1. Dimensions of mycelium and fruiting bodies of *M. anisopliae* isolated from the suppressive soil of the Boyer-Ahmad region

Fungal structure	Brady 1979	Hoog *et al.* 2000	Boyer-Ahmad region
Single-celled and double-celled conidia	1.5–2.5x4–8	2.5–3.5x5–8	2.5–3.5x2.5–7
Single-celled conidia	1.5–3.5x3.5–9	2.5x3.5	1.5–2.5x3.5–5.5
Phialide	2–3.5x6.5–13.5	9–14	2.5–3.5x10–15
Hyphal width	–	–	1–2
Width of condensed hyphae	–	–	4–10
Four-celled conidia	–	–	3–4x8–9

*isolates were cultured on CMA, the sizes were taken from 20 measurements and stated in micrometers (μm)

The morphological characters of the fungus are akin to the description of Brady (1979), Humber (1997) and Hoog *et al.* (2000). He described colonies of *Metarhizium* as broadly branched interwined conidiogenous cells which formed chains or cylindrical colonies, with conidia being ovoid and light green, and measuring < 9μm.

Determination of optimal temperature

Results for the growth of fungal colonies of *M. anisopliae* are presented in figure 4. The fungus showed maximum mycelial growth at 30°C (1.3 and 2.2 cm after two and four days, respectively). Minimum mycelial growth was observed at 20 and 37°C, and the mycelium growth was stopped at 38°C.

Optimal growth for many *M. anisopliae* isolates occur in the temperature range of 25 and 30°C (Dimbi *et al.* 2004; Devi *et al.* 2005; Bugeme *et al.* 2009). From this range, a temperature of 25°C was most frequently recorded (Quedraogo *et al.* 1997; Ekesi *et al.* 1999). Maximum growth at 30°C was observed for isolate, *M. anisopliae* V90 which showed the fastest growth (≈1.7 mm per day) at 30°C, and was surprisingly able to grow at 40°C (Hallsworth and Magan 1999).

Fungal growth at temperatures as low as 5°C, and as high as 35°C or above, has been observed in certain isolates; however, at these temperatures the growth rate is greatly reduced. It is around these extreme temperatures that most of the variation in growth among fungus isolates is most evident. Evidence of genetic groups associated with a low or high temperature growth was presented by Driver *et al*. (2000), Yip *et al*. (1992), and McCammon and Rath (1994). The growth rates of some isolates of *M. anisopliae* were evaluated at temperatures between 28 and 40°C by Brooks *et al.* (2004). They found that French and Brazilian isolates showed some growth at 37.5°C and no growth above that.

Characterization of *Metarhizium* species and varieties based on heat tolerance was carried out by Fernandes *et al.* (2010). In their study, conidial suspensions of *Metarhizium* isolates were exposed to wet heat of 45±0.2°C for 8 h, and plated on potato dextrose agar plus yeast extract (PDAY) medium. They divided the isolates into two groups: (i) all

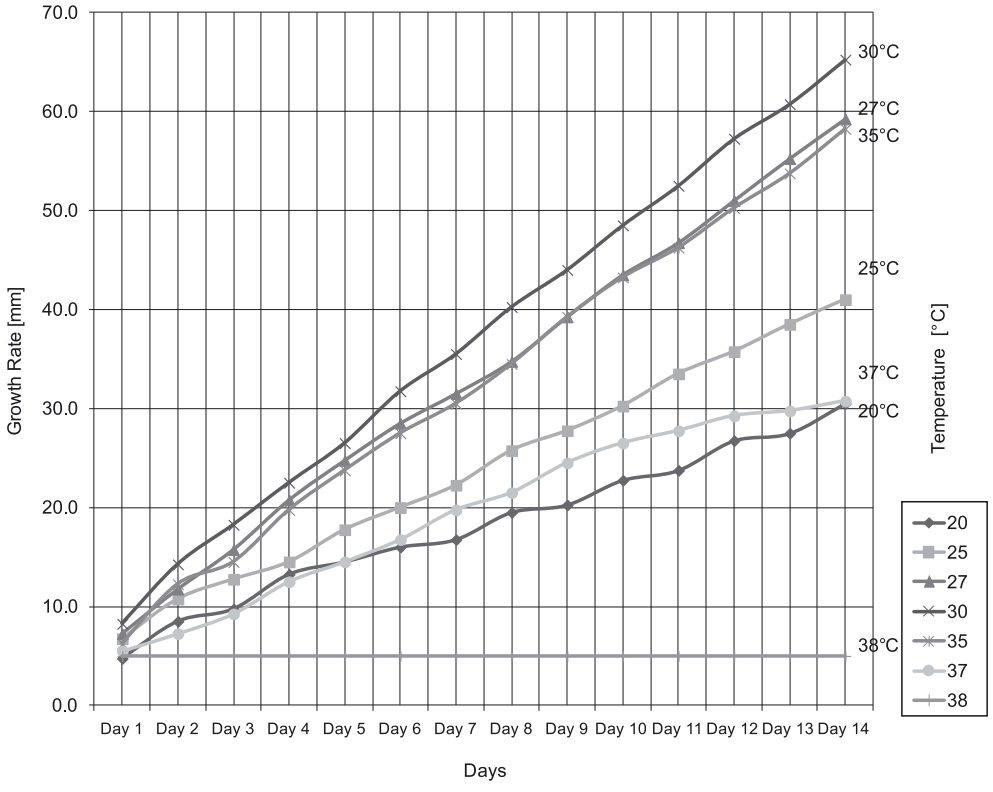

Fig. 4. Effect of different temperatures on growth rate of *M. anisopliae*, isolated from the suppressive soil of Boyer-Ahmad

isolates of *M. anisopliae* var. *anisopliae* (*Ma-an*) and *Metarhizium* from the *flavoviride* complex (*Mf*) had virtually zero conidial relative germination (RG), (ii) *M. anisopliae* var. *acridum* (*Ma-ac*) isolates demonstrated high heat tolerance (70–100% RG). Based on this study, heat and cold exposures can be used as quick tools to tentatively identify some important *Metarhizium* species and varieties.

Pathogenicity assays

According to the results obtained from the laboratory-pathogenicity-assays at seven days post-inoculation, the fungus caused mortality in second stage juveniles (Table 2, Fig. 5) but no mortality occurred in the control treatment. Fungal growth and sporulation of the isolate became manifest on the dead nematodes. Seven days after inoculation, significant differences were observed among the concentrations examined. At a concentration of 10^7, the mortality was 47.1%. At other concentrations, mortality varied between 14.9 and 29.6%, which are statistically at par with each other.

Till now, little work has been carried out investigating the effect of *M. anisopliae* on nematodes. A significant inhibitory effect of *M. anisopliae* on sugarcane nematodes has been reported by Zorilla (2001), and inhibition of the population growth of *Rotylenchus reniformis* has also been reported by Tribhuvaneshwar *et al.* (2008). These are the only records on nematophagous potential of *M. anisopliae*. Presently, some research work on this isolate has been started in the Laboratory of Nematology, Department of Plant Protection, Yasouj University in Iran, to determine the effect of the isolate on different nematodes.

The exact mode of action of *M. anisopliae* on nematodes is still unknown but it is likely similar to other fungi with sticky spores. The sticky spores will stick to the nematode at any point but mostly cause infection in the head region. They germinate, penetrate directly through the nematode cuticle, and produce infective hyphae within the body cavity. In the case of insects, the fungus spores attach to the surface of the insect, germinate and begin to grow. They then penetrate the exoskeleton of the insect and grow very rapidly inside the insect causing the insect to die.

In conclusion, our findings of a pathogenic fungal strain for soil nematodes increase the possibility for developing a commercial nematophagous fungus. The results obtained from this study showed that biological control of nematodes using *M. anisopliae* infection is feasible and can be considered an additional method of integrated management of nematodes under field conditions.

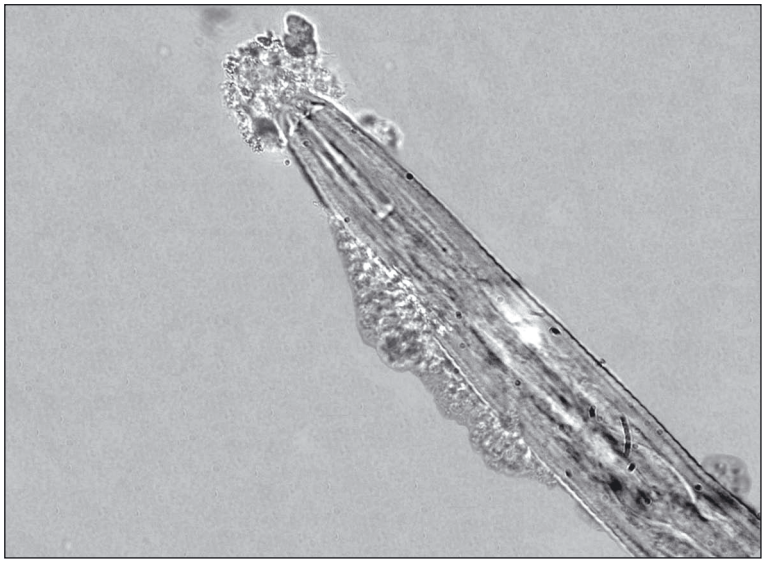

Fig. 5. J2s of *H. avenae* parasitized with *M. anisopliae*, in the pathogenicity test

Table 2. Effect of different concentrations of *M. anisopliae*, isolated from Boyer-Ahamd, against J2s of *H. avenae*

Spore Concentration	Parasitized Nematodes [%]
10^3	14.9[1]±5.4[2](8.6–18.6[3]) b
10^4	29.6±16.6(12.2–45.3) ab
10^5	20.1±8.8(12.4–29.7) b
10^6	26.7±8.9(18.3–36.2) b
10^7	47.1±0.58(46.6–47.7) a

*[1] mean, [2] CV%, [3] range

** figures followed by the same letter are not significantly different (p = 0.05) from each other

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