

Pathogenicity of Iranian isolates of *Metarhizium anisopliae* (Metschnikoff) (Ascomycota: Hypocreales) against *Trogoderma granarium* Everts (Coleoptera: Dermestidae)

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Received: 13. December 2010 / Accepted: 08. April 2011 / Available online: 10. April 2011

Abstract. The Pathogenicity of Iranian isolates of *Metarhizium anisopliae* was evaluated against adults and larvae of *Trogoderma granarium* using immersion bioassay method. For each isolate, five aqueous suspensions were prepared in a logarithmic series from 1×10^5 to 1×10^9 conidia/ml. Each concentration was replicated four times. For each replicate, 30 adults or larvae were immersed in 5 ml of the suspension for 5 s. The entire assay replicated twice. All isolates were pathogenic to adults and larvae and mortality was elevated with increasing conidial concentration. Significant differences were detected among fungal isolates at different conidial concentrations. The cumulative mortality 10 days after immersion in adults ranged between 3.33 and 20.83%, 15.83 and 70.83%, 5.93 and 80.5, 10.18 and 83.9% for different concentrations of IRAN 437C, IRAN 715C, IRAN 1018C and DEMI001, respectively. The cumulative mortality among larvae indicates greater efficacies for DEMI001 isolate (100% at 1×10^9 conidia/ml). For different concentrations of IRAN 437C, IRAN 715C and IRAN 1018C cumulative larval mortality ranged between 5.83 and 30.83%, 17.64 and 82.35%, 19.16 and 90%, respectively. LT_{50} values varied from 5.43 to 18.78 and 4.78 to 14.68 days for adults and larvae, respectively and IRAN 437C isolate for both adults and larvae demonstrated the longest LT_{50} . Probit analysis showed that there was no significant difference among IRAN 715C, IRAN 1018C and DEMI001 isolates and isolates of IRAN 437C with the LC_{50} of 1.2×10^{12} conidia/ml had lowest effect on adults. For larvae, DEMI001 isolate demonstrated the lowest LC_{50} (1×10^6 conidia/ml) and was significantly effective than other *M. anisopliae* isolates. Our research shows suitable susceptibility of *T. granarium* adults and larvae to Iranian isolates of *M. anisopliae* other than IRAN 437C and further experiments are recommended to determine details about other isolates for biocontrol of this storage pest.

Key words: *Metarhizium anisopliae*, Iranian isolates, *Trogoderma granarium*, immersion bioassay.

Introduction

Trogoderma granarium Everts (Coleoptera: Dermestidae) commonly known as khapra beetle is one of the serious pest of stored product (Burgess 2008). It is originated in India but has been distributed worldwide by trade. It occurs especially on cereals and cereal products including malt and noodles, oilseeds and oilcakes especially groundnuts, linseed, seeds of tomato, sorghum, copra, pulses and pulse products, various nut meals, dried fruits and compound animal feeding stuffs. This pest may also be present on empty sacks and other packing material. *T. granarium* infestation can be recognized by the presence of the pest (especially feeding larvae) on stored products. Early instar larvae feed on broken or damaged kernels, whereas older larvae feed on whole kernels. Larvae can occasionally be found as deep as 3–6m inside bulk grain. The larvae usually feed first on the germ portion of a cereal seed and then on the endosperm. Near the top 30 cm of the infested mass of stored products, numerous cast skins of larvae covered with long hairs are present. Infested material is also polluted with frass (Anonimus 2001, Hasan et al. 2006, Burgess 2008).

Larvae are known for their hardy nature and can withstand adverse conditions of insufficient food supply, long periods of starvation, high temperatures and low humidities. Larvae are also resistant to normal insecticide concentrations which are fatal to almost all the insect species living in the similar environment. Though polyphagous in habit, they prefer food rich in carbohydrates. Larvae can make use of large varieties of food grains including those that are not nutritious to many of the stored-product beetles (Banks 1977; Bell & Wilson 1995).

Application of insecticides is one means of preventing some losses during storage. However, the choice of insecticides for storage pest control is very limited because of the strict requirements imposed for the safe use of synthetic insecticides on or near food (Padin et al. 2002). The continuous use of chemical insecticides for control of storage grain pests has also resulted in serious problems such as resistance to the insecticides, pest resurgence, elimination of economically beneficial insects, and toxicity to humans and wildlife (Hendrawan & Ibrahim 2006).

Biological control agents are being considered as supplements or alternatives to synthetic chemical insecticides which are known to have toxic effects on nontarget organisms, including animals and humans. Entomopathogenic fungi are key regulatory factors of insect populations in nature and are attracting attention as biocontrol agents of insect pests (Weiguo et al. 2005). These organisms were the first group to be considered as biological control agents. Entomopathogenic fungi's potential in producing natural epizootics caused some attempts in improving them in IPM programs (Safavi et al. 2010)

Metarhizium anisopliae (Metschnikoff) Sorokin (Ascomycota: Hypocreales) is a mitosporic haploid fungus with a global distribution. It represents a pathogen for many insect species including a wide range of important agricultural pests, and therefore holds great potential for use as biological control agent (Butt et al. 2001).

The potential of entomopathogenic fungi *Beauveria bassiana* and especially *M. anisopliae* to control insect pests of stored products, particularly Coleopteran insects, has been investigated in several studies in recent years (Adane et al. 1996, Batta & Abu Safieh 2005, Kassa et al. 2002). Until pre-

sent, a limited number of published articles are available on biocontrol of stored-grain insects using Iranian isolates of entomopathogenic fungi, and all of these studies were carried out in the past three years. Some of Iranian isolates, such as DEMI001 (*M. anisopliae*) and IRAN 441C (*B. bassiana*), were very efficacious against different species of storage pests (Khashaveh et al. 2008a,b, Mahdneshin et al. 2008a,b, Mahdneshin et al. 2009).

To our best knowledge, this is the first paper evaluating the potential of entomopathogenic fungi especially, *M. anisopliae* for the control of *T. granarium*. The present study research was carried out to observe susceptibility of adult and larvae of *T. granarium* to Iranian isolates of *M. anisopliae* under controlled laboratory conditions.

Materials and Methods

Insect Rearing

Adults and larvae of *T. granarium* were used in the tests. Adults and larvae were taken from a culture that has been kept in the laboratory in Department of Entomology, Urmia University, Iran on wheat (mixture of whole and broken kernels) ($27 \pm 1^\circ\text{C}$ and $65 \pm 5\%$ RH) since 2005 with no history of exposure to insecticides. Adults of < 3 d old and larvae of uniform size with an average age of 20 ± 1 d old were used in the tests (Yinon & Shulov 1966).

Fungal Isolates

Four Iranian isolates of entomopathogenic fungus, *M. anisopliae* were used in experiments. The fungi were obtained from the collection maintained by the Department of Botany, Plant Pests and Diseases Research Institute, Tehran. Details of these isolates are given in Table 1.

Preparation of Conidial Suspension

Fungal isolates were cultured on Potato Dextrose Agar (PDA, Merck & Co., Inc, Germany) in 9 cm diameter Petri dishes and incubated in the dark at $25 \pm 1^\circ\text{C}$ for 15 days until cultures had sporulated. After this period, a mixture of conidia and hyphae were harvested by flooding the Petri dishes with sterile distilled water containing 0.05% (v/v) Tween 80 (Sigma Chemical, St. Louis, MO, USA) and agitating with glass rod. All samples were vortexed for 3 minutes to break up the conidial chains or clumps. Conidia were separated from hyphae and substrate materials by filtration of the suspension through five layers of cheese-cloth. The conidial concentrations were determined with a Haemocytometer (Improved Neubauer, 0.1 mm depth) (Kassa et al. 2002).

Viability of conidia was determined by spreading a drop of conidial suspensions onto the surface of glass slides held in Petri dishes lined with moistened sterile filter paper. Three glass slides per isolates representing three replicates were used and scored for germination after 24 h at $25 \pm 2^\circ\text{C}$. Conidia with germ tubes equal or greater than the width were considered to have germinated.

Dose - mortality bioassay by immersion method

For each isolate, on the basis preliminary tests, five aqueous suspensions were prepared in a logarithmic series from 1×10^5 to 1×10^9 conidia ml^{-1} in Tween 80 (0.05% v/v). Each concentration was replicated four times. For each replicate, 30 adults or larvae were treated by immersion for 5 s in 5 ml of the suspension. The insects were dipped into suspension together. The control insects were immersed in sterile distilled water with Tween 80 (0.05% v/v). The treated insects and the suspension (1 ml) were subsequently poured into a plate containing sterilized filter paper (9 cm diameter) and sealed with parafilm to prevent insects from escaping. The control insects were treated in the same way. The filter paper helped to absorb the excess moisture and increased conidial load on each insect by allowing secondary spore pick up (Adane et al. 1996). The treated insects were kept without food for 24 h at $27 \pm 1^\circ\text{C}$ and $70 \pm 5\%$ r.h. After 24 h, the treated insects in each replicate were transferred into glass pots (7cm diameter and 8.5cm height) with perforated lids containing 30 g wheat grains (variety Zarrin) and then kept at $27 \pm 1^\circ\text{C}$ and $70 \pm 5\%$ r.h. Mortality was recorded at every 48-h interval for 10 days. After the mortality count, dead insects from each immersion were washed in 70% ethanol, rinsed in sterile distilled water three times and kept separately in Petri dishes. These plates were then incubated in a plastic box with high relative hu-

midity (approximately 100%) to observe the outgrowth of fungus. These works were done for the control insects, too. The entire assay replicated twice.

A single concentration bioassay with five replicates and 20 insects for each replicate was conducted to determine LT_{50} values with 95% confidence limits (CL) for each of the 4 isolates. For each, an aqueous suspension containing 1×10^9 conidia/ml was prepared in Tween 80 (0.05% v/v)

Statistical Analysis

Control mortality was corrected for by using Abbott's (1925) formula. For dose-mortality bioassays, cumulative mortality percentage was normalized using arcsine transformation and subjected to analysis of variance (ANOVA) using SAS (2000) with insect mortality as the response variable and isolates and conidial concentrations as main effects. Means were separated by using the Duncan's Multiple Range Test at $P = 0.05$. Probit analysis was carried out by Minitab 14 to estimate both LC_{50} and LT_{50} of the isolates with 95% CL (Blair & Taylor 2007).

Results

Results of this study showed that the mean viability of conidia of all *M. anisopliae* isolates ranged from 89 to 95% (Table 1). The mortality within the control group was very low in both adults and larvae of *T. granarium* (ranged from 0.87% to 2.31%) and no fungal growth was observed on the dead control insects. In all isolates, *M. anisopliae* was pathogenic to adults and larvae of *T. granarium* in immersion bioassays but strain of IRAN 437C was less virulent to the adults and larvae of khapra beetle in comparison with other isolates. Mean percentage mortality of adults and larvae was elevated with increasing conidial concentration (Figs 1 & 2) and significant differences were detected among fungal isolates at different conidial concentrations. For adults, all main effects and associated interaction were significant for isolates ($\text{df} = 3, 79; F = 73.01; P = 0.0001$), concentrations ($\text{df} = 4, 79; F = 92.67; P = 0.0001$), isolates \times concentrations ($\text{df} = 12, 79; F = 3.63; P = 0.0004$). In the case of larvae, all main effects and associated interaction were significant for isolates ($\text{df} = 3, 79; F = 116.31; P = 0.0001$), concentrations ($\text{df} = 4, 79; F = 123.79; P = 0.0001$), isolates \times concentrations ($\text{df} = 12, 79; F = 5.23; P = 0.0001$). The cumulative mortality in adults exposed to isolates of *M. anisopliae* ranged between 3.33 and 20.83%, 15.83 and 70.83%, 5.93 and 80.5, 10.18 and 83.9% for different concentrations of IRAN 437C, IRAN 715C, IRAN 1018C and DEMI001, 10 days after immersion, respectively. The cumulative mortality among *T. granarium* larvae indicates greater efficacy for DEMI001 isolate. This is most noticeable at 1×10^9 conidia/ml where 100% mortality was achieved with DEMI001 isolate 10 days after immersion. At each concentration, mortality among larvae treated by DEMI001 isolate was higher than among larvae treated with other isolates. For different concentrations of IRAN 437C, IRAN 715C and IRAN 1018C cumulative mortality ranged between 5.83 and 30.83%, 17.64 and 82.35%, 19.16 and 90%, respectively.

For *M. anisopliae* isolates, LT_{50} values varied from 5.43 to 18.78 and 4.78 to 14.68 days for adults and larvae, respectively. Among *M. anisopliae* isolates, IRAN 437C isolate for both adults and larvae demonstrated the longest LT_{50} and based on non-overlap of 95% fiducial limits, significantly slow activity was observed rather than other *M. anisopliae* isolates and there was no significant difference among three other isolates (Table 2 & 3).

The linear relationship between the conidial concentrations of each fungal isolate and the adults and larvae mortal-

Table 1. The host, location and germination percentage of the isolates of *M. anisopliae* used in this study.

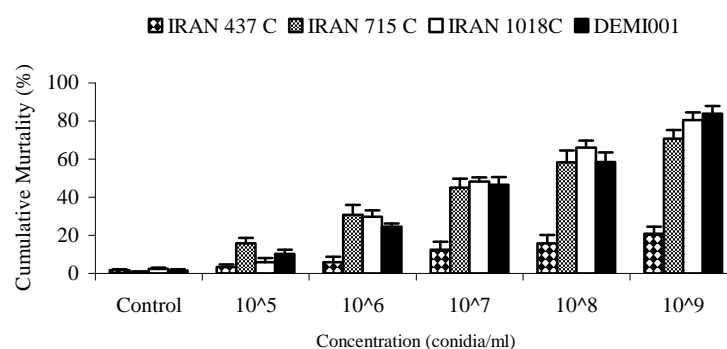
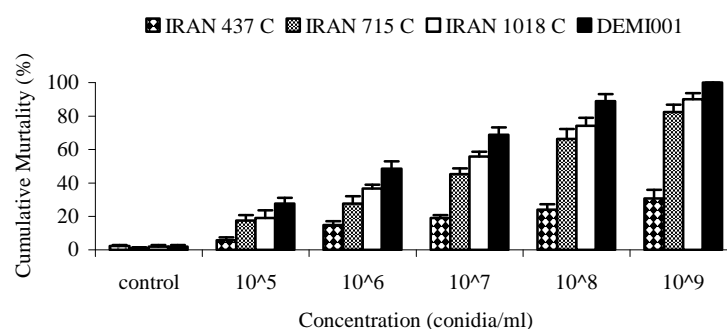
Isolate	Host (Order: Family)	Location (Country)	Germination (%)
IRAN 437C	<i>Chilo suppressalis</i> (Lep.: Pyralidae)	Rasht (Iran)	93 ± 3.3
IRAN 715C	Locust (Orth.: Acrididae)	Ahwaz (Iran)	89 ± 2.7
IRAN 1018C	<i>Parandra caspica</i> (Col.: Cerambycidae)	Nour (Iran)	91 ± 1.8
DEMI001	<i>Rhynchophorus ferrugineus</i> (Col.: Curculionidae)	Saravan (Iran)	95 ± 1.2

Table 2. LT₅₀ values with 95% confidence limits following immersion of *T. granarium* adults in aqueous suspension (1×10^9 conidia/ml) of *M. anisopliae* isolates.

Isolates	LT ₅₀ (days)	95% Confidence Limit	
		Lower	Upper
IRAN 437C	18.78	14.34	32.7
IRAN 715C	7.18	6.39	7.95
IRAN 1018C	6.94	5.62	8.84
DEMI001	5.43	5.21	6.57

Table 3. LT₅₀ values with 95% confidence limits following immersion of *T. granarium* larvae in aqueous suspension (1×10^9 conidia/ml) of *M. anisopliae* isolates.

Isolates	LT ₅₀ (days)	95% Confidence Limit	
		Lower	Upper
IRAN 437C	14.68	12.18	20.21
IRAN 715C	6.52	5.99	6.96
IRAN 1018C	5.59	4.03	7.46
DEMI001	4.78	3.5	6.12

**Figure 1.** Cumulative corrected mortality (%) ± S.E. of *T. granarium* adults within 10 days after immersion in aqueous conidial suspensions of *M. anisopliae* isolates.**Figure 2.** Cumulative corrected mortality (%) ± S.E. of *T. granarium* larvae within 10 days after immersion in aqueous conidial suspensions of *M. anisopliae* isolates.

ity was attained using probit analysis (Table 4 & 5). The test for the goodness of fit indicates no significant heterogeneity in the linear relationships for all fungal isolates tested (small or very small χ^2 values, $P > 0.05$). For Adults, based on the estimates of the LC₅₀ and associated 95% CL and observed overlap, there was no significant difference between the three isolates IRAN 715C, IRAN 1018C and DEMI001 with the LC₅₀ of 2.1×10^7 , 1.7×10^7 , and 2.8×10^7 conidia/ml, respectively. In contrast, the isolates of IRAN 437C with the LC₅₀ of 1.2×10^{12} had lowest effect on adults mortality of khapra beetle (Table 4). Among *M. anisopliae* isolates, the iso-

late DEMI001 demonstrated the lowest LC₅₀ (1×10^6 conidia/ml) and was significantly effective than other *M. anisopliae* isolates against *T. granarium* larvae based on non-overlap of 95% CL (Table 5).

Discussion

Insects suffer from a range of diseases, including some caused by fungi, which can be used in a very direct way, similar to the use of contact insecticides. When spores land

Table 4. LC₅₀ values with 95% confidence limit (CL) and probit analysis parameters for adults of *T. granarium* 10th day after immersion in aqueous conidial suspensions of different isolates of *M. anisopliae*.

Isolates	LC ₅₀	95% CL	Probit Parameters ± S.E.		P	χ ²
			Intercept	Slope		
IRAN 437C	1.2 × 10 ¹²	4.6 × 10 ¹⁰ – 2.2 × 10 ¹⁵	1.99 ± 0.4	0.25 ± 0.05	0.81	0.95
IRAN 715C	2.8 × 10 ⁷	1.4 × 10 ⁷ – 5.7 × 10 ⁷	2.18 ± 0.28	0.37 ± 0.04	0.87	0.69
IRAN 1018	1.7 × 10 ⁷	1 × 10 ⁷ – 2.8 × 10 ⁷	1.05 ± 0.32	0.54 ± 0.04	0.15	5.29
DEMI001	2.1 × 10 ⁷	1.2 × 10 ⁷ – 3.4 × 10 ⁷	1.1 ± 0.32	0.53 ± 0.04	0.44	2.96

Table 5. LC₅₀ values with 95% confidence limit (CL) and probit analysis parameters for larvae of *T. granarium* 10th day after immersion in aqueous conidial suspensions of different isolates of *M. anisopliae*.

Isolates	LC ₅₀	95% CL	Probit Parameters ± S.E.		P	χ ²
			Intercept	Slope		
IRAN 437C	1 × 10 ¹¹	7.9 × 10 ⁹ – 2.1 × 10 ¹³	2.46 ± 0.33	0.23 ± 0.04	0.58	1.96
IRAN 715C	1.3 × 10 ⁷	7.9 × 10 ⁶ – 2.3 × 10 ⁷	1.62 ± 0.3	0.47 ± 0.04	0.83	0.88
IRAN 1018	4.7 × 10 ⁶	2.8 × 10 ⁶ – 7.7 × 10 ⁶	1.5 ± 0.31	0.52 ± 0.04	0.95	0.35
DEMI001	1 × 10 ⁶	6.6 × 10 ⁵ – 1.7 × 10 ⁶	1.02 ± 0.34	0.66 ± 0.05	0.15	5.13

on the cuticle of an insect to which they are adapted, they will germinate and force their way through the cuticle of the insect into the body, thus killing the insects. If conditions are suitable, the fungus will sporulate and release more spores, continuing the cycle. These spores can be used as biological pesticides, to produce high levels of kill with materials that are very much safer than many conventional insecticides (Anonymous 1998). Laboratory assessment of entomopathogenic fungi is an essential step in identifying virulent strain prior to field or large scale use. Entomopathogenic fungi are being developed worldwide for the control of insect pests and some products are already available commercially (Ekessi et al. 2001).

Post-mortem mycelial and conidial growth demonstrated that most of the insects had died due to the pathogens. Obtained results indicated that isolate IRAN 437C was less virulent to the *T. granarium* in comparison with other isolates and only 20.83 and 30.83% mortality were recorded for adults and larvae, respectively, at 1 × 10⁹ conidia/ml 10 days after immersion. Probit analysis of data from dose-mortality bioassay by adults of *T. granarium* indicates that apart from IRAN 437C, there was no significant difference between the three isolates IRAN 715C, IRAN 1018C and DEMI001 and these isolates have the same efficacy. In contrast, for larvae, significant difference was observed between isolates and lowest LC₅₀ (1 × 10⁶) was noted for DEMI001 isolate. Therefore, it can provide better control of *T. granarium* larvae.

Several published data are available on the efficacy of the entomopathogenic fungi against stored-grain insect pests (Batta 2004a,b, Cherry et al. 2005, Ekesi et al. 2001, Rodrigues & Pratisoli 1990) but there are not any references about susceptibility of *T. granarium* to these fungi to be compared with results obtained in this study. Potential of Iranian isolates of *M. anisopliae* were investigated in several studies. Mahdeshin et al. (2009) studied the efficacy of Iranian isolates of *M. anisopliae* against adults of *Rhyzopertha domonica* Fabricius (Coleoptera: Bostrichidae). They reported that isolates DEMI001 and IRAN 715C were effective against this pest and recorded 79 and 75% mortality, respectively, at the concentrations of 4.6 × 10⁹ conidia/ml. They also demonstrated that isolates DEMI001 and IRAN 715C were virulent for

adult of *Callosobruchus maculatus* F. (Col.: Bruchidae). The mortality due to fungal infections was 81% and 84% for DEMI001 (5.2 × 10¹⁰ conidia/ml) and IRAN 715C (1 × 10¹¹ conidia/ml) (Mahdeshin et al. 2008a). In the same study, Khashaveh et al. (2008a) indicates that *Sitophilus granarius* (Col.: Corcolionidae) was susceptible to Iranian Isolates of *M. anisopliae*.

Study on Iranian isolates of *B. bassiana*, *M. anisopliae* and *Lecanicillium psalliotae* by Pirali-Kheirabadi et al. (2007) for biological control of *Rhipicephalus annulatus* demonstrated that all isolates were pathogenic and 90% adult's mortality, 89.1% decrease in egg hatchability and 88.69% reduction in reproductive efficiency of the ticks were observed using 1 × 10⁷ conidia/ml of IRAN 437C isolate. These results revealed that this isolate was very virulent to various stages of tick developmental cycle. But primary study on the pathogenicity of Iranian isolates of *M. anisopliae* by Khashaveh et al. (2008b) against three species of storage pests indicate IRAN 437C isolate was less virulent than other isolates to storage pests and mortality that did not exceed 20% even at 1 × 10⁹ conidia/ml 11 days after immersion. These results confirmed our results and represented this isolate as not being suitable for biological control of Coleopteran storage pests.

In conclusion, the presented data showed that native entomopathogenic fungi can infect the adults and larvae of *T. granarium* but their efficiency varies considerably according to the fungal isolates and conidial concentrations. This is the first report that demonstrates the pathogenic effect of an entomopathogenic fungus against *T. granarius*. Our research shows the adequate susceptibility of *T. granarium* adults and larvae to Iranian isolates of *M. anisopliae* other than IRAN 437C. However, further experiments are recommended to determine details about other isolates which maybe appropriate for biocontrol of this storage pest.

Acknowledgments. The authors are grateful to Dr. Rasol Zare and Dr. Mehran Ghazavi for providing the fungal isolates for experimentation. The resources made available by Urmia University are also gratefully acknowledged.

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