Laboratory Bioassay of Iranian Isolates of Entomopathogenic Fungus *Metarhizium anisopliae* (Metsch.) Sorokin (Ascomycota: Hypocreales) against two Species of Storage Pest

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Summary

The susceptibility of adults of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) and *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae) to three Iranian Isolates of the entomopathogenic fungi *Metarhizium anisopliae* (Metsch.) Sorokin was evaluated through bioassays with direct immersion under laboratory conditions. For each isolates, five aqueous suspensions were prepared in a logarithmic series in Tween 80 (0.05% v/v). Results showed that adult of both species were susceptible to all isolates of *M. anisopliae*. For all three isolates, mortality percentage of the two species increased with increasing conidial concentration and significant difference was observed between concentrations. The corrected cumulative mortality of adult of *O. surinamensis* 10 days after immersion ranged from 12.38 to 85.84%, 18.6 to 62.83% and 10.63 to 77.87% for different concentrations of DEMI001, IRAN 715C and IRAN 1018C, respectively. These amounts for *T. castaneum* varied from 31.07 to 74.78%, 26.02 to 75.61% and 23.33 to 89.99% for different concentrations of DEMI001, IRAN 715C and IRAN 1018C, respectively. The parameters of probit analysis demonstrated non-overlap of 95% confidence limits of LC_{50} and LC_{95} and significant difference was observed among three isolates tested against each insect. The lowest and the highest LC_{50} and LC_{95} values were observed in the isolates DEMI001 for *O. surinamensis* ($3/1\times10^{5}$ and $1/5\times10^{8)}$ and IRAN 715C for *T. castaneum* (6.2×10^{8} and 6.9×10^{14}), respectively. This observation highlights the importance and need of screening for more virulent isolates against storage pests for use in the management of these pests.

Key words

Merarhizium anisopliae, Biological control, Storage pest, Immersion bioassay

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Introduction

Cereals are an important component of both human and livestock diets. Stored cereals are vulnerable to attack by a range of insect and mite pests. Stored-product insects can have a large economic impact on stored bulk grain and processed commodities (Hagstrum and Flinn, 1995). Control of these insect populations in stored food, feedstuffs and other agricultural commodities around the world is primarily dependent upon continued applications of organophosphorus and pyrethroid insecticides and the fumigants methyl bromide and phosphine. Although effective, their repeated use for decades has disrupted biological control by natural enemies and led to outbreaks of other insect species and sometimes resulted in the development of resistance to pesticides. It has had undesirable effects on non-target organisms, and fostered environmental and human health concerns (Salunke et al., 2005). Public awareness of the risks inherent to the use of pesticides stimulated the search for and development of environmentally friendly alternatives for the control of insect pests (Sadeghi et al., 2006).

Biological control has received increased attention over the past few decades as an alternative to chemical treatment or as a component of integrated pest management (IPM) strategies. Entomopathogenic fungi have been shown to be effective biological control agents against several insect pests (Kassa et al., 2002). Several studies have shown the potential for entomopathogenic fungi to control a range of stored product insects (Searle and Doberski, 1984; Adane et al., 1996; Moino et al., 1998; Rice and Cogburn, 1999; Sheeba et al., 2001; Dal Bello et al., 2001; Cherry et al., 2005; Khashaveh et al., 2011a, b). Biological control with entomopathogenic fungi, apart from being environmentally benign, is likely to be cheap in the long-term as the bio-control agent will be self-perpetuating (Mazodze and Zvoutete, 1999).

Metarhizium anisopliae (Metsch.) Sorokin (Ascomycota: Hypocreales) is a broad host range entomopathogenic fungus first recognized as a potential candidate for biological control of agriculture pests in the 1880s (Bittencourt et al., 2004). This fungus invades host generally through the cuticle. After attaching to a susceptible insect, conidia swell, then germinate and form germ tube and an aspersoria structure. Fungus finally penetrates the insect cuticle and host invasion involves mechanic and enzymatic activity by the fungus (Michalaki et al., 2006). Entomopathogenic fungi such as *M. anisopliae* are considered safe to nontarget organisms and beneficial insects such as predators, parasitoids, and honey bees in the field that make them more attractive (Ekesi et al., 1999; Kanga et al., 2003).

In this paper, the potential of three Iranian isolates of *M. anisopliae* was evaluated against two important species of storage pest under controlled laboratory conditions. The present study research was carried out to observe adult susceptibility of Red Flour Beetle, *Tribolium castaneum* Herbst (Tenebrionidae: Coleoptera) and Saw-Toothed Grain Beetle, *Oryzaephilus surinamensis* L. (Silvanidae: Coleoptera) to *M. anisopliae*.

Materials and methods

Source and rearing of insect: Adults of *T. castaneum* and *O. surinamensis* were used in the tests. Adult of these insects were taken from a culture that has been kept in the laboratory in Department of Entomology, Urmia University, Iran since 2005 with no history of exposure to insecticides. The *T. castaneum* adults were reared on wheat flour plus 5% brewer's yeast (by weight) and adults of *O. surinamensis* were reared on pearl barley and 5% brewer's yeast (by weight); at 27±1°C, 70%±5% rh and continuous darkness.

Source of fungal isolates: Three Iranian isolates of entomopathogenic fungus, *M. anisopliae* were obtained from the collection maintained by the Plant Protection Research Institute, Tehran, Iran. Details of these isolates are given in Table 1.

Production of conidial suspension: All fungal isolates were cultured on Potato Dextrose Agar (PDA, Merck & Co., Inc, Germany) in 9 cm diameter Petri dishes and incubated in a photoperiod of 16:8 hours (L:D) at 25±1°C and 75±5% relative humidity (rh) for 15 days for complete sporulation. After this period, a mixture of conidia and hyphae was 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. Tween 80 is a polyethylene sorbitol ester that is used for emulsifying and dispersing conidia in distilled water. All samples vortexed for 3 min. to break up the conidial chains or clumps. Conidia were separated from hyphae and substrate materials by filtration of the suspension through a filter cloth (Calbiochem, Darmstadt, Germany). The conidia concentration was counted with 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±2°C. Conidia with germ tubes equal or greater than the width were considered to have germinated.

Bioassay by immersion method: Five aqueous suspension were prepared from 1×10^4 to 1×10^8 conidia mL^{-1} in Tween 80 (0.05% v/v) for primary experiments. On the basis of preliminary tests, for each isolate and insect, five concentrations of conidia were prepareted for main experiments (Robertson and Preisler, 1992). Each concentration was replicated four times. For each replicate, thirty older than three days old adults treated by im-

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 (%)
DEMI001	*Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)	Saravan (Iran)	93 ± 2.2
IRAN 715C	Locust (Orthoptera: Acrididae)	Ahwaz (Iran)	90 ± 1.9
IRAN 1018C	*Parandra caspica* (Coleoptera: Cerambycidae)	Nour (Iran)	91 ± 1.8

mersion for 5 s in 10 mL suspension. The control insect treated 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 filter paper (9 cm diameter) and sealed with parafilm to prevent insects from escaping. The filter paper helped to absorb the excess moisture and increased conidial load in each insect by allowing secondary spore to pick up (Adane et al., 1996). The treated insects were kept without food for 24 h at 25±1° C and 75±5% rh. After 24 h, the treated insects in each replicate were transferred into glass pots (7 cm diameter and 8.5 cm height) with perforated lid containing 30 g wheat flour and then kept at 25±1° C and 75±5% rh for 10 days. The mortality was recorded at 48 h interval for 10 days. Dead insects from each treatment 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 r.h. (approximately 100%) to observe the outgrowth of fungus. These works were done for the control insects, too. The entire assay repeated twice and average data obtained from them were introduced into the statistical software for analysis.

Statistical analysis: Control mortality was corrected by using Abbott's (1925) formula. For dose-mortality bioassay, cumulative mortality percentage was normalized using arcsine transformation and subjected to analysis of variance (ANOVA) using SAS (1999). Means were separated by using the Tukey-Kramer honestly significant difference test at $P = 0.05$. Probit analysis was used to estimate both LC_{50} and LC_{95} of the isolates with 95% Confidence Limits (CL) after 10 days (SPSS, 2002).

Result and discussion

Mean viability of conidia of all *M. anisopliae* isolates ranged from 90% to 93% (Table 1). The mortality within the control group was very low in each of two species and no fungal growth was observed on the control insects (*T. castaneum* = 0.83±0.83%, *O. surinamensis* = 5.83±1.59%).

At all isolates, *M. anisopliae* was pathogenic and post-mortem mycelia and conidial growth demonstrated that most of the tested insects died due to the pathogens (Figure 1 and 2). Mortality percentage for adults of each of two species increased with increasing conidial concentration and significant difference was observed between concentrations (DEMI001-*T. castaneum*: df=4, F=19.819, P<0.0001), IRAN 715C-*T. castaneum*: df=4, F=37.518, P<0.0001), (IRAN 1018C-*T. castaneum*: df=4, F=22.307, P<0.0001), (DEMI001-*O. surinamensis*: df=4, F= 50.998, P<0.0001), (IRAN 715C-*O. surinamensis*: df=4, F=16.864, P<0.0001) and (IRAN 1018C-*O. surinamensis*: df=4, F=45.532, P<0.0001). The percentage of cadavers that supported fungal sporulation in different isolates at high and low concentration ranged from 62.83% to 89.99% and 10.63% to 32.47%, respectively (Tables 2 and 3).

The parameters of the probit analysis and LC_{50} and LC_{95} are given in Tables 4 and 5. The lowest LC_{50} ($3/1\times10^5$ Conidia mL^{-1}) and LC_{95} ($1/5\times10^8$ Conidia mL^{-1}) values were observed in the isolate DEMI001 for *O. surinamensis*. In contrast, the highest LC_{50} (6.2×10^8 Conidia mL^{-1}) and LC_{95} (6.9×10^{14} Conidia mL^{-1}) values were recorded in the isolate IRAN 715C for *T. castaneum*. The parameters of the probit analysis for *O. surinamensis* and *T. castaneum* indicate no overlap between 95% confidence limit of LC_{50} and LC_{95} values. This means that the efficacy of three isolates against *O. surinamensis* and *T. castaneum* is different. These results demonstrated that the isolates DEMI001 and IRAN 1018C had better efficacy on *O. surinamensis* and *T. castaneum*, respectively. Also, the results obtained from mean separation indicate that significant difference was observed among conidia concentrations and high concentration was more virulent that is rational. This observation highlights the importance and need of screening for more virulent isolates against storage pests for use in the management of these pests.

Figure 1. Post-mortem mycelia and conidial growth in *Oryzaephilus surinamensis* infected by *Metarhizium anisopliae* (isolate IRAN 1018C)

Figure 2. Post-mortem mycelia and conidial growth in *Tribolium castaneum* infected by *Metarhizium anisopliae* (isolate DEMI001)

Table 2. Cumulative mortality percentage (corrected) ± S.E. of *O. surinamensis* adults 10th day after immersion in aqueous conidial suspensions of three isolates of *M. anisopliae*[*]

Isolates	Concentration (Conidia mL^{-1})					F	P
DEMI001	5.8×10^{3}	4.5×10^{4}	3.4×10^{5}	2.7×10^{6}	2.1×10^{7}		
	12.3±1.6 d	32.7±3.2 c	50.4±2.5 bc	72.5±5.4 ab	85.8±4.3 a	50.99	<0.0001
IRAN 715C	1.1×10^{5}	1.1×10^{6}	1.1×10^{7}	1.1×10^{8}	1×10^{9}		
	18.6±3.2 c	32.7±3.2 bc	41.5±4.6 b	47.7±0.8 ab	62.8±5.8 a	16.86	<0.0001
IRAN 1018C	4.3×10^{4}	2.8×10^{5}	1.9×10^{6}	1.3×10^{7}	8.5×10^{7}		
	10.6±3.0d	29.2±3.2 c	47.7±4.18b	73.4±4.6 a	77.8±4.65a	45.53	<0.0001

[*]Mean within a row followed by the same letter do not differ significantly by Tukey-Kramer test at P=0.05.

Table 3. Cumulative mortality percentage (corrected) ± S.E. of *T. castaneum* adults 10th day after immersion in aqueous conidial suspensions of three isolates of *M. anisopliae**

Isolates	Concentration (Conidia mL^{-1})					F	P
DEMI001	3.3×10^{5}	2.3×10^{6}	1.6×10^{7}	1×10^{8}	7.4×10^{8}		
	31.0±2.1 d	37.8±3.2 cd	50.4±2.1 bc	59.6±5.3b	74.7±4.8 a	19.81	<0.0001
IRAN 715C	2.8×10^{6}	3.9×10^{7}	5.4×10^{8}	7.5×10^{9}	1.1×10^{11}		
	26.0±1.9 d	41.2±2.1 c	44.5±0.9 c	59.6±2.3 b	75.6±5.3 a	37.51	<0.0001
IRAN 1018C	1.8×10^{5}	7.2×10^{5}	2.7×10^{6}	1.7×10^{7}	4.7×10^{7}		
	23.3±4.0 d	41.6±3.9 cd	54.1±2.8 bc	80.8±2.8 ab	89.9±7.0 a	22.30	<0.0001

*Mean within a row followed by the same letter do not differ significantly by Tukey-Kramer test at P=0.05.

Table 4. LC_{50} and LC_{95} values (Conidia/mL) (with 95% confidence limit) and probit analysis parameters for adults of *O. surinamensis* 10th day after immersion in aqueous conidial suspensions of three isolates of *M. anisopliae*

Isolate	LC_{50}	LC_{95}	χ^2	P	Slop (b)	Intercept (a)
DEMI001	3.1×10^{5} ($1.96\text{-}4.92\times10^{5}$)	1.5×10^{8} ($5.8\times10^{7}\text{-}5.7\times10^{8}$)	0.713	0.87	0.611	1.638
IRAN 715C	8.5×10^{7} ($3.3\times10^{7}\text{-}3\times10^{8}$)	6.18×10^{13} ($3.5\times10^{12}\text{-}1.3\times10^{17}$)	1.292	0.731	0.280	2.775
IRAN 1018C	2.4×10^{6} ($1.58\text{-}3.9\times10^{6}$)	1.1×10^{9} ($1.7\times10^{8}\text{-}7.7\times10^{9}$)	4.707	0.195	0.615	1.062

Table 5. LC_{50} and LC_{95} values (Conidia/mL) (with 95% confidence limit) and probit analysis parameters for adults of *T. castaneum* 10th day after immersion in aqueous conidial suspensions of three isolates of *M. anisopliae*

Isolate	LC_{50}	LC_{95}	χ^2	P	Slop (b)	Intercept (a)
DEMI001	1.3×10^{7} ($4.6\times10^{6}\text{-}2.7\times10^{7}$)	8×10^{11} ($7.6\times10^{11}\text{-}4.2\times10^{13}$)	0.94	0.816	0.344	2.548
IRAN 715C	6.2×10^{8} ($2.5\times10^{8}\text{-}1.5\times10^{9}$)	6.9×10^{14} ($3.9\times10^{13}\text{-}7.3\times10^{16}$)	2.503	0.475	0.271	2.61
IRAN 1018C	1.5×10^{6} ($1.1\text{-}2.1\times10^{6}$)	1.4×10^{8} ($7.4\times10^{7}\text{-}3.4\times10^{8}$)	1.631	0.652	0.835	-0.168

According with our scrutiny, since 1984, all investigations for efficacy determination of entomopathogenic fungi against *T. castaneum* and *O. surinamensis* were carried out with *Beauveria bassiana* (Balsamo) Vuillemin (Searle and Doberski, 1984; Padin et al., 1997; Akbar et al., 2004; Wakefield et al., 2005; Khashaveh et al., 2011a) and only a few published works are available on efficacy of *M. anisopliae* against *T. castaneum*. Batta and Abu Safieh (2005) reported that the *M. anisopliae* (Isolate Meta 1) at the rate of 6.5×10^{8} conidia/gram of wheat grains caused 46.7% mortality in adults of *T. castaneum*. The efficacy of isolate Meta 1 also was examined against the other flour beetle, *Tribolium confusum* Du Val by Michalaki et al., (2006). They recorded <55% mortality for larvae of this pest at the rate of 8×10^{10} conidia/kg wheat and flour 7 d after treatment. Both of these experiments represent the relative efficacy of *M. anisopliae* against genus *Tribolium*.

Unfortunately, there are not any references about susceptibility of *O. surinamensis* to *M. anisopliae* to be compared with results obtained in this study. This is the first report that characterizes the pathogenicity of *M. anisopliae* on adults of *O. surinamensis* in laboratory. The obtained results indicate admissible susceptibility of adults of this pest to all tested isolates. Approximately 86%, 63% and 78% mortalities were recorded in bioassay with high concentration of isolates DEMI001, IRAN 715C and IRAN 1018C, respectively.

Other investigators have reported that treatment of these two species of stored grain pests with entomopathogenic fungus, *B. bassiana* can be effective. Searle and Doberski (1984) used an isolate of *B. bassiana* for control of adults of *O. surinamensis*. They recorded 100% mortality with 10^{6} conidia/mL and noted that effective treatment requires a period of high humidity near

to dew point. Also Wakefield et al. (2005) in their investigation indicated that some *B. bassiana* isolates can provide 100% mortality in *Oryzaephilus surinamensis* (organophosphate resistant strain) 10 d after treatment in 1×10^8 conidia/mL. Padin et al. (1997) and Rice and Cogburn (1999) reported up to 80-100% mortality at 10 to 20 days post-treatment in *T. castaneum* with *B. bassiana*.

As mentioned above, there is not any adequate data about efficacy of *M. anisopliae* on these two species of storage pest but potential of Iranian isolates of *M. anisopliae* against different storage pests was investigated in several studies (Khashaveh et al., 2008; Mahdneshin et al., 2009; Mahdneshin et al., 2011; Khashaveh et al., 2011b). The high performance of Iranian isolate of *M. anisopliae* against larvae and adults of khapra beetle, *Trogoderma granarium* (Coloptera: Dermestidae) was demonstrated in another study. One hundred percents and 83.9 % mortality were achieved by isolate DEMI001 10 days after treatment with the same condition as in the present study by the conidial concentration of 1×10^9 for larvae and adults, respectively (Khashaveh et al., 2011b). Also, Mahdneshin et al. (2009, 2011) proved that isolates DEMI001 and IRAN 715C had good proficiency for control of Cowpea Weevil, *Callosobruchus maculatus* F. (Coloptera: Bruchidae) and Lesser Grain Borer, *Rhyzopertha domonica* F. (Coleoptera: Bostrichidae).

Conclusion

As a conclusion, the efficiency of Iranian isolates of *M. anisopliae* against storage pests can be strongly asserted. Based on the results of our study, we think that fungal entomopathogens could be used in the control of storage pests. However, further investigations are strongly recommended to be carried out on the possibility of field application of highly virulent isolates as well as finding other isolates of entomopathogenic fungi that have potential as biopesticides against important storage pests.

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