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Full Length Research Paper

Laboratory evaluation of the entomopathogenic fungi

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In this study, the pathogenicity of five Iranian isolates of *Beauveria bassiana* and *Metarhizium anisopliae* was evaluated against adults of *Callosobruchus maculatus* adults by immersion bioassay method at 27 ± 1°C and 60 ± 5% R.H. under laboratory conditions. Cumulative mortality of the cowpea weevil at day 11 after treatment with *B. bassiana* isolates IRAN 187C, IRAN 429C, IRAN 441C and *M. anisopliae* isolates IRAN 715C and DEM I001 by conidial suspensions at low and high concentration ranged between 29.4 to 86.2, 9 to 88.3, 21.1 to 96.3, 24.8 to 84.4 and 20.9 to 80.9%, respectively. The LC_{50} and LT_{50} values for IRAN 187C, IRAN 429C, IRAN 441C, DEMI001 and IRAN 715C were 1.9×10^{7}, 9.8×10^{6}, 2.4×10^{7}, 2.6×10^{8} and 1.2×10^{8} conidia/ml and 7.8, 6.7, 7.2, 7.7 and 7.8 day, respectively. IRAN 441C of *B. bassiana* had the highest virulence against adult cowpea weevil because it had lower LC_{50} and LT_{50} and caused the highest mortality (76%) in treatment by suspensions containing 1×10^{8} conidia/ml. *B. bassiana* had higher virulence than *M. anisopliae* against adult of cowpea weevil.

Key word: Biological control, immersion bioassay, virulence, cowpea weevil.

INTRODUCTION

Stored-products are vulnerable to attack by arthropod pests which may result in damage to stored-products and subsequent economic losses (Talukder et al., 2004). The stored-product pests cause 10 to 25% annual damages in stored-products in Iran (Bagheri, 1986). The cowpea weevil, *Callosobruchus maculatus* F. (Col: Bruchidae) causes both quantitative and qualitative damages to legume. Quantitative damage is due to grain weight loss caused by insect feeding (Padin et al., 2002). Qualitative damage is due to product alterations such as loss of nutritional and aesthetic values. The development of a single larva in a grain can lead to weight losses of 8 to 22% (Credland et al., 1986).

The control of *C. maculatus* currently has been made by using of fumigants and residual chemical insecticides (Jackai and Adalla, 1997). However, the choice of pesticides for storage pest control is very limited because of the strict requirements imposed for the safe use of synthetic insecticides on or near food the continuous use of chemical pesticides for control of stored pests has resulted in serious problems such as food contamination and insecticide resistance (Padin et al., 2002).

Under these conditions, it is necessary to find out safer

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alternative control strategies such as the use of microbial control agents against stored-product insect pests. Using fungal biocontrol agents and selected insecticides can potentially reduce the use of chemical insecticides and their subsequent their residues side effects in agriculture. *Beauveria bassiana* and *Metarhizium anisopliae* are naturally occurring entomopathogenic fungi with a wide host range (Tanada and Kaya, 1993). Several studies have shown that entomopathogenic fungi *B. bassiana* and *M. anisopliae* are effective on stored products pests such as S*itophillus oryzae* (L.)*, Rhyzopertha dominica* (F.) and *Acanthoscelides obtectus* Say (Batta, 2004; Batta and Abu, 2005; Michalaki et al., 2006; Kavallieratos et al., 2006; Dal Bello et al., 2001).

In this study, the effects of 3 isolates of *B*. *bassiana*, and two isolates of *M. anisopliae* were evaluated against *C. maculatus* under laboratory conditions.

MATERIALS AND METHODS

Insect rearing

Adults of *C. maculatus* were reared under laboratory conditions (27 ± 1°C and 60 ± 5% RH.) in glass jars (250 ml) covered with a piece of fine cloth. Insects were from a laboratory population maintained on untreated cowpea seeds since 2005. Cowpea grains of local variety Urmia were stored at -8^{o}C for one week to eliminate natural infestations. Cowpea grains were distributed in glass jars covered with metal gauze lids at a rate of 150g per jar. Several male and female adults of *C. maculatus* of mixed sex and age were placed in each jar to allow oviposition and then removed by sieving 72 h later. Infested grains were incubated at 27 ± 1°C and 60 ± 5% R.H. with a natural photoperiod.

Fungal isolates

Two Iranian isolates of *M. anisopliae* and three isolates of *B. bassiana* were obtained from the collection maintained by the Plant Protection Institute, Tehran, Iran (Table 1).

Preparation of conidial suspension and conidial germination

Fungal isolates were grown on Potato Dextrose Agar (PDA, Merck Co., Inc., Germany) in 8 cm diameter Petri dishes and incubated under dark conditions at 27°C for 14 days for complete sporulation. Fungal conidia were collected by scraping away of conidial layer using sterilized scalpel. 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. All sample vortexes 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 five layers of cheese-cloth.

The conidial concentration was counted with haemocytometer (Improved Neubauer, 0/1 mm depth). 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 isolate 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.

Dose mortality bioassay by immersion method

The dose setting bioassay was carried out to determine 5 different conidial concentrations in distilled water plus Tween 80 (0.05% v/v) based on the logarithmic scale. For each replicate, thirty individuals of one day adults of *C. maculatus* were treated by immersion for 5 sec in 5 ml of conidial suspensions. The control insects were treated with sterile distilled water plus Tween 80 (0.05% v/v).

The treated insects and the suspension (1 ml) were subsequently poured into a plate containing a sterile filter paper (9cm diameter) and sealed with parafilm to prevent insects to escape. 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 ± 1^{o}C and 60 ± 5% R.H. After 24 h, the treated insects were transferred into glass pots (7 cm diameter and 8.5cm height) with perforated lid containing 30g cowpea for simulating the natural condition and then kept at 27 ± 1^{o}C and 60 ± 5% R.H. for 11 days.

The experiment was arranged in a completely randomized design with four replicates. Mortality was recorded at 1, 3, 5, 7, 9 and 11 days. Dead insects from each treatment were washed 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 fungus outgrowth.

Statistical analysis

Cumulative percentage mortality data from experiments were corrected for natural mortality using Abbott's formula (Abbott, 1925). For dose–mortality bioassay, cumulative percentage mortality was normalized using arcsine transformation and subjected to analysis of variance (ANOVA) using SAS (2002). Means were separated by using the LSD Multiple range test at P = 0.05 level. Probit analysis was used to calculate values of the lethal concentration (LC_{50} and LC_{90}) and the lethal time (LT_{50} and LT_{90}) for each isolate (SAS Institute, 2002).

RESULTS

Germination of *B. bassiana* and *M. anisopliae* isolates tested varied from 80 to 94% (Table 1). Cumulative percentage mortality of cowpea weevil adults after exposure to different concentrations of tested isolates was shown in Figure 1. Differences between lethal effects established after treatments by different conidial concentrations of fungal isolates for different time were signifficant (P < 0.01) (Table 2). All tested isolates were pathogenic to adults of cowpea weevil in immersion bioassay but they had different virulence. Mortality rates increased with increasing conidial concentration and time of exposure (Figure 1). Final mortalities observed on day 11 after exposure were 29.4 to 86.2% in the treatments with IRAN 187C (*B. bassiana*), 9 to 88.3% in IRAN 429C (*B. bassiana*), 21.1 to 96.3% in IRAN 441C (*B. bassiana*), 24.8 to 84.4% in IRAN 715C (*M. anisopliae*) and 20.9 to 80.9% in DEM I001 (*M. anisopliae*). Virulence of the isolates was signi-ficantly different to adults of *C. maculatus* using conidial suspensions with different concentrations (Table 2).

At the same concentration, rate virulence of all isolates was significantly different after 11d (df = 4, F = 88.8,

Table 1. Origin and conidial germination of *Beauveria bassiana* and *Metarhizium anisopliae* isolates used in the study.

Fungi	Isolate	Host (Order: Family)	Location-IRAN	Germination (%)
B. bassiana	IRAN 429C	*Chilo supperssalis* (Lep: Pyralidae)	Rasht-IRAN	86.97 ± 2.26
	IRAN 187C	*Leptinotarsa decemlineata* (Col: Chrysomelidae)	Ardebil-IRAN	80.00 ± 3.62
	IRAN 441C	*Rhynchophorus ferrugineus* (Col.: Curculionidae)	Saravan -IRAN	90.14 ± 0.69
M. anisopliae	IRAN 715 C	*locust* (Orthoptera: Acrididae)	Ahvaz -IRAN	81.15 ± 6.35
	DEMI001	*Rhynchophorus ferrugineus* (Col.: Curculionidae)	Saravan-IRAN	94.00 ± 2.20

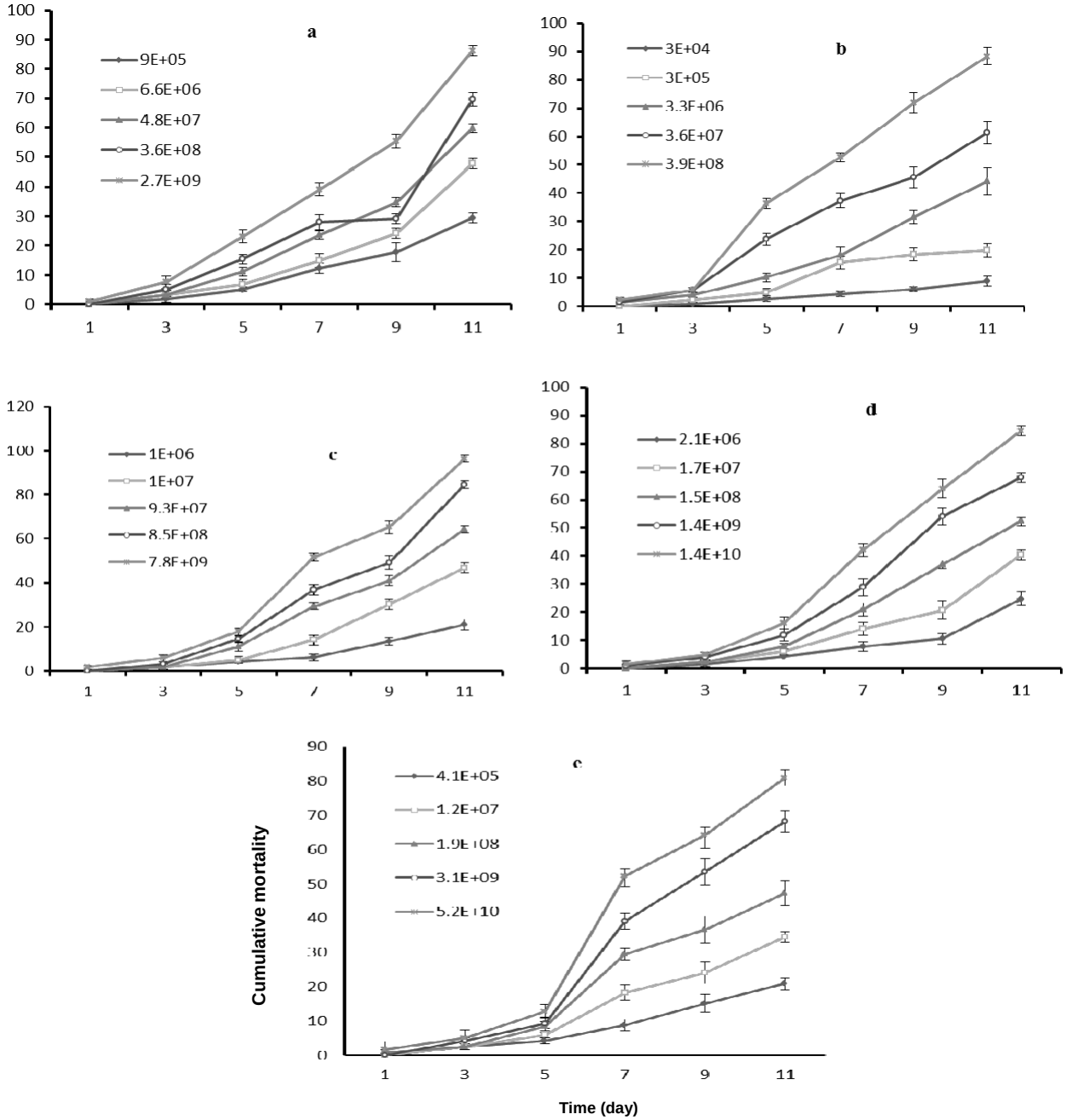

Figure 1. Cumulative percentage mortality (corrected ± SE) of *C. maculatus* adults after exposure to different concentrations (number of conidia ml^{-1}) of *B. bassiana* (Bb) and *M. anisopliae* (Ma) isolates. (a): Bb IRAN 187C, (b): Bb IRAN 429C, (c): Bb IRAN 441C, (d): Ma IRAN 715C and (e): Ma DEMI 001.

Table 2. ANOVA parameters for main effects and associated interactions for adults mortality counts.

Source	df	*Beauveria. bassiana*			*Metarhizium anisopliae*	
		IRAN 187C	IRAN 429C	IRAN 441C	DEMI001	IRAN 715C
		F	F	F	F	F
Concentration	4	110.2**	223**	168.2**	112.9**	134.2**
Time	5	622.5**	282.9**	628.2**	459.6**	544.2**
Concentration × time	20	8**	14.2**	17.2**	10.7**	11.8**

**Indicate significant difference (P ≤ 0.01).

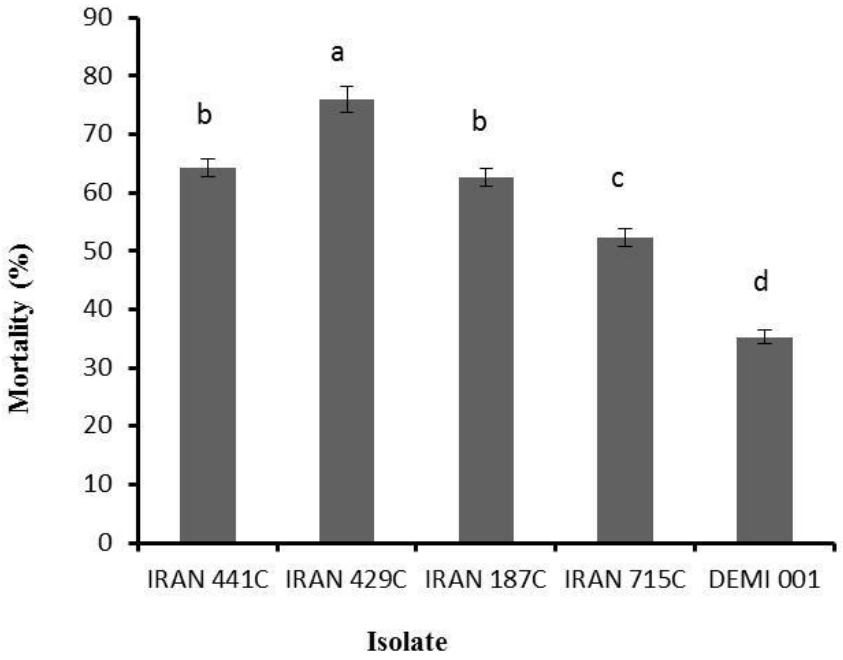

Figure 2. Comparative virulence of *B. bassiana* and *M. anisopliae* isolates against *C. maculatus* at the rates of 1×10^8 conidia/ml. Means within a column followed by different letters are significantly different (LSD), (*P*=0.05).

P < 0.0001) (Figure 2). At a rate of 1×10^8 conidia/ml, there was significant difference among the isolates except IRAN 187C and IRAN 441C (df = 4, F = 88.8, P<0.0001). The results showed that IRAN 429C was the most infective against adults of the cowpea weevil compared to the others.

The LC_{50}, LC_{90} and LT_{50}, LT_{90} values, 95% fiducial limits, slope and Chi–square ($\chi 2$) of different tested isolates of fungi against adults of the cowpea weevil are presented in Table 3 and Table 4. The lowest LC_{50} values 9.8×10^6 conidia/ml were observed in the treatment IRAN 429C (*B. bassiana*). Based on the LC_{50} values, the isolate IRAN 429C (*B. bassiana*) was the most effective to cowpea weevil adults followed by IRAN 187C (*B. bassiana*), IRAN 441C (*B. bassiana*), IRAN 715C (*M. anisopliae*) and DEM I001 (*M. anisopliae*). The LC_{50} values showed that isolates of *B. bassiana* in the current research were more virulent than isolates of *M. anisopliae*. Comparison among the estimated LT_{50} values of *B. bassiana* and *M. anisopliae* isolates indicated that IRAN 429C had the lowest LT_{50} values.

DISCUSSION

Entomopathogenic fungi are being developed worldwide for the control of insect pests and some products are already available commercially (Ekessi et al., 2001). Other investigators have reported that treatment of storage pests with entomopathogenic fungi especially *M. anisopliae* and *B. bassiana* can be effective.

The results presented indicate that all tested isolates of *B. bassiana* and *M. anisopliae* were infective to adults of *C. maculatus*, and at high concentrations caused over

Table 3. Virulence of different isolates of *Beauveria bassiana* and *Metarhizium anisopliae* against adults of *Callosobruchus maculates.*

Fungi	Isolate	Day	Slope±SE	χ^2	Lethal concentrations (conidia /ml)	
					LC_{50} (95% FL)	LC_{90} (95% FL)
B. bassiana	IRAN 187C	11	0.45±0.05	72.2	1.9×10^{7} (9.3×10^{6}-3.5×10^{7})	1.2×10^{10} (3.7×10^{9}-7.6×10^{10})
	IRAN 429C	11	0.65±0.07	88.2	9.7×10^{6} (5.7×10^{6}-1.6×10^{7})	8.5×10^{8} (3.5×10^{8}-2.9×10^{9})
	IRAN 441C	11	0.63±0.07	79	2.4×10^{7} (1.4×10^{7}-4.1×10^{7})	2.5×10^{9} (1×10^{9}-1×10^{10})
M. anisopliae	DEMI001	11	0.35±0.03	84.3	2.6×10^{8} (1.2×10^{8}-6.2×10^{8})	9.9×10^{11} (1.9×10^{11}-1.1×10^{13})
	IRAN 715C	11	0.44±0.05	81.3	1.2×10^{8} (6.3×10^{7}-2.3×10^{8})	9×10^{10} (2.5×10^{10}-5.9×10^{11})

[a] Lethal concentrations and 95% fiducial limits (FL) were estimated using logistic regression (SAS Institute, 2002).

Table 4. LT_{50} and LT_{90} values (days) of *Beauveria bassiana* and *Metarhizium anisopliae* isolates against adults of *Callosobruchus maculates.*

Fungi	Isolate	Concentrations	Slope ±SE	χ^2	Lethal time (days)	
					LT_{50} (95% FL)	LT_{90} (95% FL)
B. bassiana	IRAN 187C	2.7×10^{9}	5.2 ± 1.04	24.2	7.8 (6.5-9.4)	13.8 (10.9-28.7)
	IRAN 429C	3.9×10^{8}	4.3 ± 0.4	94	6.7 (6.1-7.2)	13 (11.5-15.5)
	IRAN 441C	7.8×10^{9}	7.1 ± 1.3	30.3	7.2 (6.1-8.3)	11 (9.4-16.3)
M. anisopliae	DEMI001	5.2×10^{10}	5.9 ± 0.6	89	7.7 (7.3-8.1)	12.7 (11.5-14.5)
	IRAN 715C	1.4×10^{10}	6.2 ± 0.65	89	7.8 (7.4-8.3)	12.5 (11.5- 14.35)

[a] Lethal concentrations and 95% fiducial limits (FL) were estimated using logistic regression (SAS Institute, 2002).

80% mortality to adults of cowpea weevil after 11 day exposure. At the same concentration, virulence rate of all isolates was significantly different. The IRAN 441C of *B. bassiana* had the highest virulence against adults of cowpea weevil because it had lower LC_{50} and LT_{50} and caused the highest mortality (76%) at 1×10^{8} conidia/ml concentration as compare with other isolates. Evidence from these experiments indicates that *B. bassiana* IRAN 441C could significantly reduce *C. maculatus* adults in stored cowpea.

Our results were in agreement with Shams et al. (2011), who reported that the LC_{50} values of *B. bassiana* on day 9 post-treatment were 3.17×10^{6} and 6.08×10^{7} conidia/ml for *C. maculatus* and *S. granarius*, respectively. Cherry et al. (2005) also have demonstrated that different isolates of *M. anisopliae* and *B. bassiana* can provide good control of *C.maculatus* by immersion bioassay at 12d. As well as, they reported *B. bassiana* to be more virulent than *M. anisopliae* against *C. maculatus*. Khashaveh et al. (2011) reported that *B*. *bassiana* can be used with success against pest's injurious stored wheat.

This finding is consistent with the results of this study. External white mycelial growth from all cadavers was evident within 24 to 48 h of death. Post- mortem mycelial and conidial growth demonstrated that the fungal pathogens were the reason of the insect's death.

Tanada and Kaya (1993) argued that infection and sporulation of several entomopathogenic fungi are influenced by environmental factors, especially temperature and humidity, and to lesser extent photoperiod. Environmental factors such as the solar rays, temperature and humidity affect the performance of insect pathogenic fungi. The fungal mycelium and conidia are very sensitive to solar radiation and high light intensities. Because most storage houses are in dark conditions away from direct solar radiation, therefore, insect pathogenic fungi such as *B. bassiana* and *M. anisopliae* can remain infective for longer periods in store. Brower et al. (1995) state that fungi have not been developed as microbial control agents of stored-product pests because a high R.H. is an essential factor in the development of fungal infection (Benz, 1987).

In this study, a high level of mortality was observed in *C. maculatus* at moderate R.H. Several authors have also reported that reduced atmospheric and grain moisture, could increase the efficacy of entomopathogenic fungi especially *B. bassiana* in storage facilities (Lord, 2005; Athanassiou and Steenberg, 2007). Results of this study suggested that entomopathogenic fungi *B. bassiana* and *M. anisopliae* are good choice for control of *C. maculatus*. Also, we need some additional studies for formulating and improving application methods.

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