Comparison of the virulence of some Iranian isolates of *Beauveria bassiana* to *Eurygaster integriceps* (Hem.: Scutelleridae) and production of the selected isolate

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Abstract

The virulence of four Iranian isolates and one exotic isolate of *Beauveria bassiana* on the fifth instar nymphs and also five Iranian isolates and one exotic isolate of it on the adults of *Eurygaster integriceps* Puton was studied using dipping and topical micro-application techniques, respectively. Nymphs were highly susceptible to the isolates. Comparison of LC_{50} values showed no significant difference among the isolates to nymphs. In case of the adults, LD_{50} values of DEBI 002, DEBI 006 and DEBI 008 were comparatively lower than those of other isolates (0.05% Tween 80® solution treatment). Coincidentally, DEBI 002 showed the lowest LD_{50} value among the others (odorless kerosene treatment). In addition, DEBI 002 showed the lowest LT_{50} value to adults. In production phase, the effects of different liquid culture media (microbiological glucose, biochemical glucose, chemistry sucrose and sugar) supplemented with yeast extract and solid media (rice, wheat and barley) were studied on blastospore and conidial production, respectively. The highest total yield was obtained 0.801×10^7 blastospores/ml media after 4 days for microbiological glucose plus yeast extract. Maximum conidial production was achieved 1.17×10^9 conidia/gr substrate using rice as medium. Viability of produced conidia on different solid media showed no significant difference among treatments.

Key words: *Beauveria*, *Eurygaster integriceps*, virulence, production, liquid cultures, solid media

چکیده

زهرآگینی چهار جدایه‌ی ایرانی و یک جدایه‌ی غیربومی *Beauveria bassiana* روی پوره‌های سن پنج سن گندم به روش زیست‌سنجی غوطه‌وری و زهرآگینی پنج جدایه‌ی ایرانی و یک جدایه‌ی غیربومی آن به روش موضعی روی حشرات کامل سن گندم مقایسه شد. پوره‌ها حساسیت بالایی را به جدایه‌ها نشان دادند. مقایسه‌ی غلظت کشنده‌ی ۵۰٪ پوره‌ها نشان داد که اختلاف معنی‌داری بین جدایه‌ها وجود ندارد. در مورد حشرات کامل، جدایه‌های DEBI 002، DEBI 006 و DEBI 008 مقادیر دز کشنده‌ی ۵۰٪ پایین‌تری در مقایسه با جدایه‌های دیگر داشتند (تیمار 0.05% Tween 80®). جدایه‌ی DEBI 002، هم‌زمان پایین‌ترین مقدار دز کشنده‌ی ۵۰٪ را در بین بقیه‌ی جدایه‌ها داشت (تیمار نفت بی‌بو). همچنین، این جدایه کمترین مقدار زمان کشنده‌ی ۵۰٪ را برای حشرات کامل نشان داد. در مرحله‌ی تولید، اثرات محیط‌های کشت مایع مختلف (گلوکز میکروبیولوژی، گلوکز بیوشیمیایی، ساکارز آزمایشگاهی و شکر) همراه با عصاره‌ی مخمر روی میزان تولید بلاستوسپورها و محیط‌های کشت جامد (برنج، گندم و جو) روی تولید کنیدی‌ها مورد بررسی قرار گرفت. بیشترین میزان تولید، $۰/۸۰۱ \times ۱۰^{۷}$ بلاستوسپور در میلی‌لیتر در مورد گلوکز میکروبیولوژی + عصاره‌ی مخمر بعد از ٤ روز تلقیح و بیشترین میزان تولید کنیدی، روی برنج به عنوان ماده‌ی زمینه‌ای ($۱/۱۷ \times ۱۰^{۹}$ کنیدی در هرگرم) به دست آمد. اثر محیط کشت جامد در زنده‌مانی کنیدی‌ها معنی‌دار نبود.

واژگان کلیدی: *Beauveria*، *Eurygaster integriceps*، زهرآگینی، تولید، محیط‌های کشت مایع، محیط‌های کشت جامد

Introduction

The sunn pest, *Eurygaster integriceps* Puton, is the most damaging and economically important pest of wheat and barley in the Middle-East. Sunn pest affects around 15 million hectares annually (Moore & Edgington, 2006). In 2005, the treated area for sunn pest control was dramatically about 1.8 million hectares in Iran (Moein Namini, 2006). The most conventional insecticides for chemical control of sunn pest are fenitrothion (sumithion®) and deltamethrin (decis®). Other insecticides such as fenthion (lebaycid®) and trichlorfon (dipterex®) may also be used (Mossalinejad *et al.*, 2002).

The white muscardine fungus, *Beauveria bassiana* (Bals.) Vuill. has been shown to be a promising control agent against sunn pest (Parker *et al.*, 2003; Skinner *et al.*, 2004). Determining the effectiveness of introducing fungi to the overwintering localities of the sunn pest, scientists recorded significant mortality in plots treated with *B. bassiana* and *Metarrhizium anisopliae* than in the control (Parker *et al.*, 2003). Using standardized bioassay methods for sunn pest, it was proved that the *Paecilomyces farinosus* (Holm ex S.F. Gray) Brown and Smith isolates were not particularly effective but three of the Iranian *B. bassiana* isolates tested, showed great potential for sunn pest control (ICARDA Annual Report, 2003).

Trials during 2004 resulted strong indications that biopesticides based on a Syrian strain of *B. bassiana* could be an effective IPM component for control of the summer generation of sunn pest. Very effective laboratory mortality had been achieved, as well as minor reductions in field numbers (Edgington & Moore, 2005).

One hundred isolates of *Beauveria* spp. obtained from sunn pest and other insects at overwintering sites in 7 countries were selected and *B. bassiana* was mass produced and used in field trials. Field trials recorded insignificant adult mortality. Hence, it is quite likely that many, if not most, of the isolates to be casual associations. Consequently, in the wheat fields, isolates were sought from summer populations of sunn pest. The results were encouraging with nymph mortality reaching 87% (Moore & Edgington, 2006).

An important consideration in selecting of a strain is its virulence (Inglis *et al.*, 2001). Since the previous studies mostly have been carried out with non Iranian isolates on the sunn pest that might have different biophysiological characteristics from the indigenous ones, the objectives of this study were: (1) calculating the LD_{50}, LC_{50} and LT_{50} of some Iranian isolates from different sources and regions, (2) selecting the most virulent isolate, and (3) evaluating the influence of two carriers on infection. Another objective of this study was evaluation of the potential of some liquid media and solid substrates for blastospores and conidial production, respectively. The appropriate time of adding liquid culture to solid medium to mass produce the fungus was also investigated.

Biphasic fermentation system combines the benefits of high biomass production in liquid fermentation and production of stable, hydrophobic aerial conidia on a solid substrate (Jenkins & Goettel, 1997). Conidial production of two isolates of *B. bassiana* and one of *M. anisopliae* using corn, wheat and millet was studied. For *B. bassiana* isolates, conidial production on wheat was higher (El Damir, 2006).

Optimization of the production system encouraged us to perform a low-tech, easy-to-manipulate mass production technique for fine tuning media and timing.

Materials and methods

Fungal isolates

The pathogenicity tests on the basis of Koch's postulates were performed for adults and nymphs. After preliminary tests, five isolates for nymph bioassays and six isolates for adult bioassays were selected. The isolates (table 1) are now preserved (according to Humber, 1997) at the Department of Agricultural Entomology, Iranian Research Institute of Plant Protection, Tehran, Iran.

Table 1. The accession number, host or source and region of collection of *Beauveria* isolates.

Accession number	Host or source	Region of collection
DEBI 001	soil	Fashand (Karaj)
DEBI 002	soil	Atashgaah (Karaj)
DEBI 006	Coleopteran adult	Kurdkooy (Golestan)
DEBI 008	*Chorthipus brunneus*	Evin (Tehran)
ARSEF201	*Diabrotica undecimpunctata*	Corvallis (Oregon)
DEBI 013	*Coccinella septempunctata*	Ghareaghaach (Varamin)

Maintenance of field-collected insects

The *E. integriceps* nymphs and adults were collected from wheat fields of Varamin (Tehran, Iran) and its vicinity using a sweep net. The samples were maintained at 25 ± 2 °C, 60% R.H. and a L:D 16:8 photoperiod on wheat ears. The insects were kept one week at these conditions before they underwent the bioassays to remove the disturbed individuals.

Bioassay - nymphs

The isolates DEBI 001, DEBI 002, DEBI 006, DEBI 008 and ARSEF 201 were studied on fifth instar nymphs. Conidia produced on SDAY plates were gently harvested into 0.05% Tween 80® solution (MERCK, Germany). After bracketing tests, which determined the minimum (10^2 conidia/µl) and the maximum (10^4 conidia/µl) concentration, the nymphs were treated with dilutions of 10^2, 3.2×10^2, 10^3, 3.2×10^3 and 10^4 conidia/µl. The control treatment included distilled water containing 0.05% Tween 80®.

There were ten nymphs in each treatment (replicated three times). The nymphs were put in a buchner funnel fitted to a vacuum flask and covered with a Whatmann # 1 filter paper. The spore suspension (30 ml) was poured onto the nymphs in the buchner funnel. In this way, they were dipped in the suspension for 5 s before exhausting the suspension by a hand-operated vacuum pump. Both nymphs and adults maintained at 25 ± 2 °C, 60% R.H. and a L:D 16:8 photoperiod on wheat grains. After 24 hours, mortality was recorded daily for 10 days. Abbot's formula was applied to correct the mortality percentage in the control (Butt & Goettel, 2000). The LC_{50}, LD_{50} and LT_{50} were calculated by POLO-PC. Pairwise comparison was performed using confidence intervals.

Bioassay - adults

The isolates DEBI 001, DEBI 002, DEBI 006, DEBI 008, DEBI 013 (*Beauveria brongniartii*) and ARSEF 201 were investigated using (i) distilled water containing 0.05% Tween 80® and (ii) odorless kerosene as carriers. The suspension (1 µl) was applied between the second coxae of the insects. There were ten adults in each treatment (replicated three times). Six spore concentrations (10^2, 3.2×10^2, 10^3, 3.2×10^3, 10^4, and 3.2×10^4 conidia/µl) were tested and mortality counts were taken for 17 days.

Biphasic production - liquid phase

For some isolates, the observed efficacy of submerged spores/conidia may or may not out way the aerial conidia and is entirely dependent on the type of liquid medium used for the production (Kassa, 2003). To select an optimized carbon source, liquid media containing sugar, chemical sucrose (BDH chemicals, England), biochemical glucose (MERCK, Germany) and microbiological glucose (MERCK, Germany) were compared. The mixtures of yeast extract (30 g) with either of the above mentioned carbon source nutrients (30 g) in distilled water (1 lit) were used (Jenkins & Prior, 1993; Jenkins & Lomer, 1994). The liquid media (75 ml) were distributed into 250 ml conical flasks (Jenkins *et al.*, 1998), where 37.5 µl of 0.05% Tween 80® was also added to obtain 0.05% solution. One ml of the spore suspension, concentration of 5.2×10^6 conidia/ml was poured into each flask. The flasks were incubated in a shaking incubator (US-848DSRNL, VISION SCIENTIFIC, Korea) at 150 rpm, 24 °C. After 24 h, samples were taken from the inoculated liquid cultures to count the blastospores every day.

Biphasic production - solid phase

The grains (1 kg of rice, wheat and barley) were added to boiling distilled water (700 ml) and let to parboil for 1 h. After getting cool, they were distributed in autoclavable propylene bags. The bags were autoclaved at 1 atm and 120 °C for 1 h and transferred to a laminar air cabinet. The liquid culture was diluted by 50% with sterile cold water and the resulted liquid inoculum was added to the bags (150 ml/kg rice) (Jenkins *et al.*, 1998). The stiff necks were covered with two layers of sterile paper towels and one layer of aluminium foil. The bags were set inside disinfected large containers and incubated at 24 °C. After 47 days, conidia were harvested. Before extraction, opened bags were incubated in room temperature for 5 days. Cereal clumps were separated by hand and sterile distilled water (100 ml) containing 0.05% Tween 80® was added to each bag. The spore suspension was extracted and its volume was measured. Viability tests were carried out using the agar slide technique (Hall & Menn, 1999) with two replicates for each bag along with the contamination monitoring (Jenkins & Grzywacz, 2000). Data were analyzed using a one-way ANOVA followed by Duncan's Multiple Range test (SAS INSTITUTE).

Results

Bioassay - nymphs

Comparison of LC_{50} values (overlap of confidence intervals) showed no significant difference among treatments (fig. 1). The *B. bassiana* isolates were almost equally virulent to the fifth instar nymphs of *E. integriceps*. Nymphs were highly susceptible to all the isolates (table 2).

Table 2. Values of LC_{50}, 95% confidence intervals, probit regression slopes and probit intercepts of *B. bassiana* isolates to the fifth instar nymphs of *E. integriceps*.

***B. bassiana* isolates**	**Log LC_{50} (95% CI)**	**Slope ± SE**	**Intercept**	**X^2**	**P**
DEBI 001	3.00 (2.43 - 3.56)	0.46 ± 0.15	-1.39 ± 0.46	0.76	0.87
DEBI 002	2.98 (2.71 - 3.24)	0.87 ± 0.16	-2.60 ± 0.50	2.13	0.58
DEBI 006	2.74 (2.53 - 2.92)	1.28 ± 0.19	-3.53 ± 0.57	2.11	0.66
DEBI 008	2.61 (2.34 - 2.81)	1.09 ± 0.18	-2.85 ± 0.53	1.45	0.76
ARSEF 201	2.86 (2.61 - 3.08)	1.00 ± 0.17	-2.87 ± 0.52	1.92	0.59

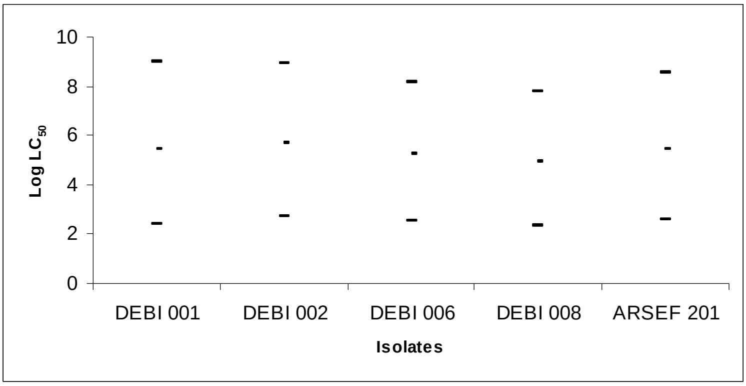

Figure 1. Intervals overlapping of log LC_{50}s of different isolates of *B. bassiana* to the fifth instar nymphs of *E. integriceps*.

Bioassay - adults

Comparison of LD_{50} values (overlap of confidence intervals) proved a significant difference among the isolates and the carriers (figs 2, 3 and 4). Induced mortality by DEBI 002, DEBI 006 and DEBI 008 was higher than those of ARSEF 201, DEBI 013 and DEBI 001, showing the first group was more efficacious than the latter. The most virulent isolates DEBI 002, DEBI 006 and DEBI 008 had smaller LD_{50} values while ARSEF 201, DEBI 013 and DEBI 001 had comparatively larger LD_{50} values (0.05% Tween 80® + distilled water) (table 3). The LD_{50} values of DEBI 001, DEBI 006, DEBI 008, ARSEF 201 and DEBI 013 were different from the DEBI 002 exhibiting the lowest (odorless kerosene) (table 4).

Table 3. Values of LD_{50}, 95% confidence intervals, probit regression slopes and probit intercepts of *Beauveria* isolates to the adults of *E. integriceps* (0.05% Tween 80®).

***Beauveria* isolates**	**Log LD_{50} (95% CI)**	**Slope ± SE**	**Intercept**	**X^2**	**P**
DEBI 001	5.11 (4.50 - 6.79)	0.58 ± 0.15	-2.98 ± 0.55	0.41	0.97
DEBI 002	4.03 (3.72 - 4.53)	0.69 ± 0.13	-2.80 ± 0.46	1.93	0.68
DEBI 006	4.03 (3.75 - 4.47)	0.76 ± 0.13	-3.07 ± 0.48	0.58	0.94
DEBI 008	4.11 (3.80 - 4.62)	0.73 ± 0.13	-2.99 ± 0.48	1.19	0.80
ARSEF 201	4.81 (4.76 - 9.23)	0.47 ± 0.15	-2.69 ± 0.54	0.48	0.96
DEBI 013	4.93 (4.22 - 7.44)	0.40 ± 0.12	-1.99 ± 0.44	1.66	0.80

Table 4. Values of LD_{50}, 95% confidence intervals, probit regression slopes and probit intercepts of *Beauveria* isolates to the adults of *E. integriceps* (odorless kerosene).

Beauveria isolates	Log LD_{50} (95% CI)	Slope ± SE	Intercept	X^2	P
DEBI 001	3.64 (3.39-3.95)	0.79 ± 0.13	-2.90 ± 0.44	0.53	0.98
DEBI 002	3.39 (3.14-3.65)	0.83 ± 0.13	-2.80 ± 0.43	3.35	0.56
DEBI 006	4.05 (3.54-5.37)	1.02 ± 0.15	-4.14 ± 0.57	10.48	0.05
DEBI 008	3.63 (3.37-3.97)	0.75 ± 0.13	-2.72 ± 0.44	2.37	0.70
ARSEF 201	4.34 (3.97-5.04)	0.66 ± 0.13	-2.86 ± 0.48	0.42	0.98
DEBI 013	4.29 (3.90-5.03)	0.61 ± 0.13	-2.61 ± 0.45	1.76	0.87

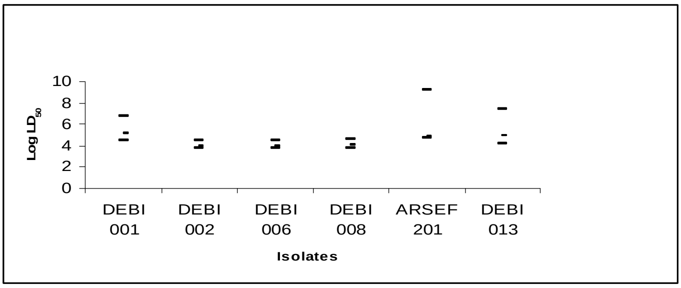

Figure 2. Checking the overlap of confidence intervals of log LD_{50}s of different isolates of *Beauveria* to adults of *E. integriceps* (Tween 80® solution).

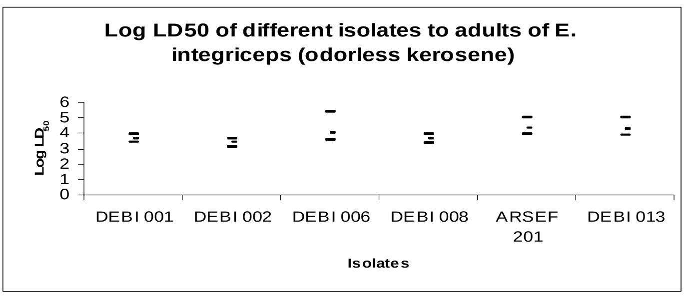

Figure 3. Checking the overlap of confidence intervals of log LD_{50}s of different isolates of *Beauveria* to the adults of *E. integriceps* (odorless kerosene).

Figure 4. Examination of overlap between confidence intervals of log LD_{50}s of two different carriers.

There was statistical difference among LT_{50}s of the isolates. At concentration of 10^4 conidia/µl, the lowest LT_{50} obtained by DEBI 002, followed by DEBI 001, DEBI 008, a group of DEBI 006, ARSEF 201 and DEBI 013 listed in ascending order (table 5 and fig. 5). In the same way, DEBI 002 had the lowest LT_{50} at concentration of 3.2×10^4 conidia/µl (fig. 6).

Table 5. Values of LT_{50} and confidence intervals of *Beauveria* isolates to the adults of *E. integriceps* at two concentrations.

***Beauveria* isolates**	**10^4 conidia/µl**	**3.2×10^4 conidia/µl**
DEBI 001	13.47 (12.61-14.48)	11.15 (10.60-11.74)
DEBI 002	11.10 (11.34-12.73)	8.73 (8.22-9.24)
DEBI 006	20.16 (18.81-21.95)	10.17 (9.65-10.70)
DEBI 008	16.24 (15.29-17.39)	11.14 (10.66-11.64)
ARSEF 201	21.94 (19.79-24.96)	-
DEBI 013	23.40 (21.04-26.79)	16.77 (15.99-17.70)

Additionally, the LT_{50} values decreased whenever the concentration increased. According to Todorova *et al.* (2002), an inverse relationship is observed between LT_{50} and the concentration of conidia used.

DEBI 002, having an acceptable LT_{50} with optimum growth qualities and noticeable sporulation (Haji Allahverdi Pour, unpublished data), was nominated to optimize the production system in laboratory.

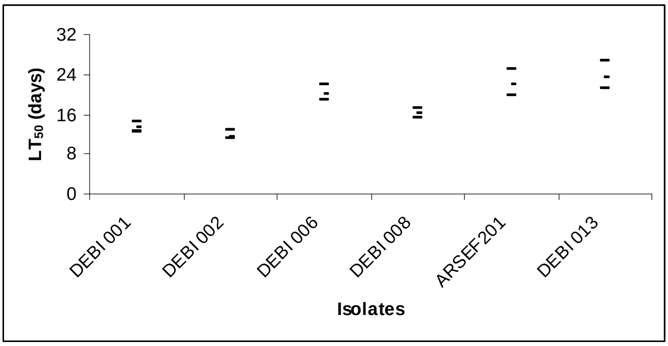

Figure 5. Examination of overlap between confidence intervals of LT_{50}s of different isolates of *Beauveria* to the adults of *E. integriceps* at concentration of 10^4 conidia/µl.

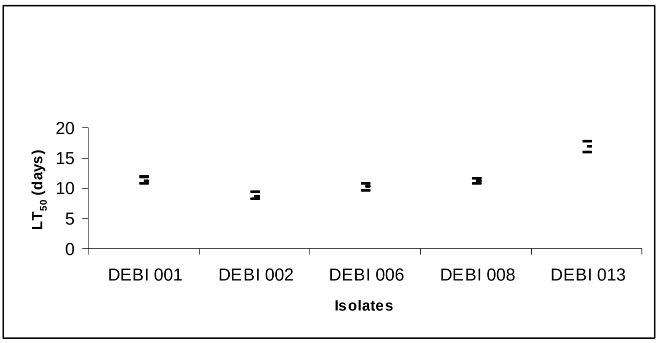

Figure 6. Examination of overlap between confidence intervals of LT_{50}s of different isolates of *Beauveria* to the adults of *E. integriceps* at concentration of $10^{4.5}$ conidia/µl.

Comparison of the effect of the two different carriers on infection proved that odorless kerosene treatment significantly produced more infection in adults than 0.05% Tween 80® solution treatment (table 6). It shows oil-based formulation are more effective than aqueous formulation.

Table 6. Values of LD_{50} and confidence intervals for used carriers.

Carrier	Log LD_{50} (95% CI)	Slope ± SE	Intercept
0.05%Tween 80®	4.52 (4.28-4.86)	0.60 ± 0.05	-542.7
Odorless kerosene	3.87 (3.71-4.06)	0.75 ± 0.05	-586.5

Production - liquid phase

Analysis of variance of mean number of blastospores in different liquid media showed statistical differences among treatments ($F_{3,47}$ = 6.34, P = 0.0017). Also, there was statistical difference in blastospore production on different days ($F_{3,47}$ = 115.55, P = 0.0001). Interaction between medium and day had a significant effect on total spore yield ($F_{9,47}$ = 6.78, P = 0.0001).

Comparison of the means using Duncan's Multiple Range test showed that medium containing microbiological glucose (level a) produced more conidia than the others (table 7).

Table 7. Mean comparison of number of blastospores/ml produced in liquid media for DEBI 002 (Duncan's test).

Media	Mean of # spores/ml
Microbiological glucose + Yeast extract	0.801×10^7a
Chemistry sucrose + Yeast extract	0.649×10^7b
Sugar + Yeast extract	0.676×10^7b
Biochemistry glucose + Yeast extract	0.645×10^7b

Mean values followed by the same letter are not statistically significant (α = 5%). Means are of 12 observations.

Comparison of means classified the fourth day after inoculation the liquid media as "level a" (table 8), but because of the enhanced growth of mycelia on the fourth day, the third day was chosen for inoculating solid substrate.

Table 8. Mean comparison of number of blastospores/ml on different days for DEBI 002 (Duncan's test).

Day	Mean of # spores/ml
4th	1.18×10^7a
3rd	0.93×10^7b
2nd	0.64×10^7c
1st	0.50×10^7d

Means are of 12 observations (α = 5%).

Production - solid phase

The effect of solid substrates (rice, wheat and barley) on the quantity of conidial production showed a significant difference ($F_{2,17}$ = 55.09, P = 0.0001). Comparing the average number of produced conidia per gram of substrate proved that the highest level yield (1.17×10^9 conidia/gr substrate) was achieved on rice; whilst 0.68×10^9 and 0.60×10^9 conidia/gr substrate were harvested from barley and wheat (level b), respectively (table 9).

Means of conidial viability on barley, wheat and rice was achieved 97.83%, 96.00% and 95.00% on the harvest day, respectively. Analysis of variances showed no significant difference among treatments ($F_{2,15}$ = 3.39, P = 0.06). These data provides useful information for developing a simple and pragmatic approach for mass production of entomopathogenic fungi.

Table 9. Mean comparison of number of conidia produced on the grains for DEBI 002 (Duncan's test).

Substrate	Mean of # conidia/gr substrate
Rice	1.17×10^9a
Barley	0.68×10^9b
Wheat	0.60×10^9b

Means are of 6 observations (α = 5%).

Discussion

The most virulent isolate, the DEBI 002, with optimum growth qualities and noticeable sporulation (Haji Allahverdi Pour, unpublished data), was chosen for mass production in laboratory. In comparison to the other isolates, DEBI 002 also showed the lowest LT_{50} and highest mortality on the fifth instar nymphs. It was isolated from a soil sample collected from Atashgaah (Karaj, Iran), a sunn pest overwintering locality. These characteristics put DEBI 002 forward as an appropriate and promising agent in IPM of the sunn pest.

The DEBI 002 caused more than 82% mortality, whilst the only exotic isolate, ARSEF 201 caused only 30% mortality (applying 3.2×10^4 conidia/μl). The DEBI 002's higher virulence to sunn pest adults than the isolates from insect source, agrees with the observation of Talaei-Hassanloui (1999) that the isolate from soil origin caused higher mortality than those from insect origin.

The present study supports the results of Ghazavi (2003) that odorless kerosene had significant effects on the efficacy of *B. bassiana* on *Locusta migratoria* (L.). According to Locke (1984), the entomopathogenic fungi may gain entry into the host insect by replacing epicuticular lipids with an aqueous phase. He stated that this would be happen in the presence of oil because the lipids in the insect cuticle may rush out, followed by an aqueous cuticular fluid, covering the surface with watery droplets. Under these conditions, conidial germination would be expected to increase. Ibrahim *et al.* (1999) suggested that better spread of conidia and better germination rates in oil, improved transportation of conidia to areas of thinner cuticle.

Dorta *et al.* (1990) tested conidial production of *M. anisopliae* on rice bran, rice husk mixtures and rice. They found that *M. anisopliae* produced 5-15 times more conidia on rice bran and rice husk mixtures than on rice. Puzari *et al.* (1997) reported that a total amount of 39.33×10^7 conidia/ml water of *B. bassiana* were produced using a medium of rice hulls, saw dust and rice at a ratio of 75:25:100.

By far the most commonly selected substrate for production of fungal conidia has been white rice. This is probably due to a combination of factors including nutritional balance, cost, worldwide availability, and physical characteristics such as grain size and shape, hydration properties and structural integrity even after colonization by fungi (Jenkins *et al.*, 1998). Our results agree with observation of Nelson *et al.*, (1996) that demonstrated maximum yield was achieved when fungi were grown on *rice* for 3 weeks at 23 °C, under natural day light. They studied the effects of solid media (rice, wheat and barley) with additives (glucose and yeast extract), temperature and length of incubation on conidial production.

However, the highest yield achieved on rice doesn't mean that rice is the most appropriate grain to mass produce the entomopathogenic fungi.

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Received: 16 July 2007
Accepted: 7 July 2008