

Virulence of Some Isolates of Entomopathogenic Fungus, *Beauveria bassiana* on *Ostrinia nubilalis* (Lepidoptera: Pyralidae) Larvae

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ABSTRACT

The European Corn Borer (ECB) is one of the most important insect pests of corn and some other crops such as rice in Iran. This pest is one of the most important hosts of the entomopathogenic fungus *Beauveria bassiana*, a well known fungal entomopathogen with high host range and considerable potential in insect pest control. In an isolate selection program of fungal isolates against ECB, ten isolates consisting of eight isolates from Iran and two from other countries were assayed using the dipping method on third instar larvae of ECB. Inoculum concentrations were 10^4 , 10^5 , 10^6 , 10^7 , and 10^8 conidia ml⁻¹. For each concentration, 30 larvae were dipped into the conidial suspension for 30 seconds. Control larvae were treated with distilled water containing 0.03 percent Tween-80 as surfactant. Each experiment was repeated three times. Results showed that BEH isolate which was isolated from the soil of insects living in the field, caused the highest mortality in larvae in comparison with other isolates with a mean of 57.67 percent mortality using 10^8 conidia ml⁻¹. Other isolates, such as DEBI007 and EVIN I, were scored in the lower position with producing 53.43 and 42.67 percent mortality, respectively. EVIN II, DEBI002, and DEBI008 caused the lowest mortality in assayed larvae. A decrease in larval feeding was detectable a few days before death. Possible causes for the low mortality in isolates are discussed.

Keywords: *Beauveria bassiana*, Bioassay, Entomopathogenic fungi, *Ostrinia nubilalis*.

INTRODUCTION

The European corn borer (ECB), *Ostrinia nubilalis* Hübner is one of the most important pests of corn plantations in North America (Wagner and Lewis, 2000) and in the humid conditions of northern Iran (Esmaili *et al.*, 1996). However, it can also be seen in the corn plantations in the Southeast of Iran.

Problems with synthetic chemical insecticides have given rise to a sense of urgency in the development of biological control agents as supplements or alternatives to these chemicals. Entomopathogenic fungi

(EPF) are key regulatory factors in pest insect populations and are considered very promising biological control agents (St. Leger *et al.*, 1996). These organisms were the first group to be considered as biological control agents (BCAs). EPF's potential in producing natural epizootics caused some attempts in improving them in IPM programs (Carrutters and Soper, 1981). More than 700 species belonging to 90 genera are pathogenic to insects (Roberts and Humber, 1981). Most Deutromycetes are generalist pathogens and some isolates have been formulated and developed as BCAs and are applied on different pests in developed countries (Chanley, 2003). However, there is

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nearly no use of formulated entomopathogenic fungi in less developed countries such as Iran. Fortunately, the mycopathogens of insects have been the subject of many experiments for assessing their efficacy for use in the biological control of some important insect pests in Iran. Lack of robust and reliable field effects, restrictions on the use of exotic fungal biological control agents, lack of information of most growers about methods of using them (Butt *et al.*, 2001), narrow host range, and inconsistent and poor control in field trials (Butt *et al.*, 1999) are some of the restrictions placed on BCAs market. Therefore, mycoinsecticides have a toe-hold in the biological crop protection market (Charnley, 1997), although some fungal species such as *Beauveria bassiana* (Balsamo) Vuillemin have a wide host range (Clarkson and Charnley, 1996). It occurs worldwide with large host list and in soil as a ubiquitous saprophyte (Tanada and Kaya, 1993). There is relatively considerable investment in research on and formulation improvement of *B. bassiana*. Numerous formulations of this fungus have been registered for control of different pests in various orders of insects (Milner, 1997; Burges, 1998; Butt and Copping, 2000).

Because of the importance of *O. nubilalis* in North America, various investigations have been made to suppress its populations on corn (Bartlett and Lefebvre, 1934; Stirrett *et al.*, 1937; Beall *et al.*, 1939; York, 1958). Feng *et al.* (1985) studied the age-specific dose-mortality effects of *B. bassiana* on ECB. An interesting part of that research was directed towards the relationship between *B. bassiana* and corn plant, starting with the studies of Bartlett and Lefebvre (1934). They stated that the succulent tissues of cornstalk were an ideal environment for *B. bassiana* growth. Complementary evidence for this relationship and colonization of corn plant by the entomopathogen has been reported by numerous authors (Lewis and Cossentine, 1986; Lewis and Bing, 1991; Vakili, 1990; Wagner and Lewis, 2000). These research

studies suggest the potential for season-long suppression of ECB by *B. bassiana* (Bing and Lewis, 1991) and have culminated in the improvement of two products including Ostrinil® and CornGuard® for biological control of ECB in France and US, respectively (Milner, 1997; Burges, 1998).

With regards to the environmental hazards of chemicals and importance of ECB in Iran, some bioassays were arranged for evaluating the virulence of some Iranian isolates of *B. bassiana* towards it. Two foreign isolates of *B. bassiana* were included in the experiments for further comparison.

MATERIALS AND METHODS

Rearing of ECB

Ostrinia nubilalis larvae and pupae were collected from the Tirtash region in Behshar, Mazandaran Province in the North of Iran to the South of the Caspian Sea. This region is relatively warm and humid in summer and is one of the most important dispersion areas of ECB in Iran. After identification of insect species in the laboratory (Department of Plant Protection, Tehran University), a pair of newly emerged adults were placed in a transparent octagonal plastic container (h= 10 cm, d= 16.5 cm). Eggs were kept at 25°C. Larvae were transferred to an artificial diet. This diet was prepared according to Poitout and Bues (1972) with some modifications including the removal of Fumidyl-B (anti-nosemose) and Uromycin (anti-bacterial) and the addition of 1% fructose for attracting larvae to the artificial diet. Most larvae pupated in the corners of the plastic container, although some pupae were formed inside the semi-dried diet. Ten newly emerged male and female adults were transferred to a new container and fed on cotton buds soaked with 7% fructose for stimulation of oviposition. The whole rearing process was carried out in a chamber at 25±1°C, 72±5% relative humidity and over a photoperiod of 16:8 hours (L:D) with

an equal combination of white and yellow bulbs.

Fungal Isolates

Ten isolates of the entomopathogenic fungus *B. bassiana* were obtained from different insect hosts and geographical areas. Table 1 shows some characteristics of used fungal isolates. Before original bioassays all isolates were passed from *Tenebrio molitor* larvae to adjust their virulence. A single spore of each isolate was used for generation of a new colony. Fungal colonies were cultured on SDYA (Sabouraud's Dextrose Yeast Agar) medium (1% yeast extract) in 90 mm Petri dishes sealed with Parafilm® M. SDA and yeast extract were purchased from Merck®. Fungal cultures were kept in an incubator at 25±1°C and a photoperiod of 16:8 hours (L:D) for two weeks.

minutes for homogenisation of the hydrophobic conidia. Resulting suspensions were then filtrated through three layers of muslin to eliminate hyphae and unsuspended conidia. Spore concentration was estimated using an improved Neubauer haemocytometer in filtrates under a light microscope (×400). Conidial viability was tested in a water-agar medium. Droplets were taken from each suspension in sterile conditions and spread over the medium. Cultures were kept at room temperature for 10 hours and the viability percentage was determined by counting 100 random spores in a microscope field (×400). Three different microscopic fields were counted for each isolate. Only conidia with a germ tube as long as the conidium's width were considered to have germinated. Spore suspensions were sealed with Parafilm and kept at 4°C in a refrigerator until use.

Bioassays

Conidial Suspensions and Viability

Conidia were harvested from each culture surface by scraping them off the medium using a scalpel and transferring them to a 100 ml beaker containing 15 mL 0.03% Tween-80. Suspensions were agitated for 5

The concentration of each isolate was adjusted to 10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ conidia ml⁻¹. Third instar larvae of *O. nubilalis* were used for bioassays. In the control, larvae were treated with sterile distilled water containing 0.03% Tween-80. Thirty larvae were used for bioassay of each

Table 1. Details of *Beauveria bassiana* isolates used in bioassays.

Accession Number	Host or Source	Origin
DEBI001 ^a	Soil	Iran
DEBI002	Soil	Iran
DEBI003	<i>Rhynchophorus ferugineus</i> (Col.: Curculionidae)	Iran
DEBI007	Soil	Iran
DEBI008	<i>Chorthippus brunneus</i> (Orth.: Acrididae)	Iran
LRC107 ^b	<i>Leptinotarsa decemlineata</i> (Col.: Chrysomelidae)	Portugal
LRC137	<i>Leptinotarsa decemlineata</i>	Canada
EVIN I ^a	Soil	Iran
EVIN II	Soil	Iran
BEH	Soil	Iran

^a DEBI and EVIN isolates kindly supplied by Dr. M. Ghazavi, Plant Pests and Diseases Research Institute, Iran.

^b LRC isolates kindly supplied by Dr. M. Goettel, Lethbridge Research Centre, Agriculture and Agri-Food Canada, Alberta, Canada.



concentration of different isolates. Larvae immersed in conidial suspensions for 30 seconds and excess suspension were then removed using a Buchnel funnel. Ten larvae were kept in a transparent plastic container (d= 12 cm, h= 4 cm). A 1.2 cm diameter hole was placed in each container for proper ventilation and covered with a polyester net. Some of the artificial diet was placed in each container for larval consumption. Larvae were kept in an incubator at $25\pm1^{\circ}\text{C}$, nearly saturated relative humidity, and a photoperiod of 16:8 hours (L:D). The containers were observed daily for 12 days and dead insects were counted and transferred to plastic Petri dishes (90 mm) covered with wet No. 1 Whatman filter paper for observation of fungal growth in dead insects. All the experiments were repeated three times.

Statistical Analysis

Mortality data were corrected using Abbott's formula as explained by Goettel and Inglis (1997). Normality of the mortality data was investigated using a Chi square (χ^2) test prior to data analysis (Statgraphic Plus, Version 3.0). Analysis of variance (ANOVA) was conducted using a factorial test (PROC ANOVA) in SAS (1989) program. Isolate and conidial concentrations were considered as factors that influence the mortality of larvae. LC_{50} and LC_{25} estimations were carried out using PROC Probit in SAS program. When F Values were significant, means were compared using Duncan's test.

RESULTS

Mortality in the control was 5-10 percent in different treatments, corrected using Abbott formula. Results of the normality test showed that data came from a normal test ($\chi^2= 7.58$, $P= 0.1805$). Therefore, analysis of variance was done without any transformation of the corrected mortality data. Analysis of variance of mortality data showed significant differences in treatments ($F= 10.8$, $df= 9$, $P< 0.01$) both from the isolate ($F= 25.02$, $df= 9$, $P< 0.01$) and conidial concentration ($F= 64.99$, $df= 4$, $P< 0.01$) aspects. Figure 1 shows the mortality percentage in different concentrations of various isolates of *B. bassiana* on *O. nubilalis* larvae. As can be seen from Figure 1, there were no mortality percentages above 50% in most cases. Therefore, LC_{50} and LT_{50} values were not within tested doses, and so LC_{50} values were estimated with response curves in the SAS program (Table 2). Duncan's multiple range tests for comparison of mean mortality data among isolates of *B. bassiana* showed significant differences among them. In 10^4 conidia ml^{-1} , DEBI001 imposed higher mortality on larvae in comparison with other isolates (mean percentage 19 ± 4.21); but DEBI002 showed the lowest mortality within the tested population of ECB larvae (1.26 ± 1.12). In all other fungal concentrations BEH exhibited a higher mean mortality on insects compared with other isolates. The highest mean mortality was recorded at 57 ± 6.05 in 10^8 conidia ml^{-1} of BEH. DEBI001 and EVIN I showed statistically similar amounts of mortality in 10^8 conidia ml^{-1} . In comparison, larval mortality caused by DEBI001 was 19 (±4.21) percent in 10^4 conidia ml^{-1} that reached 41.69 (± 8.93) percent in 10^8 conidia ml^{-1} . But EVIN I imposed a more than four times lower mortality (4.34 ± 1.15)

Table 2. Significance of mean mortality percentage among different isolates using Duncan multiple range test.

Isolates	BEH	DEBI007	DEBI001	LRC107	EVIN I	LRC137	DEBI008	DEBI003	EVIN II	DEBI002
Mean mortality	34.93	28.73	28.13	25	22.93	21.20	12.73	11.80	8.20	7.67
Duncan grouping	A	B	BC	BCD	CD	D	E	E	E	E

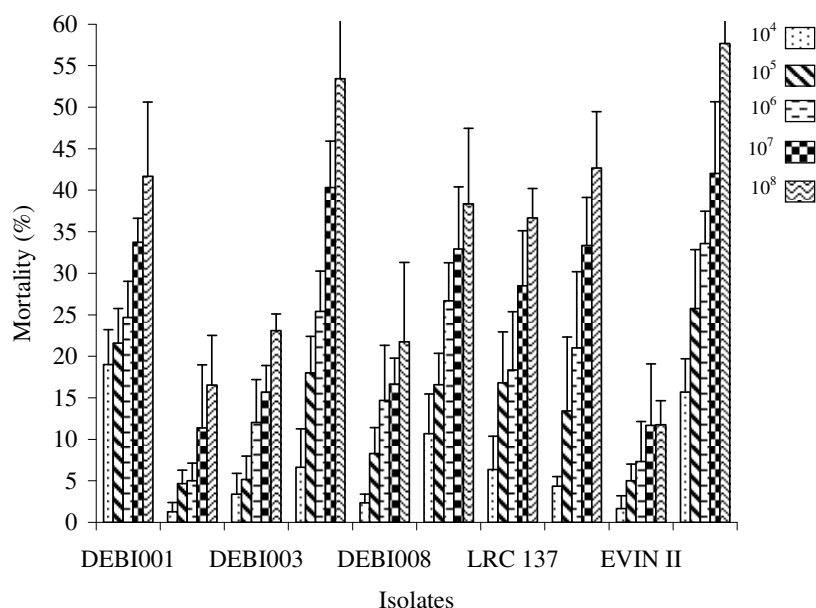


Figure 1. Mean percentage mortality ($\pm$ SE) caused by different *Beauveria bassiana* isolates at concentrations of 10^4 - 10^8 conidia ml^{-1} on third instar larvae of European corn borer.

in larvae at 10^4 conidia ml^{-1} . Larval mortality by this isolate reached 42.67 (± 6.80) percent at 10^8 conidia ml^{-1} .

Significance of the mean mortality percentage among different isolates using the Duncan test showed that there was not statistically any difference among DEBI008, DEBI003, EVIN II and DEBI002 (group E). The highest mortality in BEH was statistically different from other isolates (group A). The rest of the isolates grouped between these two extremes (Table 2).

DISCUSSION

Some soil derived isolates imposed higher mortality in comparison with other isolates from species of coleopteran and orthopteran. BEH was from soil from an insect pest habitat and demonstrate the highest mortality among tested isolates. Goettel *et al.* (1990) noted that the isolates most virulent to a host are isolated from the same or related host species. In our experiments the isolate that had derived

from the host environment (soil) showed to be the most aggressive one among the isolates used. DEBI007 was an isolate derived far from the insect habitat but showed high mortality on the third instar *O. nubilalis* larvae. DEBI001 and EVIN I isolates were moderately lethal compared with the most highly effective doses. Although EVIN II was from the same origin as EVIN I, it imposed the least mortality on insects. Among beetle-derived fungal isolates, two foreign isolates (LRC107 and LRC137) demonstrated moderate mortality on larvae. But weevil and locust-derived isolates (DEBI003 and DEBI008, respectively) showed less infectivity.

It can be inferred from our data that there were considerable variations in fungal isolates' virulence towards ECB larvae. These variations may result from various sources. All fungi use a combination of enzymes and mechanical force to penetrate the host cuticle (Butt, 2002). Since proteins constitute a major component of the insect cuticle it follows that proteases must play an important role in the penetration process



(Butt, 2002). Relationships between enzyme activities and the virulence of *B. bassiana* towards *Galleria mellonella* L. and *Trichoplusia ni* (Hübner) has been demonstrated by Gupta *et al.* (1994) and many other researchers, but this may not be the case in all instances (Gillespie *et al.*, 1998). First author's unpublished data has shown the relationships between subtilisin Protease1 (Pr1) production and virulence of some *B. bassiana* isolates against ECB larvae. Cuticle-degrading enzymes (CDEs) may determine not only virulence but also the host specificity of fungal isolate (Gupta *et al.*, 1994). Cuticle types in various insects differ in their protein composition and degree of sclerotization (Charnley, 2003) and many CDEs are induced by cuticular components (Paterson *et al.*, 1994; Butt *et al.*, 1998) and some of them are produced under nutrient-poor conditions and repressed by excess nutrients (St. Leger *et al.*, 1992). Conidial growth and nutritional conditions and especially their carbon:nitrogen content can considerably influence the virulence of some hyphomycetous fungi such as *B. bassiana* (Safavi *et al.*, 2007) and *Metarhizium anisopliae* (Metsch.) Sorok. (Shah *et al.*, 2005).

During the experiment, larvae continued feeding from the artificial diet. However, food consumption was lower in more effective isolates such as BEH and DEBI007 compared with the control. In these isolates and some other isolates feeding activity reduced approaching their time of death the artificial diet remained longer in highly effective isolates than in the control and less effective isolates. Larvae left the artificial diet and food consumption was generally corrupted around 24 hours before death. Host food can be an effective agent in the development of fungal pathogens besides the host immune system (Tefera and Pringle, 2003). It is suggested that total low susceptibility observed in our experiments using *B. bassiana* may be related to the suppressing effects of some antimicrobials

used in an artificial diet for *O. nubilalis* larvae. Although in this research there was not any comparison between natural and artificial diets, some researchers have confirmed this idea. Tefera and Pringle (2003) confirmed the high rate of mortality and mycosis in *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) larvae reared on a natural diet (maize leaves) compared with those on the artificial diet. Conversely, Goettel *et al.* (1993) working on alfalfa leafcutter bees, *Megachile rotundata* (Fab.) demonstrated that bees reared on a natural diet were generally less susceptible to *Ascosphaera aggregata* Skou. compared with bees reared on the artificial diet. Likewise, adult chinch bugs, *Blissus leucopterus* Say, inoculated with *Beauveria bassiana*, showed higher mortality when fed wheat, barley, or the artificial diet compared with corn or sorghum. This confirms the inhibitory effect of these foods, presumably from fungistatic secondary plant chemicals (Ramoska and Todd, 1985). The artificial diet contains antimicrobial substances (methyl 4-hydroxybenzoate, ascorbic acid, sorbic acid, and ethyl alcohol) (Kfir, 1992), which probably inhibit infectivity of the fungal isolates.

Although bioassays are the most important component in fungal isolate selection, understanding the virulence determinants and identifying enzymes and their real role in pathogenesis will help in the production of more efficient fungal biopesticides. Our data showed some isolates such as BEH and DEBI007 are more effective in controlling ECB populations. We suggest more work using these two and other potential isolates on some other important pests, for example *Chilo suppressalis* Wall., that not only is systematically close to ECB, but also has more or less similar habitation. The most effective isolate can be selected by these works and improved by genetic research and manipulation. Working on improvement in the formulations is the next

step that has been done on non-native isolates in North America and France.

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بیمارگری چند جدایه‌ی قارچ بیمارگر حشرات *Beauveria bassiana* (Bals.) Vuill. روی کرم ساقه‌خوار اروپایی ذرت *Ostrinia nubilalis* (Lepidoptera: Pyralidae)

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چکیده

ساقه‌خوار اروپایی ذرت (*Ostrinia nubilalis*) یکی از آفات مهم ذرت و چند محصول دیگر از جمله برنج در ایران است. این حشره یکی از میزبان‌های قارچ *Beauveria bassiana* است. بنابراین این آفت در محیط غذایی مصنوعی توصیه شده پرورش یافته و در یک برنامه‌ی انتخاب بهترین جدایه قارچ در کنترل آفت مذکور، تأثیر ده جدایه‌ی قارچ شامل هشت جدایه‌ی داخلی همراه با دو جدایه‌ی خارجی با روش غوطه‌وری روی لاروهای سن سوم ساقه‌خوار بررسی گردید. غلظت‌های بکار رفته قارچ بیمارگر در زیست سنجی شامل 10^4 ، 10^5 ، 10^6 ، 10^7 و 10^8 کنیدی در هر میلی‌لیتر بود. در هر غلظت از هر جدایه‌ی قارچی تعداد ۳۰ لارو به مدت ۳۰ ثانیه در سوسپانسیون کنیدی غوطه‌ور شد. لاروهای شاهد با آب مقطر حاوی ۰/۰۳ درصد Tween-80 تیمار شدند. آزمایش سه بار تکرار گردید. نتایج نشان داد که جدایه‌ی BEH – که از خاک محل جمع‌آوری آفت جداسازی گردید – در مقایسه بیشترین درصد تلفات ($6/05 \pm 57/67$) در غلظت 10^8 کنیدی در هر میلی‌لیتر را در لاروهای سن سوم ساقه‌خوار اروپایی ذرت در غلظت‌های مختلف ماده‌ی تلقیحی موجب گردید. جدایه‌های دیگر مانند DEBI007 و EVIN I به ترتیب با ۵۳/۴۳ و ۴۲/۶۷ درصد از نظر ایجاد مرگ و میر بالاتر در رده‌های بعدی قرار گرفتند. در نقطه‌ی مقابل، جدایه‌هایی چون EVIN II، DEBI002 و DEBI008 کمترین درصد تلفات را در لاروهای مورد آزمایش ساقه‌خوار اروپایی ذرت نشان دادند. کاهش تغذیه لاروها یکی از نشانه‌های آلودگی با قارچ بود. علت پایین بودن درصد تلفات در جدایه‌های مختلف مورد بحث قرار گرفته است.