Original Contribution

EFFECT OF SUBLETHAL CONCENTRATION OF *BEAUVERIA BASSIANA* FUNGUS ON DEMOGRAPHIC AND SOME BIOCHEMICAL PARAMETERS OF PREDATORY BUG, *ANDRALLUS SPINIDENS* FABRICIUS (HEMIPTERA: PENTATOMIDAE) IN LABORATORY CONDITIONS

M. Gholamzadeh-Chitgar^{1,2*}, J. Hajizadeh², M. Ghadamyari², A. Karimi-Malati², H. Hoda³

¹Plant Protection Research Department, Mazandaran Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Sari, Iran

²Department of Plant Protection, Faculty of Agricultural Science, University of Guilan, Rasht, Iran

³Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

ABSTRACT

Effects of *Beauveria bassiana* on *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae), a predator of lepidopterous larvae in rice fields were investigated. The experiments were conducted at a concentration of 3×10^5 conidia ml^{-1} (LC_{30}) of *B. bassiana* on life table and some biochemical parameters of fifth instar nymphs. Insects were infected by dipping in the conidial suspension for 10s. The results showed that pre-oviposition period, fecundity, percent egg hatchability and longevity of treated bugs were not significantly affected compared with the control. Esterase (using β -naphthyl as substrate) and glutathione S-transferase activity in *A. spinidens* increased 48 h after treatment. The fungus did not cause adverse effect on acetylcholinesterase activity. *B. bassiana* did not cause significant difference on life table parameters of *A. spinidens* compared to the control. According to the present study, it can be concluded that *B. bassiana* is compatible with the predatory bug at sublethal concentration (LC_{30}).

Key Words: *Andrallus spinidens*, fungus, suspension, sublethal, life table, biochemical parameters

INTRODUCTION

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) is a well known entomopathogenic fungus that has great potential as biological control agent against a wide range of insects. Entomopathogenic fungi are one important component within integrated pest management systems. As a result of the excessive use of insecticides and the associated problems of them, there is an increasing demand for sustainable, environmentally friendly control methods. The success of fungal entomopathogens as biological control agents in the field depends not only on high efficacy against insect pests, but also on low virulence against non target insects (1).

Andrallus spinidens Fabricius is a non-specific predator of lepidopterous larvae in rice fields (2). Second to fifth instar nymphs and adults of

A. spinidens have predatory activity on larvae of rice pests such as: *Chilo suppressalis* Walker (Pyralidae), *Naranga aeneascens* Moore (Noctuidae) and *Mythimna unipunctata* Haworth (Noctuidae) (3, 4). This pentatomid bug is distributed worldwide (5) and recognized as a potential biological control agent in rice fields in India, Malaysia and Iran (2, 3, 6). There are three factors which favor *A. spinidens* as potentially useful biological control agent of rice pests: relatively short life cycle, aggressive feeding behavior and ability to feed continually for several hours (2). This natural enemy would be directly exposed to the *B. bassiana* in the field when this fungus was used as a biopesticide.

Many reports have described the detrimental effects of entomopathogenic fungi on insects (7, 8, 9). Majidishilar, (10) reported that *B. bassiana* has great potential as biological agent for control of *C. suppressalis*. Therefore, *A. spinidens* would be exposed to field-applied entomopathogenic fungi if *B. bassiana* are

*Correspondence to: Moloud Gholamzadeh-Chitgar, Fax: 011-54262418; Email: b_gh.chitgar60@yahoo.com

used as a microbial control of *C. suppressalis*. If the fungus has negative effect on *A. spinidens*, predation of lepidopteran pests by the predatory bug may be limited in rice fields. Research has recently focused on the epizootiology and resultant effects of entomopathogenic fungi on predatory arthropods (1, 9, 11, 12, 13). Lethal and sublethal effects of entomopathogens on beneficial insects with regard to fecundity, longevity and survivorship are worth evaluating (13). Life table study is the best approach to combine the lethal and sublethal effects (14).

Xenobiotics can affect biochemical activity of detoxification enzymes in the exposed insects. In insects, general esterases (EST) and glutathione S-transferase (GST) are detoxifying enzymes to react against xenobiotics. Acetylcholinesterase (AChE) is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine in the nervous system. Glutathione S-transferase (E.C.2.5.1.18) belongs to the class of phase II detoxifying enzymes that catalyze the conjugation of glutathione with xenobiotics. The changes in the activity or spectrum of detoxification enzymes can induce infections in insects (15). Biochemical parameters seem quite promising to assess and predict the effects of toxicants on beneficial species (16). There is a relationship between toxicant-induced biochemical alternations and subsequent demographic changes as a pressing problem in Ecotoxicology (17).

There is no data about the effect of *B. bassiana* on the life table parameters of *A. spinidens*. The goal of current research was to assess the sublethal effects of *B. bassiana* on the life table and some biochemical parameters of *A. spinidens* to predict the potential of the pathogen in combination with *A. spinidens* for the control of rice pests.

MATERIALS AND METHODS

Insect rearing

The adults and nymphs of *A. spinidens* were collected from rice fields in Amol city, Mazandaran province (north of Iran 36°28'11"N 52°21'03"E), in late September 2012. The predators were reared on last larval instars of *Galleria mellonella* L. (Lep.: Pyralidae) in laboratory condition (25 ± 2 °C, 60±10% RH (Relative humidity) and a photoperiod of 16:8 h L:D). The second generation of *A. spinidens* was used for the experiments.

Chemicals

Acetylthiocholine iodide (ATC), 5, 5'-dithiobis-(2-nitrobenzoic acid, DTNB), triton X-100 were purchased from Sigma. Fast blue RR salt, α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA) were obtained from Fluka. 1-chloro-2, 4-dinitrobenzene (CDNB) and acetone were purchased from Merck, Germany.

Fungal culture and preparation of conidial suspension

A strain of *B. bassiana* (IR-403) which was originally isolated from *Chorthippus brunneus* (Orthoptera: Acrididae) (supplied by Dr. M. Ghazavi, Plant Pests and Diseases Research Institute, Iran) was cultured on Sabouraud dextrose agar (SDA) at 25°C under continuous light. After 14 days, the conidia were removed from culture medium using a sterile spatula and suspended in 10 ml sterile distilled water containing 0.05% Tween 80. Suspensions were agitated for 5 minutes for homogenisation of the hydrophobic conidia. Then the suspension was passed through a two-folded mesh cloth to separate mycelium. In order to determine the number of spores, hemacytometer (Neubauer) was used. For bioassay, the suspensions were prepared at concentrations of 10⁴, 10⁵, 10⁶, 10⁷ and 10⁸ conidia ml⁻¹.

Bioassays

For bioassay, newly molted 5th-instar nymphs of *A. spinidens* immersed in conidial suspension of each concentration for 10 seconds and then excess suspension was removed using a filter paper. In the control, nymphs were treated with sterile distilled water containing 0.05% Tween-80. A total of 40 insects were used for each concentration and control. The treated insects were kept in a transparent plastic container (d= 7 cm, h= 10 cm). A 6 cm diameter hole was placed in the door of container for proper ventilation and covered with a mesh cloth. A piece of moistened cotton wool was placed in the bottom of each container to maintain the humidity. Then, these containers were kept in a chamber at 25 ± 2 °C, 70 ± 10% RH with a photoperiod of 16:8 hours (L: D) and were monitored daily for 8 days. The dead insects were counted and transferred to plastic Petri dishes (90 mm) covered with wet cotton wool in order to allow fungi to sporulate in dead insects. The LC₃₀ of *B. bassiana* were determined.

Life table parameters

In order to study the sublethal effects of *B. bassiana* on the life table parameters of *A. spinidens*, 1- day- old fifth-instars nymphs of

A. spinidens were used. The nymphs were treated with the LC_{30} of *B. bassiana* as mentioned above. The surviving nymphs were removed and kept in a transparent plastic container (d= 7 cm, h= 10 cm). A 6 cm diameter hole was placed on the container lids for proper ventilation and covered with a mesh cloth. A piece of moistened cotton wool was placed in the bottom of each container to maintain the humidity. Then, these containers were kept in an incubator set at $25 \pm 2^\circ\text{C}$, $60 \pm 10\%$ RH with a photoperiod of 16:8 hours (L: D) and were monitored daily until the adults appeared. After mating, the deposited eggs were kept until hatching. A cohort of 30 eggs was used for constructing life table. After emergence of first instars nymphs, they were provided with moistened cotton and left in the container. After molting, they were moved to individual Petri dishes (90 mm) covered with moistened cotton wool and were provided with the last larval instar of *G. mellonella*. The nymphs were checked daily until they reached the adult stage. The emerged adults were kept in pairs of one male and one female, and their mortality and the oviposition rate of female were recorded daily. In addition, life table parameters including the net reproductive rate (R_0), intrinsic rate of increase (r), finite rate of increase (λ), doubling time (DT) and mean generation time (T) were determined.

Biochemical analysis

24- 48 and 72 hours after treatment with the sublethal concentration, i.e. the LC_{30} of *B. bassiana*, the surviving nymphs were used for the enzymes activity determination according to the following methods based on the previous work (18).

Sample preparation

Each *A. spinidens* nymph was homogenized in 250 μl of 0.2 M phosphate buffer (pH 7.0) containing 0.05 % triton X-100 using a plastic pestle. Next, the homogenate was centrifuged at 12000 g for 10 min at 4°C . The supernatant was used as an enzyme solution for assessing the esterase, glutathione S-transferase and AChE activity. For determination of head AChE activity, each head of *A. spinidens* nymph was homogenized in 30 μl of 0.2 M phosphate buffer (pH 7.0) containing 0.05 % triton X-100 using a plastic pestle. The homogenate was centrifuged at 12000 g for 10 min at 4°C .

Esterase activity

The esterase activity was measured according to van Asperen's method (19). The substrates were α -NA and β -NA. 12.5 μl of supernatant was added to a microplate containing 112.5 μl

0.2 M, pH 7.0, phosphate buffer per well. The addition of 50 μl substrate per well (0.65 mM in buffer) initiated a reaction. After incubation for exactly 15 min at room temperature, 50 μl of fast blue RR salt was added and the microplate left in the dark for 30 min. Absorbance at 450 nm (OD_{450}) was then measured continuously for 10 min with a microplate reader (Awareness Stat Fax® 3200).

Glutathione S-transferase (GST) activity

GST activity was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced GSH as substrates with slight modifications according to Habig *et al.* (20). 15 μl of supernatant was added to 70 μl 0.2 M, pH 7.0, phosphate buffer, 100 μl CDNB and 100 μl GSH in buffer. The non-enzymatic reaction of CDNB with GSH measured without supernatant served as control. The change in absorbance was measured continuously for 10 min at 340 nm.

Acetylcholinesterase (AChE) activity

AChE activity was measured according to the methods of Ellman *et al.* (21). Fifty microliters of the enzyme source was added to each well of microplate containing 140 μl of 0.2 M phosphate buffer (pH 7.0) and 20 μl DTNB solution. Then 40 μl of ATC was added to each well. Optical density was measured continuously for 10 min at 415 nm.

Data analysis

The LC_{30} value and 95% confidence intervals were calculated from probit regressions using the POLO-PC computer program (22). Life table parameters were determined according to Chi (23). Difference between the treatments was determined by *t*-test (24).

Protein assay

Total protein was determined according to Bradford method (25) with bovine serum albumin as standard.

RESULTS

Log concentration probit-mortality of *A. spinidens* to *B. bassiana* is shown in **Figure 1**. The LC_{30} value was found at concentration of 3×10^5 conidia ml^{-1} for fifth-instars nymphs of *A. spinidens*. Sublethal effects of *B. bassiana* on pre-oviposition period, fecundity, percent egg hatchability and longevity of *A. spinidens* are presented in **Table 1**. There was not significantly difference between treated insects and control in pre-oviposition period ($T = -0.21$, $df=19$, $p=0.836$), fecundity ($T=0.78$, $df=26$, $p=0.442$), percent egg hatchability ($T=0.76$, $df=19$, $p=0.456$), female longevity ($T=0.52$, $df=26$, $p=0.60$) and male longevity

($T=0.36$, $df=26$, $p=0.72$). Sublethal effects of *B. bassiana* on life table parameters of *A. spinidens* are shown in **Table 2**. The r value was not significantly affected in offspring of the treated predator ($T=0.38$, $df=58$, $p=0.704$). There were not significant differences between R_0 values for *B. bassiana* in comparison with the control ($T=0.57$, $df=58$, $p=0.573$). The mean generation time ($T=0.36$, $df=58$, $p=0.92$), the doubling time ($T=0.80$, $df=58$, $p=0.427$),

and the finite rate of increase ($T=0.38$, $df=58$, $p=0.703$) did not vary significantly between treatments. The course of the age-specific survival rate (l_x) and the age-specific number of progeny per day (m_x) of *A. spinidens* for treated and untreated females are presented in **Figure 2**. The last female remained alive on 77th day was seen in control, however in *B. bassiana* treatment the death of last female occurred approximately at the age of 71 days.

Figure 1. Log concentration probit–mortality of fifth-instar nymphs of *Andrallus spinidens* to *Beauveria bassiana*

Table 1. Effects of *Beauveria bassiana* on biological parameters of *Andrallus spinidens*

Treatments	Fecundity	Egg hatch%	pre-oviposition period (day)	Longevity(day)	
				Female	Male
Control	108.2±21.3a	98.4±18.2a	8.36±0.9a	30.92±4.4a	17.83±3.1a
<i>B. bassiana</i>	86±18.7a	95±15.3a	8.6±0.5a	27.78±4.1a	16.27±2.9a

Means ± SE in the columns with the same letter are not significantly different ($p \leq 0.05$)

Table 2. Effects of *Beauveria bassiana* on the life table parameters of *Andrallus spinidens*

Treatments	r (day ⁻¹)	λ (day ⁻¹)	DT (day)	T (day)	R_0 (females offspring/female)
Control	0.085±0.006a	1.088±0.006a	8.15±0.50a	46.51±1.01a	50.53±13.99a
<i>B. bassiana</i>	0.081±0.006a	1.085±0.007a	8.55±0.57a	45.76±0.93a	40.2±11.7a

Means ± SE in the columns with the same letter are not significantly different ($p \leq 0.05$)

Figure 2. Effect of *Beauveria bassiana* (B) in comparison with the control (A) on age specific survival rate (l_x) and age specific fecundity (m_x) of *Andrallus spinidens*

Effect of *B. bassiana* on the esterase activity in *A. spinidens* is shown in **Table 3**. Using α -naphthyl as substrate, esterase activity had not significantly difference between *B. bassiana* and control in 24 h ($T = -2.10$, $df=4$, $p=0.103$), 48 h ($T = -3.6$, $df=4$, $p=0.022$) and 72 h ($T=0.95$, $df=4$, $p=0.396$) after treatment. But the enzyme activity showed significant changes between treated and untreated bugs when β -naphthyl was used as substrate. The results showed significantly difference in 24 h ($T = 5.34$, $df=4$, $p=0.0059$), 48 h ($T = -9.93$, $df=4$, $p=0.006$) and 72 h ($T=4.82$, $df=4$, $p=0.0085$) after treatment between treated insects and control. Effect of *B. bassiana* on the AChE activity in *A. spinidens* is shown in

Table 4. Head AChE activity in 24 h ($T = 0.27$, $df=4$, $p = 0.813$), 48 h ($T = -1.47$, $df=4$, $p = 0.279$) and 72 h ($T = -1.75$, $df=4$, $p = 0.222$) after treatment was not significant. Also, body AChE activity in 24 h ($T = -19$, $df=4$, $p=0.028$), 48 h ($T = -5.62$, $df=4$, $p = 0.03$) and 72 h ($T = 2.7$, $df=4$, $p = 0.114$) after treatment had not significant difference in treated *A. spinidens* in comparison with the control. Effect of *B. bassiana* on the GST activity in *A. spinidens* is shown in **Table 5**. GST activity was affected by *B. bassiana* in 24 h ($T = 6.48$, $df=4$, $p=0.0029$), 48 h ($T = -11.44$, $df=4$, $p=0.0003$) and 72 h ($T = -1.15$, $df=4$, $p=0.0031$) after treatment.

Table 3. Effects of *Beauveria bassiana* on the esterase activity of *Andrallus spinidens* (nmol min⁻¹mg protein⁻¹)

	Esterase activity (nmol min ⁻¹ mg protein ⁻¹)		
	Time (h)	Control	<i>B. bassiana</i>
α -naphthyl as substrate	24	1082.14 $\pm$ 0.002	2118.77 $\pm$ 0.195
	48	927.08 $\pm$ 0.009	1345.87 $\pm$ 0.55
	72	2729.15 $\pm$ 0.016	2253.78 $\pm$ 0.152
β -naphthyl as substrate	24	350.19 $\pm$ 0.011	339.33 $\pm$ 0.003*
	48	149.12 $\pm$ 0.007	404.37 $\pm$ 0.006*
	72	418.6 $\pm$ 0.01	290.49 $\pm$ 0.01*

Means $\pm$ SE in rows followed by (*) differ significantly ($p \leq 0.05$)

Table 4. Effects of *Beauveria bassiana* on the AchE activity of *Andrallus spinidens* (nmol min⁻¹mg protein⁻¹)

	AchE activity (nmol min ⁻¹ mg protein ⁻¹)		
	Time (h)	Control	<i>B. bassiana</i>
Head	24	78.22 $\pm$ 0.005a	55.94 $\pm$ 0.01a
	48	22.12 $\pm$ 0.002a	56.16 $\pm$ 0.01a
	72	22.38 $\pm$ 0.0005a	61.5 $\pm$ 0.01a
Body	24	30.79 $\pm$ 0.002a	58.1 $\pm$ 0.0015a
	48	23.12 $\pm$ 0.006a	49 $\pm$ 0.0025a
	72	72.57 $\pm$ 0.0008a	35 $\pm$ 0.0005a

Means $\pm$ SE in the rows with the same letter are not significantly different ($p \leq 0.05$)

Table 5. Effects of *Beauveria bassiana* on the GST activity of *Andrallus spinidens* (μ mol min⁻¹mg protein⁻¹)

Treatment	GST activity (μ mol min ⁻¹ mg protein ⁻¹)		
	24h	48h	72h
Control	10.95 $\pm$ 0.009	8.10 $\pm$ 0.011	9.731 $\pm$ 0.035
<i>B. bassiana</i>	10.60 $\pm$ 0.02*	11.24 $\pm$ 0.014*	10.11 $\pm$ 0.031*

Note: Means $\pm$ SE in columns followed by (*) differ significantly ($p \leq 0.05$)

DISCUSSION

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.

B. bassiana is the main entomopathogenic fungus in Iran, which has been used against many insect pests including rice lepidopter pests (10). Therefore, it is necessary to investigate its effect on non target insects prior

to their release. In theory, if the predatory insect species are susceptible to *B. bassiana* after conidia are directly applied, or the insect is indirectly infected via feeding on contaminated surfaces, the potential of the fungal pathogen for integrated pest management would be compromised. However, if their effects on natural enemies are low compared with currently used synthetic insecticides, entomopathogenic fungi could be a compatible component with natural enemies in an IPM programme (26).

There are a lot of works about the effect of *B. bassiana* on non target insects (1, 27, 28) but, only a few studies have reported the side-effects of the fungus on life table parameters (13).

The results of the present study clearly demonstrated that the reproduction, longevity and the percentage of hatched eggs of *A. spinidens* was not affected significantly after exposure to *B. bassiana* at a concentration of 3×10^5 conidia ml^{-1} . In agreement with our findings, fecundity, egg viability and longevity of *Prynocaria congener* Billberg (Coleoptera: Coccinellidae) females did not differ significantly by *B. bassiana* treatment at a concentration of 10^4 conidia ml^{-1} (13). Strain Bb21 of *B. bassiana* at concentration of 5×10^8 conidia ml^{-1} had not important effect on egg production of *Chrysoperla carnea* (Chrysopidae) and *Harmonia axyridis* (Col.: Coccinellidae) (29). While, some authors did not find a significant change in the fecundity and or longevity in the exposed insect by pathogens, others reported the opposite result. For example, adult longevity of *Orius sauteri* (Hemiptera: Anthicidae) decreased by 9-13% when feeding on *Frankliniella occidentalis* Pergande (Tysanoptera: Thripidae) larvae contaminated by *B. bassiana* at concentrations of 10^4 and 10^8 conidia ml^{-1} compared with those fed *F. occidentalis* larvae not treated with *B. bassiana* (9 Gao et al. 2012). Also, *B. bassiana* at concentrations of 10^5 , 10^7 and 10^9 conidia ml^{-1} reduced *H. axyridis* egg production dramatically and at a concentration of 10^9 conidia ml^{-1} reduced the fecundity of *Adalia bipunctata* (Col.: Coccinellidae) to zero (29). Hajek and St. Leger (30) reported that entomopathogenic fungi affected the host insect through a combination of events including mechanical damage by hyphal growth, nutrient depletion, and the production of toxins. Any of these processes at a sublethal level could negatively affect the reproductive system of the host. A fungal infection initiated at this time and the resulting physiological

stress could interfere with egg development (31).

In the current study, the life table parameters of *A. spinidens* were not significantly affected by *B. bassiana*. These results are in agreement with *B. bassiana* treatment (10^4 and 10^8 conidia ml^{-1}) in *P. congener* where r , T , R_0 and λ did not differ significantly (13). Also, life table parameters of *Serangium japonicum* Chapin (Col.: Coccinellidae) were not affected by *V. lecanii* at concentrations of 10^4 , 10^5 , 10^6 , 10^7 and 10^8 conidia ml^{-1} (32). Xu et al. (33) showed no significant effects of *V. lecanii* (10^7 conidia ml^{-1}) on life table parameters of *Axinoscymnus cardilobus* (Col.: Coccinellidae).

Xenobiotics are largely detoxified by various enzymes. Glutathione S-transferases and nonspecific esterases are most commonly involved in this process in insects (15).

Esterase activity (using α and β -naphthyl) in the untreated bugs increased in the 3th day after treatment. It is reported that general esterase activity increases with aging, and newly emerged insects have a much lower level of general esterase activity than mature insects (34). Using β -naphthyl as substrate, enzyme activity was increased significantly 48 h after treatment by *B. bassiana*. A 2–3-fold increase in esterase activity was observed in the hemolymph of *G. mellonella* two days after infection by *B. bassiana* at concentration of 10^7 conidia ml^{-1} (15). Similar results were found on esterase activity in treated *G. mellonella* by *Paecilomyces fumosoroseus* Wize and *Metarhizium anisopliae* (35 Serebrov et al. 2001). Increased activity of detoxification enzymes in mycoses and other infections represents the insect response to body intoxication with metabolites of the pathogen or with products of host tissue degradation (15). Acetylcholinesterase (AChE) is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine in the nervous system. In this study, *B. bassiana* had not significant effect on AChE activity in both head and body of *A. spinidens*. GST activity was affected by *B. bassiana* at concentration of 3×10^5 conidia ml^{-1} . The enzyme activity in *A. spinidens* was increased 48 h after treatment by *B. bassiana*. Fungal infection of insect increases glutathione S-transferase activity (15). Similar results were reported by Fan et al. (36) in the treated larvae of *Dendrolimus tabulaeformis* Tsai et Liu (Lep.: Lasiocampidae) by *B. brongniartii* Saccardo Petch at concentrations of 6×10^6 and 6×10^7

conidia ml⁻¹. Induction of GST activity is an indication of a detoxification process (37). According to the present study, *B. bassiana* at concentration of 3×10⁵ conidia ml⁻¹ had not detrimental effects on life table parameters and enzymes activity of *A. spinidens*. It can be concluded that *B. bassiana* is compatible with the predatory bug at sublethal concentration (LC₃₀).

ACKNOWLEDGMENTS

The authors express their gratitude to the Research Council of the University of Guilan and the Ministry of Science, Research and Technology for financial support during the course of this project.

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