

Assessment of *Beauveria bassiana* and Their Enzymatic Extracts against *Metamasius spinolae* and *Cyclocephala lunulata* in Laboratory

Lluvia Sánchez-Pérez¹, Silvia Rodríguez-Navarro², Victor Hugo Marín-Cruz², Miguel Ángel Ramos-López³, Alejandro Palma Ramos⁴, Juan Esteban Barranco-Florado⁴

¹Masters Program in Agricultural Sciences, Division of Biological and Health, Metropolitan Autonomous University-Xochimilco, Coyoacán, México

²Department of Agricultural and Animal Production, Metropolitan Autonomous University-Xochimilco, Coyoacán, México

³Faculty of Chemistry, Autonomous University of Queretaro, Santiago de Querétaro, México

⁴Department of Biological Systems, Metropolitan Autonomous University-Xochimilco, Coyoacán, México

Email: barranco@correo.xoc.uam.mx

How to cite this paper: Sánchez-Pérez, L., Rodríguez-Navarro, S., Marín-Cruz, V.H., Ramos-López, M.Á., Ramos, A.P. and Barranco-Florado, J.E. (2016) Assessment of *Beauveria bassiana* and Their Enzymatic Extracts against *Metamasius spinolae* and *Cyclocephala lunulata* in Laboratory. *Advances in Enzyme Research*, 4, 98-112.

<http://dx.doi.org/10.4236/aer.2016.43010>

Received: July 1, 2016

Accepted: September 10, 2016

Published: September 13, 2016

Copyright © 2016 by authors and Scientific Research Publishing Inc. This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

Abstract

The application of enzymatic extracts and conidia of *Beauveria bassiana* in *Metamasius spinolae* and *Cyclocephala lunulata* was evaluated. The variables were mortality and time of death. In *M. spinolae*, mortality with extracts 29%, conidia 27% and the combination of both 31%, all had a time of death of four days. Although with different symptoms, used enzymatic extracts: contraction and softening of the joints; by conidia: mycelium in the joints; in the combination of conidia and enzymatic extracts: abundant aerial mycelium. In *C. lunulata*, 100% mortality in all treatments; Time of death: enzymatic extracts and extracts with conidia 1.2 days; conidia 2.8 days. Symptoms were different, enzymatic extracts: melanization and internal tissue lysis; enzymatic extract and conidia: mycelium emerged and melanization; conidia: mycelium emerged. Enzymatic extracts showed insecticidal activity in *M. spinolae* and *C. lunulata*. These results suggest the potential of enzymatic extracts as biocontrol agents to improve the use of entomopathogenic fungi.

Keywords

Insecticidal Activity, *Beauveria bassiana*, *Metamasius spinolae*, *Cyclocephala lunulata*, Enzymatic Extracts

1. Introduction

Metamasius spinolae (Gyllenhal, 1838) (Coleoptera: Curculionidae), is a main pest of

the cacti crop (*Opuntiasp*) in Mexico. It reduces crop yield [1] [2] and is distributed throughout the country with the highest incidence in Milpa Alta, D. F. with yield losses of 51.73% [3]. Larvae are inserted into the base in the older plant stalks, feeding on internal tissue, forming galleries and damaging the support structure. They also favor the entry of pathogens to the plant, and adults feed on the young pads. [4]. Due to the economic and cultural importance of cacti, it is necessary to propose alternatives to control *M. spinolae* and to replace the use of pesticides [2] [4]. *Cyclocephala lunulata* (Burmeister, 1847) (Coleoptera: Melolonthidae) white grub feeds on the roots of sugar cane (*Saccharumofficinarum* L.), maize (*Zea mays* L.), alfalfa (*Medicagosativa* L.), potato (*Solanumtuberosum* L.), asparagus (*Asparagus officinalis* L.), coffee plant (*Coffeearabica* L.), beet (*Betavulgaris* L.), lettuce (*Lactucasativa* L.), onion (*Alliumcepa* L.) and pastures (Poaceae) [5] [6]. In order to control populations of *C. lunulata*, are sprayed systemic insecticides as Furadan®, hazardous to human health and the environment [6]. Entomopathogenic fungi (EF) represent one of the most efficient biological control alternatives. *Beauveria bassiana* (Bálsamo) Vuillemin (Ascomycota: Hypocreales), which infects many insect's orders [4] [7], is a non-chemical alternative; it can degrade and penetrate the insect cuticle by synthesizing hydrolytic enzymes such as lipases, proteases and chitinases [8] [9]. Insecticidal and antifeedant activity of enzymes of *B. bassiana*, such as subtilisin type protease (Pr1), which alters the peritrophic membrane of insects, blocking the formation or regeneration, has been tested [10]. Lipases start penetration of the insect integument, degrading lipids, waxes and lipoproteins that prevent water loss and protection against pathogenic organism [11]. After degradation of the epicuticle, chitinases and proteases are secreted to degrade proteins that surround chitin, core biopolymer which forms integument of insects [12]. Degradation products serve as nutrients to the fungus. The most important characteristic of this is that its application and management do not pose human health risks and the agricultural products are safe [13]. In this study we were evaluated the insecticidal activity and reduce the time of death of *M. spinolae* and *C. lunulata* for the conidia and enzymatic extract of *B. bassiana* obtained from solid state fermentation.

2. Materials and Methods

2.1. Fungal Isolates

An isolate (11) of *B. bassiana* was cultured in potato dextrose agar (PDA) at 25°C for 15 days. The spores were suspended in a solution of Tween 80 (0.05%) in sterile distilled water and stored at 4°C.

2.2. Culture Conditions

Solid-state fermentation of *B. bassiana* was performed in 250 ml Erlenmeyer flasks. Culture medium was prepared as described by Barranco *et al.* [14]. For enzymatic induction shrimp shell was used (60 g·l⁻¹). A concentration of 1 × 10⁷ spores was inoculated per gram of humid matter. The culture conditions were RH 75%, pH 5.0 and tem-

perature of 25°C.

2.3. Extraction of Conidia

A solution Tween 80 (0.05%) was added to a solid-state fermentation from 15 days in sterile distilled water at a ratio of 1:1 (w/v). The mixture was homogenized, filtered and the suspension was stored at 4°C in sterile tubes. The conidia were counted using a hemocytometer (Neubauer, United States Patent PLANE Charles A. Neubauer, Baltimore, Md, 1970). The suspension was adjusted to a concentration of 1×10^8 conidia mL^{-1} [14].

2.4. Obtaining the Crude Enzymatic Extract

Distilled water was added to a solid-state fermentation for five days at a ratio of 1:1 (w/v). The mixture was homogenized, pressed and centrifuged at 10,000 rpm for ten minutes at 4°C. The supernatant was filtered through a membrane Millipore of 0.45 μm and the extract was stored at 4°C in sterile tubes.

2.5. Determination of Lipase Activity

For the enzyme assay, the substrate was an emulsion of olive oil and gum arabic at 10% (1:1), 1 mL of this substrate, 2 mL of phosphate buffer 50 mM, pH 6.8 and 1 mL of crude enzyme extract were mixed. The reaction mixture was incubated one hour at 37°C under constant stirring. The reaction was stopped by adding 4 mL of a solution of acetone ethanol 1:1 containing 0.09% of phenolphthalein. Enzyme activity was determined by titrating free fatty acids using 5 mL of NaOH [11]. One international unit was defined as enzyme activity that produced 1 μmol of fatty acid min^{-1} .

2.6. Determination of Protease Activity

Protease assay: 1) subtilisin (Pr1) succinyl-alanina 2 proline-phenylalanine-p-nitroaniline (Sigma-Aldrich, Inc. 3050 Spruce Street, St. Louis, USA); 2) trypsin (Pr2) Benzoyl-phenylalanine-valine-arginine-p-nitroaniline (Sigma-Aldrich, Inc. 3050 Spruce Street, St. Louis, USA). Reaction mixture: 0.05 mL of substrate (1 mmol), 0.85 mL of buffer Glycine-NaOH pH 8.5 and 0.1 mL of crude enzyme extract were mixture. The mixture was incubated at 50°C for one hour and the reaction was stopped by adding 0.25 mL of 30% acetic acid. The mixture was allowed to stand 15 min on ice, and then centrifuged at 5000 rpm for 5 min at 4°C. It was read at 410 nm [11]. One international unit was defined as enzyme activity that produced 1 nmol of nitroanilide min^{-1} .

2.7. Determination of Endochitinase Activity

0.5 ml of chitin colloidal 0.5% (w/v) was used as substrate along with 0.5 mL of enzyme extract and 1.0 mL phosphate buffer citrate 0.2 M, pH 5.6. The mixture was incubated at 50°C for one hour, and the reaction was stopped adding 3 mL of dinitrosalicylic acid (DNS). The mixture was heated in a water bath for five minutes. The solution was centrifuged at 10,000 rpm and read at 575 nm [15]. One international unit was defined as

enzyme activity that produced 1 nmol of reducing sugars min^{-1} .

2.8. Determination of Exochitinase Activity

The substrate used was p-nitrophenyl-N-acetyl- β -D-glucosamine (Sigma-Aldrich, Inc. 3050 Spruce Street, St. Louis, USA). 200 μL of a solution of 1.0 $\text{mg}\cdot\text{mL}^{-1}$ substrate, 200 μL of the enzyme extract and 200 μL of citrate phosphate buffer 0.2 M, pH 5.6 were taken and incubated at 37°C for one hour under constant stirring at 180 rpm; the reaction was stopped with 1 mL of 0.02 M NaOH. It was read at 400 nm [16]. One international unit was defined as enzyme activity required that liberated 1 μmol of p-nitrophenol min^{-1} . All assays were performed in triplicate.

2.9. Insects

Adults of *M. spinolae* were collected in San Juan Tepenahuac, Milpa Alta, México, and moved to the insectarium laboratory of the Xochimilco Metropolitan Autonomous University (UAM-X). They were kept individually in plastic cups 1 L. They were fed 20 g of spiny tender cactus, changing every two days. The larvae of *C. lunulata* were collected on the lawn of the sports area of the UAM-X, by sampling five golds, on area of 50 \times 50 cm^2 . The material was placed individually in plastic cups 1 L with 200 g of soil from the site of collection and were fed with a grass root ball of garden, changing according to the degree of wilting showed that each root ball. 20 larvae were separated until complete their development. With adults, taxonomic determination was made by Roberto Alejandro Terron Sierra from UAM-X. The individuals were acclimatized for seven days with a photoperiod of 12:12 L/D, 25°C \pm 2°C and 50% RH in the bioclimatic chamber LUMISTELL®.

2.10. Bioassay

Inoculation. Adults of *M. spinolae* were immersed in each treatment for 10 s. In order to remove excess water, they were placed on absorbent paper for 10 s [4]. Larvae of *C. lunulata*, on the back of the head capsule, were injected with 10 μL of each treatment [17] with a gas chromatography syringe (Hamilton Microliter™ Serie 700. Sigma-Aldrich).

Experimental Design. Completely randomized, with six treatments: T1: negative control (0.05% Tween 80); T2: crude enzymatic extract; T3: 1×10^8 conidia; T4: conidia more crude enzymatic extract; T5: positive control (Bea Tron®) 5×10^8 ; T6: enzymatic extract more positive control. *M. spinolae* had 48 replications with a total of 288 insects per treatment and *C. lunulata* had 10 replications per treatment with a total 60 insects. The observations were made every 24 hours for seven days. The experiments were established in the bioclimatic chamber LUMISTELL® under the conditions described above.

2.11. Statistical Analysis

Data were analysed with the software JMP® 8. The independent analyses were made for

the bioassay in *M. spinolae*. Chi square tests to mortality rate and Kruskal-Wallis to average time death, because the model did not meet the assumptions of normality and homoscedasticity. In *C. lunulata* larvae, analysis of variance and post hoc multiple comparison of mean values were carried out by Tukey test. All experiments described in this paper were for triplicate. For all analysis, differences of means were considered significant at p values <0.05.

3. Results

3.1. Enzymatic Activities

The enzymatic activities obtained of the crude extract of *B. bassiana* from five days correspond to lipases (820.83 U), subtilisin-type protease Pr1 (0.422 ± 0.077 U), trypsin-type protease Pr2 (0.095 ± 0.029 U), endochitinases (3.239 ± 0.382 U) and exochitinases (2.41 ± 0.07 U). These enzymes are responsible for breaking the integument of insects. The production of the subtilisin-type protease (Pr1) and trypsin (Pr2) was high: Pr1 activity is 4.4 times greater than the activity of Pr2. This is the first report of the use of enzymatic extracts and conidia of *B. bassiana* together to reduce the time of death of *M. spinolae* and *Clunulata* under controlled conditions.

3.2. Bioassay

Mortality. *M. spinolae*: there are significant differences among treatments, $\chi^2 (5) = 91.271$, $p < 0.001$. The best results were obtained by the positive control treatment Bea Tron® with crude enzymatic extract (T6) with 77% mortality; 75% in the positive control Tron® Bea (T5), followed by the crude enzymatic extract (T2), conidia (T3) and conidia with crude enzymatic extract (T4) with 29, 27 y 31% (**Figure 1**); there was significant differences between treatments (**Table 1**).

Mortality. In larvae *C. lunulata*, the results showed significant difference, $\chi^2 (5) = 46.614$, $p < 0.001$, among treatments: crude enzymatic extract (T2), conidia (T3) and conidia more crude enzymatic extract (T4) had 100% mortality, the positive control with crude enzymatic extract (T5) had 90% and positive control (T6) had 50% (**Figure 2**). The negative control (T1) was no mortality in organisms. 100% survival in the negative control showed that the conditions under which the bioassay was established were appropriate and the mortality in treatments was due to its insecticidal effect (**Table 2**).

Time of Death. *M. spinolae*: The results showed significant differences between treatments by applying the Kruskal-Wallis test: $\chi^2 (5) = 78,548$, $p < 0.0001$. In the treatments: crude enzymatic extract (T2), conidia (T3) and conidia more crude enzymatic extract (T4) observed that there were shorter times of death of four days on average. While, in treatments: positive control (Bea Tron®) (T5) and positive control (Bea Tron®) more crude enzymatic extracts (T6) were nine days on average (**Table 1**).

Time of Death. *C. lunulata*: In this case, the results showed significant differences between treatments by applying analysis of variance ($F (5, 298.84) = 35.93$; $p < 0.0001$). The treatments: crude enzymatic extract (T2) and conidia more crude enzymatic extract (T4) caused the death of the larvae in a time of 1.2 days on average, Respect to

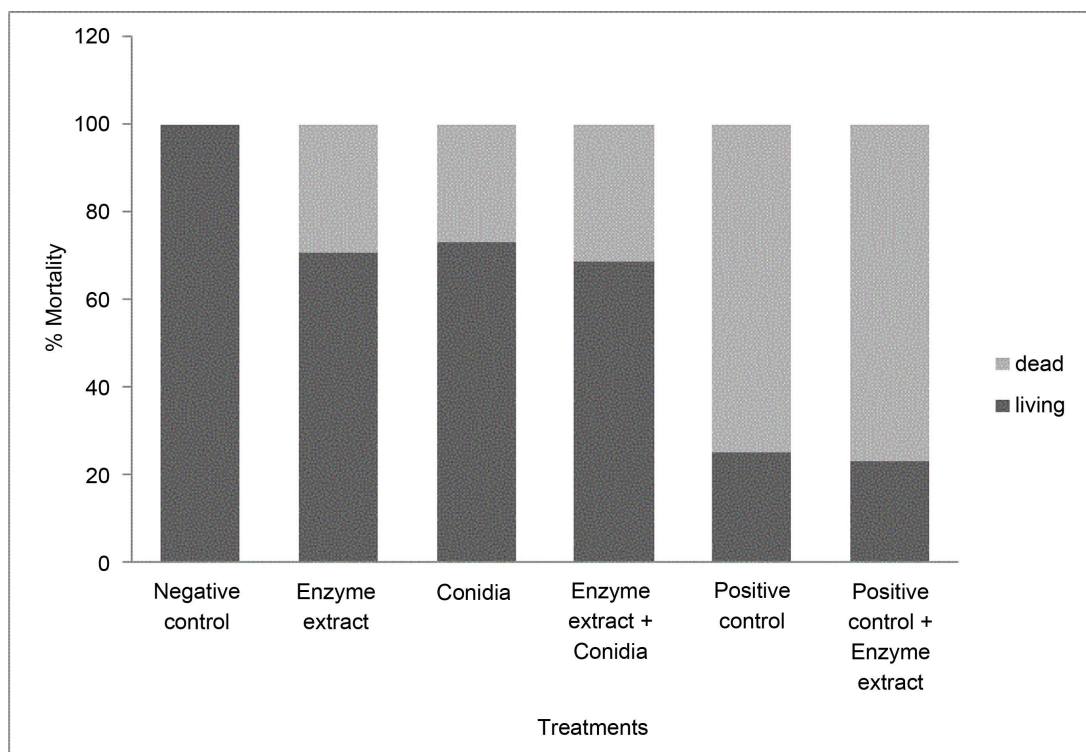


Figure 1. Mortality percentage to adult of *M. spinolae* under laboratory conditions.

Table 1. Mortality and time of death of adult *M. spinolae*.

Treatment	Mortality (%)	Time (days 9)
T1. Negative control (Tween 80 0.05%)	0	N.D.
T2. Enzymatic extract	29.17 ^a	3.87 ± 1 ^b
T3. Conidia 1 × 10 ⁸	27.08 ^a	3.93 ± 1 ^b
T4. Enzymatic extract + Conidia 1 × 10 ⁸	31.25 ^a	4.3 ± 1 ^b
T5. Positive control (Bea Tron®) 5 × 10 ⁸	75.00 ^b	9.22 ± 1 ^a
T6. Positive control + Enzymatic extract	77.08 ^b	8.89 ± 1 ^a

N.D. It was not determined. Means signed by the same letter differ not significantly according to Tukey. N = 48.

positive control (Bea Tron®) more enzymatic extracts (T6) was in 1.7 days, these treatments showed reduced the time of death. The treatments conidia (T3) and positive control (Bea Tron®) caused death in a longer time with 2.8 and 8.8 days, respectively. In both bioassays, the negative controls (T1) not cause the death of any insect (**Table 2**).

4. Discussion

In entomopathogenic fungi, enzymes that degrade insect cuticle play an important role in initiating the infectious process [18]. Shrimp shell is similar to the insect cuticle [19]. Its structure is a suitable substrate to induce the synthesis of proteases, chitinases and lipases [14] [20]. The proteolytic activities Pr1 and Pr2 were determined, being higher

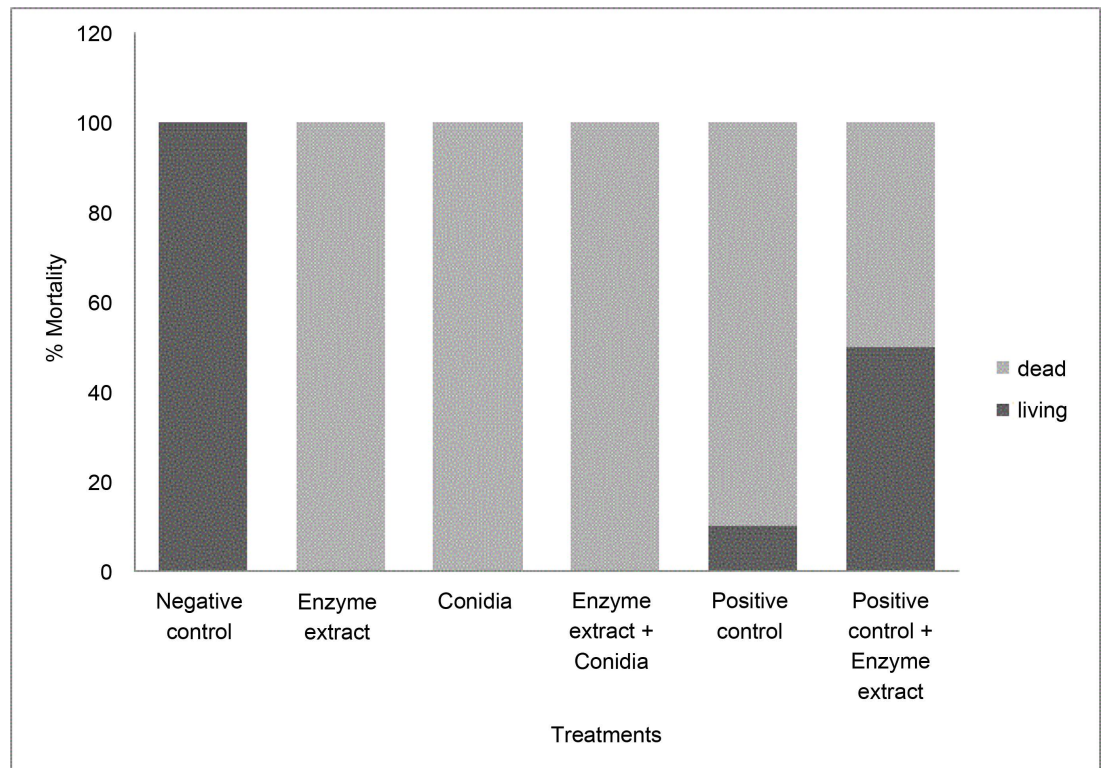


Figure 2. Mortality percentage to larvae of *C. lunulata* under laboratory conditions.

Table 2. Mortality and time of death of larvae of *C. lunulata*.

Treatment	Mortality (%)	Time (days 9)
T1. Negative control (Tween 80 0.05%)	0	N.D.
T2. Enzymatic extract	100 ^a	1.2 ± 0.4 ^{b,c}
T3. Conidia 1 × 10 ⁸	100 ^a	1.2 ± 0.4 ^{b,c}
T4. Enzymatic extract + Conidia 1 × 10 ⁸	100 ^a	2.8 ± 0.4 ^b
T5. Positive control (Bea Tron®) 5 × 10 ⁸	60 ^b	8.8 ± 0.057 ^a
T6. Positive control + Enzymatic extract	90 ^a	1.7 ± 0.42 ^b

N.D. It was not determined. Means signed by the same letter differ not significantly according to Tukey. N = 10.

activity of Pr1 in relation to Pr2. These results are similar to those obtained by Dias *et al.* [21] using cuticle of the coffee berry borer (*Hypothenemus hampei*). According to St. Leger *et al.* [22], the difference between the activity of Pr1 and Pr2 is because trypsin is a complement of the subtilisin enzyme. The ability to produce subtilisins is a virulence factor of *B. bassiana* [12] that allows infecting various orders of insects. By contrast, trypsins work as a suppressor of the immune system of the insect [23]. In addition, the lipolytic activities determined using the shell of shrimp as substrate, while not a specific inducer, and are attributed to an adjustment mechanism cAMP dependent signal transduction [24] to which the enzymes are regulate. Lipases are fundamental for starting epicuticle penetration, first barrier of protection against insects of these microorgan-

isms. Without these enzymes, infection would not be established [25] [26]. Lipases of *Isaria fumosorosea* have been used against diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) [11]. Chitinases are enzymes of interest as agents for control of pests and plant diseases [27] [28]. Exo and endo chitinase activity was determined in the extract, these enzymes act synergistically with proteases [29], even though the production of exochitinases in this solid culture was lower than that obtained by Barbosa *et al.* [30], who used silkworm pupae. However, induction of chitinases by using shrimp shell has been demonstrated [14].

The insecticidal action was mainly due to softening of the integument by the action of enzymes contained in the crude enzymatic extract, an effect that allowed the germ tube to penetrate the structure more easily to finally reach the haemocoel and promote infection in less time. So, the enzymatic extracts of *B. bassiana* showed insecticide when applied topically to adult of *M. spinolae*, alone or together with conidia, which represents the first report for this pest. Enzymes have their specific action on the cuticle of each component. Lipases hydrolyze the ester bonds of lipoproteins, fats and waxes epicuticle [11] [31], the Pr1 and Pr2 proteases degrade the proteinaceous material and finally, chitinases degrade the network of chitin [32], the main structural component of the exoskeleton in the walls, so that the cuticle lose rigidity and facilitating penetration. The insecticidal effect of enzymatic extracts was probably due to penetration by the pore channels, the base of silk, or the intersegmental membranes of the integument [33], including the mouth, spiracles and other openings outside of the insect. In dead individuals of *M. spinolae*, treated with enzymatic extract (T2), contraction of the three pairs of legs and weaker binding, abdomen and pronotal flexibility was observed, possibly due to degradation and joint intersegmental membranes, both with lower levels of sclerotization (**Figure 3(b)** and **Figure 3(c)**). Insects in the control group showed no symptoms associated with altered activity in the food or displacement or anatomy during the experiment (**Figure 3(a)**).

The bodies of *M. spinolae* treated with conidia (T3) presented mycelium of *B. bassiana*, mainly on the coxa and intersegmental space pronotum and abdomen (**Figure 3(e)** and **Figure 3(f)**). In other bodies, it also emerged from the side of the elytra and the joints between the femur and tibia (**Figure 3(d)**) which remained completely rigid and extended as a result of the invasion of the mycelium in the muscle tissue. In other orders of insects this sign has been reported [34]. On the other hand, in the bodies of treatment with conidia and enzymatic extract (T4) showed numerous groups of conidiogenous cells and aerial mycelium that emerged from the intersegmental spaces (**Figure 3(g)**). Most of the body surface of the insects was colonized by *B. bassiana* (**Figure 3(h)** and **Figure 3(i)**), unlike bodies with conidia treatment. Possibly the use of extracts with conidia facilitated the emergence of mycelium allowing their proliferation.

Use of enzymatic extracts with conidia of *B. bassiana* resulted in symptoms such as feeding activity inhibition: in three or four days the insect stops feeding and alterations in its locomotor system. Besides, the extracts could facilitate the emergence of mycelium, giving the fungus the greatest attributes in terms of persistence and spread. From

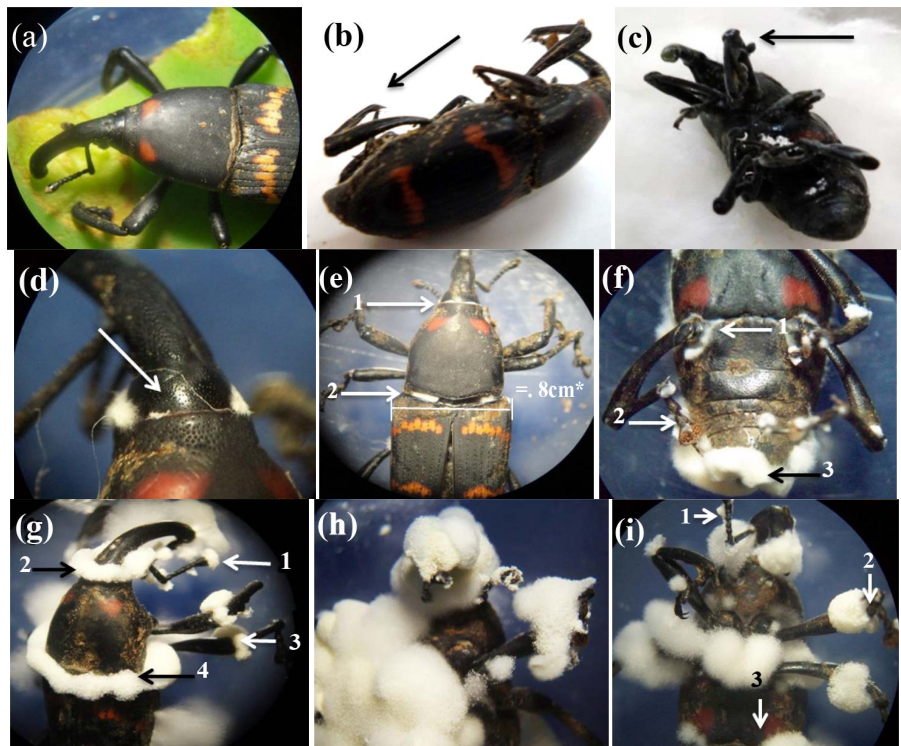


Figure 3. *M. spinolae*: (a) **Negative control** (b) and (c) **Enzymatic extract**, showing contraction of the legs; (d) **Conidia**, dorsal view of the head. (e): dorsal view with mycelium in the intersegmental space between pronotum (1) and abdomen (2), (f): ventral view from the coxa (1), knuckles, side of the elytra (2) and anus (3); **Conidia more enzymatic extract**. (g): dorsal view from the antennas (1), head (2), joints of femur and tibia (3); pronotum and abdomen (4); (h) ventral view covered by aerial mycelium; (i) ventral view from antennas and head with aerial mycelium (1); the articulation of the femur and tibia (2) and coxae (3).

an agricultural point of view, this raises their viability in the field as it becomes a new source of infection to be dispersed by abiotic factors such as the air and the breeze or contamination between individuals [35].

Injection of the enzyme extract in larvae of *C. lunulata* caused a toxic effect. The enzymes Pr1 and Pr2 contained in the enzyme extract caused pigmentation of cuticles as a response of the immune system to the presence of foreign enzymes inside the bodies (**Figure 4(b)**). The basic function of Pr1 is to degrade cuticle proteins and facilitate penetration of the integument. In addition, Pr1 is also toxic when introduced into the haemocoel to degrade proteins of hemolymph and melanization of integument [36] or secondary metabolites such as bassiacridin that cause the formation of melanised spots on tracheae and air sacs and melanised nodules in contact with the fat body [37]. The responsible compound for pigmentation of the cuticle is melanin, whose biological property is its fungicidal activity, and is synthesized by phenoloxidase in the hemolymph of arthropods, which is an inactive proenzyme called prophenoloxidase (proPO) likely to be active for the recognition of foreign agents [38]. Once proPO cascade system sets are activated in haemocytes, defense reactions as phagocytosis, encapsulation for removing foreign agent [39]; proPO insect system is activated by serum proteases

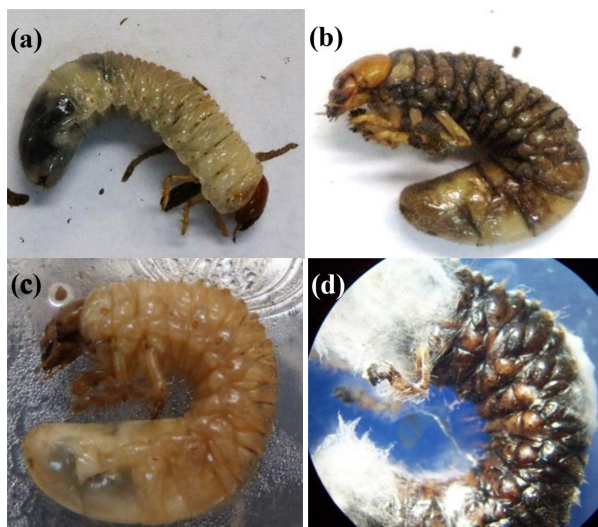


Figure 4. *C. lunulata* larvae: (a) **Negative control**. Side view with normal coloration; (b) **Enzymatic extract**, showing melanization throughout the body mainly among spaces intersegmental and regions near spiracles and loss of firmness of the tissue (side view); (c) **Conidia** (side view), the tissue became more rigid (d) **Conidia more enzymatic extract**, appearance of larvae with melanization tone dark brown throughout the body coupled with the emergence of mycelium from oral apparatus and leg joints and anal opening.

[40] present in an inactive form in haemocytes, reacting in the presence of β -1, 4 glucans from yeast, bacterial peptidoglycans or lipopolysaccharides of Gram bacteria [41]. This complex interacts with the surface of haemocytes to activate the prophenoloxidase system.

In larvae of *C. lunulata*, various pigmentary changes were observed on the tegument. Negative control larvae maintained their characteristic creamy white and soft texture and body (Figure 4(a)). However, the bodies of larvae of the enzyme extract (T2) took brownish pigmentation attributed to melanization, although the highest intensity in areas near spiracles, and body tissue lost firmness (Figure 4(b)).

In the bodies of larvae inoculated with conidia (T3), were observed changes in pigmentation to an orange color in the body. Tissue of the bodies also varied. It was observed with greater rigidity and later emerged mycelium (Figure 4(c)). The larvae inoculated with conidia more extracts (T4) acquired a dark brown pigmentation and mycelium emerged from natural openings and firm body tissue (Figure 4(d)). The synergy between extracts and conidia caused combining melanization and mycelium, in order to persist in the environment [35]. Lower mortality was observed in the commercial product probably because of *B. bassiana* strain is less virulent for *C. lunulata* larvae; however the commercial product plus the extract increased mortality. Melanization in larval cuticle has been reported by Fuguet and Vey [42] which was observed in larvae of *Galleria mellonella* (Lep: Pyralidae) by injection with conidia of different isolates of *B. bassiana*, where different tones and degrees of pigmentation were observed as well as patterns that were categorized, pointing to the processes of melanization in the integument, located at points where mycelium emerged. In this work, in the bodies of *C. lu-*

nulata inoculated with conidia of *B. bassiana*, the melanization was not in dark colors, but it was in orange pigmentation Chavez *et al.* [17] reported the same results with injecting crude extract of *B. bassiana* to *Phyllophaga* ssp. However, in larvae injected with crude enzyme extract, the death was 24 hr post inoculation.

The insecticidal activity of enzymes such as chitinases and proteases of *B. bassiana* has already been tested in some pests of agricultural importance when topically applied as a powder, it showed insecticidal activity on cotton aphid *Aphis gossypii* Glover [43] or with crude proteins of *Isaria fumosorosea* that affect the growth and metamorphosis of diamond back moth *Plutella xylostella* [44]. Similar results were reported by Quesada-Moraga *et al.* [45] for the use of crude proteins of *Metarhizium anisopliae* (01/58-His) in *Spodoptera littoralis* larvae, causing inhibition of feeding activity. These enzymes in the intestine cause damage significant to the structure of the peritrophic membrane, resulting in the inability of feeding and death [45] [46].

5. Conclusion

The crude enzymes extract of *B. bassiana* obtained by solid-state fermentation have insecticidal potential to be used in the biological control of pests of agricultural importance, given that they are constituted by a mixture of lipases, proteases and chitinases, which caused a toxic effect on *M. spinolae* and *C. lunulata*. Also, to use it as an adjuvant with conidia reduced the time of death of the insects, opening new prospects for the development of bio-insecticides with less impact to the environment, because they are a sustainable option to replace and reduce the use of chemical insecticides. It is necessary to continue studies to assess the stability and effectiveness of the enzymatic extracts in other models and different biological states and perform histological studies of insects treated, to determine the effect of these biomolecules on insect' physiology.

Acknowledgements

We are grateful to John A. Goolsby, Ph.D., Research Entomologist Biological Control and Integrated Pest Management of Ticks, Insects and Weeds. United States Department of Agriculture, Agricultural Research Service, for their valuable comments and correcting the manuscript. This research was support by Consejo Nacional de Ciencia y Tecnología (CONACYT), Lluvia de Carolina Sánchez Pérez beneficiary of a grant.

References

- [1] Tafoya, F., Zuñiga-Delgadillo, M., Alatorre, R., Cibrian-Tovar, J. and Stanley, D. (2004) Pathogenicity of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) against the Cactus Weevil, *Metamasius spinolae* (Coleoptera: Curculionidae) under Laboratory Conditions. *Florida Entomologist*, **87**, 533-536.
[http://dx.doi.org/10.1653/0015-4040\(2004\)087\[0533:POBBDH\]2.0.CO;2](http://dx.doi.org/10.1653/0015-4040(2004)087[0533:POBBDH]2.0.CO;2)
- [2] Cerón-González, C., Rodríguez-Leyva, E., Lomelí-Flores, J.R., Hernández-Olmos, C.E., Peña-Martínez, R. and Mora-Aguilera, G. (2012) Evaluación de insecticidas sintéticos sobre adultos de *Metamasius spinolae* (Coleoptera: Curculionidae) procedentes de Tlalnepantla, Morelos. *Revista Mexicana Ciencia Agrícola*, **3**, 217-229.

- [3] SEDEREC (2009) Geoestadística del nopal-verdura en el Distrito Federal. In: Alimentación, P., Ed., Secretaría de Agricultura, Ganadería, Desarrollo Rural, México, Oficina Estatal de Información para el Desarrollo Rural Sustentable del Distrito Federal (OEIDRUS D.F.).
- [4] Orduño-Cruz, N., Guzmán-Franco, A.W., Rodríguez-Leyva, E., López-Collado, J., Valdéz-Carrasco, J.M. and Mora-Aguilera, G. (2011) Susceptibility of the Cactus Weevil *Metamasius spinolaeto* *Beauveria bassiana* and *Metarhiziumanisopliae* under Laboratory and Field Conditions. *Journal of Applied Microbiology*, **11**, 939-948. <http://dx.doi.org/10.1111/j.1365-2672.2011.05097.x>
- [5] Morón, M.A. (2006) Revisión de las especies de *Phyllophaga* (*Phytalus*) grupos *obsoleta* y *pallida* (Coleoptera: Melolonthidae: Melolonthinae). *Folia Entomologia Mexicana*, **45**, 1-104.
- [6] Aragón-García, A., Morón, M.A., Damián-Huato, M.A., López-Olguín, J.F., Pinsón-Rincón, E.P. and Pérez-Quintanilla, J.N. (2012) Fauna de Coleoptera Lamellicornia de la zona cañera del ingenio de Atencingo, Puebla, México. *Acta Zoológica Mexicana*, **28**, 161-171.
- [7] Farenhorst, M., Mouatcho, J.C., Kikankie, C.K., Brooke, B.D., Hunt, R.H., Thomas, M.B., Koekemoer, L.L., Knols, B.G. and Coetzee, M. (2009) Fungal Infection Counters Insecticide Resistance in African Malaria Mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 17443-17447. <http://dx.doi.org/10.1073/pnas.0908530106>
- [8] Lubeck, I., Arruda, W., Souza, B.K., Staniscuaski, F., Carlini, C.R., Schrank, A. and Vainstein, M.H. (2008) Evaluation of *Metarhizium anisopliae* Strains as Potential Biocontrol Agents of the Tick *Rhipicephalus (Boophilus) microplus* and the Cotton Stainer *Dysdercus peruvianus*. *Fungal Ecology*, **1**, 78-88. <http://dx.doi.org/10.1016/j.funeco.2008.09.002>
- [9] Boldo, J.T., Junges, A., Amaral, K.B., Staats, C.C., Vainstein, M.H. and Scharank, A. (2009) Endochitinase CHI2 of the Biocontrol Fungus *Metarhizium anisopliae* Affects Its Virulence toward the Cotton Stainer Bug *Dysdercus peruvianus*. *Current Genetics*, **55**, 551-560. <http://dx.doi.org/10.1007/s00294-009-0267-5>
- [10] Sobotnik, J., Kudlikova-Krizkova, I., Vancova, M., Munzbergova, Z. and Hubert, J. (2008) Chitin in the Peritrophic Membrane of *Acarussiro* (Acari: Acaridae) as a Target for Novel Acaricides. *Journal of Economic Entomology*, **101**, 1028-1033. [http://dx.doi.org/10.1603/0022-0493\(2008\)101\[1028:CITPMO\]2.0.CO;2](http://dx.doi.org/10.1603/0022-0493(2008)101[1028:CITPMO]2.0.CO;2)
- [11] Ali, S., Huang, Z. and Ren, S. (2010). Production of cuticle degrading enzymes by *Isaria fumosorosea* and their evaluation as a biocontrol agent against diamondback moth. *Journal of Pest Science* **83**: 361-370. <http://dx.doi.org/10.1007/s10340-010-0305-6>
- [12] Fang, W., Feng, J., Fan, Y., Zhang, Y., Bidochka, M.J., Leger, S.T., and Pei, Y. (2009) Expressing a Fusion Protein with Protease and Chitinase Activities Increases the Virulence of the Insect Pathogen *Beauveria bassiana*. *Journal of Invertebrate Pathology*, **102**, 155-159. <http://dx.doi.org/10.1016/j.jip.2009.07.013>
- [13] Shahid, A.A., Rao, A.Q., Bakhsh, A. and Husnain, T. (2012) *Entomopathogenic fungi* as Biological Controllers: New Insights into Their Virulence and Pathogenicity. *Archives of Biological Sciences*, **64**, 21-42. <http://dx.doi.org/10.2298/ABS1201021S>
- [14] Barranco-Florido, J.E., Bustamante-Camilo, P., Mayorga-Reyes, L., Martínez-Cruz, P. and Azaola, M.A. (2009) β -N Acetylglucosaminidase Production by *Lecanicillium* (*Verticillium*) *lecanii* ATCC 26854 by Solid-State Fermentation Utilizing Shrimp Shell. *Interciencia*, **34**, 356-360.
- [15] Almeida, F.B. (2001) Mycoparasitism Studies of *Trichoderma harzianum* Strains against *Rhizoctoniasolani*: Evaluation of Coiling and Hydrolytic Enzyme Production. *Biotechnol-*

- ogy Letters, **29**, 1189-1193. <http://dx.doi.org/10.1007/s10529-007-9372-z>
- [16] Agrawal, T. and Kotasthane, A.S. (2012) Chitinolytic Assay of Indigenous *Trichoderma* Isolates Collected from Different Geographical Locations of Chhattisgarh in Central India. *SpringerPlus*, **1**, 73. <http://dx.doi.org/10.1186/2193-1801-1-73>
- [17] Chávez-Ibañez, E., Rodríguez-Navarro, S., Sánchez-Pérez, L.L.C., Partida-Hamdan, A. and Barranco-Florido, J.E. (2014) Actividad insecticida *in Vitro* de extracto crudo de *Beauveria bassiana* (Bálsamo) Vuillemin sobre larvas de *Phyllophaga* spp. (Harris). *Revista de Protección Vegetal*, **29**, 226-230.
- [18] Fernandes, E.G., Valerio, H.M., Eltrin, T. and Van Dersand, S.T. (2012) Variability in the Production of Extracellular Enzymes by Entomopathogenic Fungi Grown on Different Substrates. *Brazilian Journal of Microbiology*, **43**, 827-833. <http://dx.doi.org/10.1590/S1517-83822012000200049>
- [19] Xu, Y., Bajaj, M., Schneider, R., Grage, S.L., Ulrich, A.S., Winter, J. and Gallert, C. (2013) Transformation of the Matrix Structure of Shrimp Shells during Bacterial Deproteinization and Demineralization. *Microbial Cell Factories*, **12**, 90. <http://dx.doi.org/10.1186/1475-2859-12-90>
- [20] Rosas-García, N.M., Ávalos-De León, O., Villegas-Mendoza, J.M., Mireles-Martínez, M., Barboza-Corona, J.E. and Castañeda-Ramírez, J.C. (2014) Correlation between pr1 and pr2 Gene Content and Virulence in *Metarhizium anisopliae* Strains. *Journal of Microbiology and Biotechnology*, **24**, 1495-1502. <http://dx.doi.org/10.4014/jmb.1404.04044>
- [21] Dias, B.A., Neves, P.M.O.J., Maia, L.F. and Furlaneto, M.C. (2008) Cuticle Degrading Proteases Produced by the Entomopathogenic Fungus *Beauveria bassiana* in the Presence of Coffee Berry Borer Cuticle. *Brazilian Journal of Microbiology*, **39**, 301-306. <http://dx.doi.org/10.1590/S1517-83822008000200019>
- [22] St Leger, R.J., Joshi, L., Bidochka, M.J. and Rizzo, N.W. (1996) Biochemical Characterization and Ultrastructural Localization of Two Extracellular Trypsins Produced by *Metarhizium anisopliae* in Infected Insect Cuticles. *Applied and Environmental Microbiology*, **62**, 1257-1264.
- [23] Gao, Q., Jin, K., Ying, S.H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X.Q., Zhou, G., Peng, G., Luo, Z., Huang, W., Wang, B., Fang, W., Wang, S., Zhong, Y., Ma, L.J., St Leger, R.J., Zhao, G.P., Pei, Y., Feng, M.G., Xia, Y. and Wang, C. (2011) Genome Sequencing and Comparative Transcriptomics of the Model Entomopathogenic Fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genetics*, **7**, e1001264. <http://dx.doi.org/10.1371/journal.pgen.1001264>
- [24] Fang, W., Ripoll, M.P., Wang, S. and St Leger, R.J. (2009) Protein Kinase A Regulates Production of Virulence Determinants by the Entomopathogenic Fungus, *Metarhizium anisopliae*. *Fungal Genetics and Biology*, **46**, 277-285. <http://dx.doi.org/10.1016/j.fgb.2008.12.001>
- [25] Beysda Silva, O.W., Santi, L., Scharank, A. and Vainstein, M.H. (2010) *Metharhizium anisopliae* Lipolytic Activity Plays a Pivotal Role in Rhipicephalus (*Boophilus*) Microplus Infection. *Fungal Biology*, **144**, 10-15. <http://dx.doi.org/10.1016/j.mycres.2009.08.003>
- [26] Ali, S., Ren, S. and Huang, Z. (2014) Extracellular Lipase of an Entomopathogenic Fungus Effecting Larvae of a Scale Insect. *Journal Basic Microbiology*, **54**, 1148-1159. <http://dx.doi.org/10.1002/jobm.201300813>
- [27] Binod, P., Sukumaran, R.K., Shirke, S.V., Rajput, J.C. and Pandey, A. (2007) Evaluation of Fungal Culture Filtrate Containing Chitinase as a Biocontrol Agent against *Helicoverpa armigera*. *Journal of Applied Microbiology*, **103**, 1845-1852. <http://dx.doi.org/10.1111/j.1365-2672.2007.03428.x>

- [28] Wang, K., Yan, P.S., Cao, L.X., Ding, Q.L., Shao, C. and Zhao, T.F. (2013) Potential of Chitinolytic *Serratia marcescens* Strain JPP1 for Biological Control of *Aspergillus parasiticus* and Aflatoxin. *Biomed Research International*, **2013**, Article ID: 397142. <http://dx.doi.org/10.1155/2013/397142>
- [29] St Leger, R.J. (1991) Integument as a Barrier to Microbial Infections, In: Binnington, K. and Retnakaran, A., Eds., *Physiology of the Insect Epidermis*, Commonwealth Scientific and Industrial Research Organization, Melbourne, 286-308.
- [30] Barbosa, R.C., Atilio, J.J. and Souza, G.L.H. (2012) Optimization of the Chitinase Production by Different *Metarhizium anisopliae* Strains under Solid-State Fermentation with Silkworm Chrysalis as Substrate Using CCRD. *Advances in Applied Microbiology*, **2**, 268-276. <http://dx.doi.org/10.4236/aim.2012.23032>
- [31] Juárez, M.P. and Fernández, G.C. (2007) Cuticular Hydrocarbons of Triatomines. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **14**, 711-730. <http://dx.doi.org/10.1016/j.cbpa.2006.08.031>
- [32] Sandhu, S.S., Sharma, A.K., Beniwal, V., Goel, G., Batra, P., Kumar, A., Jaglan, S., Sharma, A.K. and Malhotra, S. (2012) Myco-Biocontrol of Insect Pests: Factors Involved, Mechanism, and Regulation. *Journal of Pathogens*, **2012**, Article ID: 126819. <http://dx.doi.org/10.1155/2012/126819>
- [33] Boevé, J.L., Ducarme, V., Mertens, T., Bouillard, P. and Angel, S. (2004) Surface Structure, Model and Mechanism of an Insect Integument Adapted to Be Damaged Easily. *Journal of Nanobiotechnology*, **2**, 10. <http://dx.doi.org/10.1186/1477-3155-2-10>
- [34] Ricaño, J., Güerri-Agulló, B., Serna-Sarriás, M.J., Rubio-Llorca, G., Asensio, L., Barranco, P. and Lopez-Llorca, L.V. (2013) Evaluation of the Pathogenicity of Multiple Isolates of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) for the Assessment of a Solid Formulation under Simulated Field Conditions. *Florida Entomologist*, **96**, 1311-1324. <http://dx.doi.org/10.1653/024.096.0410>
- [35] Meyling, N.V and Eilenberg, J. (2007) Ecology of the Entomopathogenic Fungi *Beauveria bassiana* and *Metarhizium anisopliae* in Temperate Agroecosystems: Potential for Conservation Biological Control. *Biological Control*, **43**, 145-155. <http://dx.doi.org/10.1016/j.biocontrol.2007.07.007>
- [36] Wang, C., Hu, G. and St Leger, R.J. (2005) Differential Gene Expression by *Metarhizium anisopliae* Growing in Root Exudate and Host (*Manduca sexta*) Cuticle or Hemolymph Reveals Mechanisms of Physiological Adaptation. *Fungal Genetics and Biology*, **42**, 704-718. <http://dx.doi.org/10.1016/j.fgb.2005.04.006>
- [37] Quesada-Moraga, E. and Vey, A. (2004) Bassiacridin, a Protein Toxic for Locusts Secreted by the Entomopathogenic Fungus *Beauveria bassiana*. *Mycological Research*, **108**, 441-452. <http://dx.doi.org/10.1017/S0953756204009724>
- [38] Nappi, A.J. and Christensen, B.M. (2005) Melanogenesis and Associated Cytotoxic Reactions: Applications to Insect Innate Immunity. *Insect Biochemistry and Molecular Biology*, **35**, 443-459. <http://dx.doi.org/10.1016/j.ibmb.2005.01.014>
- [39] Cerenius, L., Lee, B.L. and Söderhäll, K. (2008) The proPO-system Pros and Cons for Its Role in Invertebrate Immunity. *Trends in Immunology*, **29**, 263-271. <http://dx.doi.org/10.1016/j.it.2008.02.009>
- [40] EL Chamy, L., Leclerc, V., Caldelari, I. and Reichhart, J.M. (2008) Sensing of "Danger Signals" and Pathogen Associated Molecular Patterns Defines Binary Signaling Pathways "Upstream" of Toll. *Nature Immunology*, **9**, 1165-1170. <http://dx.doi.org/10.1038/ni.1643>
- [41] Ashida, M. and Brey, P.T. (1997) Recent Advances in Research on the Insect Phenoloxidase

- Cascade. In: Brey, P.T. and Hultmark, D., Eds., *Molecular Mechanisms of Immune Responses in Insects*, Chapman & Hall, London, 135-172.
- [42] Fuguet, R. and Vey, A. (2004) Comparative Analysis of the Production of Insecticidal and Melanizing Macromolecules by Strains of *Beauveria bassiana* spp. *in Vivo* Studies. *Journal of Invertebrate Pathology*, **85**, 152-167. <http://dx.doi.org/10.1016/j.jip.2004.03.001>
- [43] Kim, J.S., Roh, J.Y., Choi, J.Y., Wang, Y., Shim H.J. and Je, Y.H. (2010) Correlation of the Aphicidal Activity of *Beauveria bassiana* SFB-205 Supernatant with Enzymes. *Fungal Biology*, **114**, 120-128. <http://dx.doi.org/10.1016/j.mycres.2009.10.011>
- [44] Shoaib, F., Jin, F.L., Muhammad, N., Ren, S.X. and Mubshar, H. (2012) Toxicity of Proteins Secreted by Entomopathogenic Fungi against *Plutella xylostella* (Lepidoptera: Plutellidae). *International Journal of Agriculture and Biology*, **14**, 291-295.
- [45] Quesada-Moraga, E., Carrasco-Díaz, J.A. and Santiago-Álvarez, C. (2006) Insecticidal and Antifeedant Activities of Proteins Secreted by Entomopathogenic Fungi against *Spodoptera littoralis* (Lep., Noctuidae). *Journal of Applied Entomology*, **130**, 442-452. <http://dx.doi.org/10.1111/j.1439-0418.2006.01079.x>
- [46] Harrison, R.L and Bonning, B.C. (2010) Proteases as Insecticidal Agents. *Toxins*, **2**, 935-953. <http://dx.doi.org/10.3390/toxins2050935>



Submit or recommend next manuscript to SCIRP and we will provide best service for you:

Accepting pre-submission inquiries through Email, Facebook, LinkedIn, Twitter, etc.
A wide selection of journals (inclusive of 9 subjects, more than 200 journals)
Providing 24-hour high-quality service
User-friendly online submission system
Fair and swift peer-review system
Efficient typesetting and proofreading procedure
Display of the result of downloads and visits, as well as the number of cited articles
Maximum dissemination of your research work

Submit your manuscript at: <http://papersubmission.scirp.org/>