BIOCHEMICAL EFFECTS OF THE ENTOMOPATHOGENIC FUNGUS, *BEAUVERIA BASSIANA* ON THE RED PALM WEEVIL, *RHYNCHOPHORUS FERRUGINEUS*

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Abstract

Fourth instar larval was chosen to evaluate the biochemical activity (proteases, Chitinases and α-&β –esterases) of the red palm weevil treated by *Beauveria bassiana* under laboratory conditions. Biochemical analysis of the haemolymph indicated that the fungus, *B. bassiana*-9894 increased proteases, Chitinases and α- & β-esterases enzymes level compared with control. The level of total protein was decreased, meanwhile, the total lipids were increased compared with control.
Key words: *Rhynchophorus ferrugineus*, entomopathogens, *Beauveria bassiana,* enzymes.

INTRODUCTION

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) belongs to the family of Curculionidae (Coleoptera). *R. ferrugineus* is a major economic pest of coconut palm, date palm, oil palm and sago palm. It also attacks a wide range of ornamental palms. It has been known first in south east Asia until it appeared in United Arab Emirates in 1985, Kingdom of Saudi Arabic in 1987 and Egypt in 1992 (Saleh, 1992).

Entomopathogenic fungi differ from other insect pathogens in their ability to invade a host by direct penetration of the cuticle, which is essentially composed of proteins and chitin associated with lipids and phenolic compounds.

One of the advantages of entomopathogenic fungi is that the host insect need not ingest those. Entomopathogenic fungi, such as *Beauveria bassiana*, infect the host insect by penetrating the insect cuticle. The fungal conidium attaches to the cuticle by nonspecific hydrophobic mechanism and subsequently germinates. The insect cuticle consist primary of a chitin matrix embedded with proteins**.** Entomopathogenic fungi produce extracellular proteases and chitinases that degrade these proteinaaceous and chitinous components, allowing hyphal penetration through the cuticle and access to the nutrient-rich insect hemolymph (Charnley and Legar

1991). Proteases, particularly the subtilisin –like protease called Pr1, and the chitinases Bbchit1 (Fang *et al.*, 2005) are important virulence factors for *B. bassiana*. Chitinases are widely distributed and are found in viruses, bacteria, fungi, plants and animals. Some of these chitinases contain a chitin-binding domain linked to the catalytic site via an intermediate region (Beintema, 1994). Chitin is an important component of insect cuticle, which is the primary barrier against pathogens. Chitinases are required by the fungus for both penetration through the cuticle barrier and when emerging from the host (Fang *et al.* 2005). Over expression of Bbchit 1 was able to increase the ability of *B. bassiana* to digest insect cuticle, resulting increased virulence against insect (Fang *et al.*, 2005). Entomopathogenic fungi, such as *B. bassiana*, penetrate the insect cuticle utilizing a plethora of hydrolytic enzymes, including chitinases, which are important virulence factors (Yanhura *et al.,* 2007).

Insect cuticle is a composite structural material with mechanical properties that are optimal for their biological functions. The cuticle consists of a thin outer epicuticle containing lipids and proteins and a thick procuticle consisting mainly of chitin and proteins (Andersen *et al.*, 1995). Entomopathogenic fungi enter their hosts through direct penetration of the cuticle, which is a barrier against most microbes. Consequently, fungal pathogens have a potential as a biological means of controlling sap-sucking insects that have not been easily controlled with chemical pesticides. During the fungal penetration through the host cuticle, hydrolytic enzymes such as proteases, chitinases, and lipases are produced and secreted and are important for the initiation of the infection process (Yang and Yeh, 2005). Investigations on insect proteins and lipids found in both haemolymph and body fat are of particular interest because they provide us with an adequate background to investigate the synthetic activities associated with the different processes in developing organisms.

MATERIALS AND METHODS

1. Red palm weevil, *Rhynchophorus ferrugineus* (Olivier).

The red palm weevil, *R. ferrugineus* were reared on the stems of sugarcane as described by KaaKeh *et al.*, (2001) under laboratory conditions at 28±1ºC and 60-70% R.H. Adult weevils were collected from infested date palm trees at plantation locations at El-Kassasin area, Ismailia Governorate, Egypt**.**

2. *Beauveria bassiana***.**

The strain of *B. bassiana*-9894 used in this study was isolated from infected cadaver adults of *R. ferrugineus* collected from El-Kassasin area. Isolated fungus was purified and identified in Assuit University Mycological Center (AUMC).

Culture of entomopathogenic fungus, *B. bassiana*-9894:

The conidia fungus were cultured on agar Petri dishes of Dox medium (Thom and Raper., 1945). The medium consists of Sucrose 20 g/L, $KHPO_4$ 1 g/L, KNO_3 2 g/L, KCl 0.5 g/L , Yeast extract 2g/L, $MgSO_4.7H_2O$ 0.5 g/L , $FeSO_4.7H_2O$ 0.002 g/L and agar 20 g/L and incubated at 25±2 ºC and 60-70% R.H for two weeks. At the end of incubation period the conidia were harvested from the surface of the culture directly by scraping with sterile solution of 0.02% Tween-80. The conidia were separated by filtration through sterilized glass-wool. For experimental work, conidia of *B. bassiana*-9894 were suspended in 50 ml sterile distilled water with 0.02% Tween-80 and vortexes for 1 minute to break spore chains into individual spores and assure uniform mixing. Spores suspended in sterilized water were counted using haemocytometer counts technique (Goettel and Inglis 1997).

3. **Effect of *B. bassiana*-9894 on some biochemical parameters in red palm weevil *R. ferrugineus*.**

Fourth instar larval was chosen to evaluate the biochemical activity of red palm weevil (protease, chitinase, α-esterase and β –esterase) under laboratory conditions. Four replicates of 4th instar larvae were directly immersed for 60sec. in a conidia aqueous suspension of LC_{50} value (1.1×10^6 spores/ml) and transferred onto plastic boxes (5 larvae per box) containing filter paper for 24h then transferred onto plastic boxes containing 50g of moist sugar cane sawdust and succulent pieces of sugarcane stems and incubated at 28˚C. The live larvae were collected after 2, 3, 5 and 7 days post-treatment to biochemical assay.

3.1. Preparation of larval homogenates.

The collected larvae (1gm) were homogenized in distilled water (1:4w/v) at 500 rpm using a Teflon homogenizer- (MECHIANIKA PRECYZYJNA Warszawa type MPN-309-Poland)-surrounded with a jacket of crushed ice for 3minutes. Homogenates were centrifuged at 6000 rpm for 10 min at 5°C using (BECKMAN GS-6R Centrifuge). After centrifugation, the supernatant was divided into small aliquots (0.5 ml) and stored at -20°C until analysis. Three replicates were carried out for each biochemical determination.

3.1.1. Determination of chitinase activity.

Chitinase was assayed using 3, 5-dinitrosalicylic acid reagent to determine the free aldehydic groups of hexosamine liberated on chitin digestion according to the method described by Ishaaya and Casida (1974).

Preparation of 3, 5-dinitrosalicylic acid reagent (DNSA)

The dinitosalicylic acid reagent was prepared by dissolving one gram of 3, 5-dinitrosalicylic acid in 20ml of 2N NaOH solution and 50 ml of distilled water with the aid of a magnetic stirrer. Potassium sodium tartarate (30 gm) was added, and magnetic stirring was continued until a clear solution was obtained. Distilled water was then added to bring the final volume to 100 ml.

Assay of chitinase activity

The reaction mixture consisted of 0.12 ml (0.2M) phosphate buffer (pH 6.6); 0.3 ml of 0.5% colloidal chitin; 0.18 ml sample homogenate. After 60 min incubation at 37°C, enzyme activity was terminated by adding 1.2 ml 3, 5-dinitrosalycilic acid agent (DNSA). The reaction mixture was heated for 5 min at 100°C then cooled in an ice-bath, and diluted with 1.2 ml distilled water. Undigested chitin was sedimented by centrifugation for 15min at 6000 rpm and the absorbance of the supernatant was determined spectrophotometrically at 500nm. Direct reaction of   N-acetylglucosamine (NAGA) with DNSA reagent, under conditions similar to those of the enzyme reaction, gives a linear plot of absorbance value versus amount of NAGA, 1 µg of NAGA giving an absorbance value of 0.78. Chitinase activity is expressed as µg N-acetylglucosamine released/gm body weight/min.

3.1.2. Determination of protease activity.

The protease activity was determined by the casein digestion method described by Ishaaya *et al*. (1971). The reaction mixture consisted of 0.2ml (0.2M) glycine buffer (pH 10, 0.4 ml of 1.5% casein solution, and 0.2 ml homogenate sample. Enzymatic activity was terminated after 60 min incubation at 37°C by adding 1.2 ml of 5% trichloroacetic acid solution. The reaction mixture was centrifuged at 6000 rpm for 15 min and the supernatant was taken for enzymatic activity evaluation. The proteolytic activity was determined at 280nm as O.D. (Optical Density) unit for samples x10^3. Protease activity was expressed as µg tyrosine/gm body weight.

3.1.3. Determination of non- specific esterase Activities.

Alpha and beta esterases (α-β) were determined according to the method of Van Asperen (1962) using α-naphthyl acetate and β–naphthyl acetate as substrates, respectively. This method depends on the Naphthol produced as a result of substrate hydrolysis can be measured by the addition of diazoblue sodium lauryl sulphate solution which produce a strong blue color in case of α-naphthol or strong red color in the case of β – naphthol. The colour was measured spectrophotometrically.

The reaction mixture consisted of 5 ml substrate solution (3×10^{-4} M α- or β-naphthylacetate), 1% acetone and 0.04 M phosphate buffer pH 7 and 20 µl of larval homogenate. The mixture was incubated for exactly 15 min at 27 C^0. Then l ml of

diazoblue color reagent (prepared by mixing 2 parts of 1 % diazoblue β and 5 part of 5 % sodium lauryl sulphate) was added. The developed color was read at 600 and 555 nm for α-and β-naphthol, respectively. The activity was expressed as μg α- or β-naphthol released/ min/ gm body weight.

Preparation of standard Curves of α-and β-naphthol.

Stock solution were prepared by dissolving 20 mg α- or β-naphthol in 100 ml of 0.04m phosphate buffer (pH 7) ten milliliter of stock solution were diluted up to 100 ml by the phosphate buffer Aliquots containing of 2.5, 5, 10, 20 and 40μg of α-naphthol or 2.5, 5, 7.5, 10 and 20 μg β- naphthol were pipetted into test tubes and completed to 5 ml by phosphate buffer, one milliliter of diazoblue reagent was added and the developed color was measured as mentioned before. The standard curves of both α- E and β- E were blotted by O. D (Optical Density) against concentration.

3.1.4. Determination of total proteins content.

Total proteins were determined by the method of Bradford (1976) using standard of Bovine serum albumin.

preparation of protein reagent

Coomassie Brilliant Blue G-250 (100mg) was dissolved in 50 ml of 95% ethanol. To this solution, 100 ml 85% (w/v) phosphoric acid were added. The resulting solution was diluted to a final volume of 1 liter.

preparation of standard curve of protein

For preparation of standard curve serial concentrations of Bovine serum albumin solutions containing 10 to 100μg of protein were pipetted into test tubes and the volume was adjusted to 0.1 ml with phosphate buffer (pH 6.6). Five ml of protein reagent were added and the resulting color was measured spectrophotometrically at 595nm after 2 minutes. The optical densities against concentrations, thus a curve can be constructed.

Protein assay.

50μl of larval homogenate were pipetted into a test tube containing 50μl phosphate buffer (pH 6.6), then 5 ml of protein reagent were added to the test tube and the contents were vortexed. The absorbance at 595 nm was measured after 2 min against blank prepared from 0.1 ml of phosphate buffer (pH 6.6) and 5 ml of protein reagent. The content of protein was obtained from the standard curve and expressed as mg protein / gram.

3.1.5. Determination of total lipid content.

Total lipid content in *R. ferrugineus* larval homogenate was estimated according to Knight *et al.* (1972) using phosphovanillin reagent.

Pure vanillin (0.6 gm) was dissolved in 10 ml absolute ethanol and complete to 100 ml distilled water. Concentrated phosphoric acid (400 ml) was added, and the solution was stored in dark glass bottle at room temperature.

Sample solution of *R. ferrugineus* larvae (250µl) was added to concentrated sulfuric acid (5 ml) in a test tube and heated in a boiling water bath for 10 min. after cooling to room temperature, 500 µl of digested sample was added to phosphovanillin reagent (6.0 ml). After 45 min incubation in dark, the developed color was measured at 525 nm against reagent blank prepared from 500 µl distilled water and 6.0 ml phosphovanillin reagent. The result is expressed as µg lipid/gram body weight.

For standard curve, serial concentrations of oleic acid and palmitic acid mixture (7:3) from 1 to 5 mg/ ml were prepared in absolute ethanol were used and treated in the same manner as the unknown. The standard curve was blotted by O.D. (Optical Density) against concentration.

4. Analysis of results.

Experimental data were analyzed using one way analysis of variance (ANOVA) using SPSS (statistical package for social sciences, ver. 20), and the significance among the samples was compared at P≤0.05. Results were represented as mean ±SE (n=4).

RESULTS AND DISCUSSION

Effect of *B. bassiana*-9894 on some biochemical parameters in red palm weevil.

Fourth instar larval was chosen to evaluate the biochemical activity of red palm weevil (Proteases, Chitinases and α-&β –esterases) under laboratory conditions. *B. bassiana*-9894 isolate was found to be the most effective isolate against the different stages of the red palm weevil, *R. ferrugineus* (Ali *et al.*, 2015).

1. Effect on proteases, chitinases and α & β-esterase.

The changes in activity of haemolymph proteases, chitinases and α-&β-esterase in 4th instar larvae of red palm weevil, *R. ferrugineus* treated by LC_{50} (i.e., 1.1×10^6 spores/ml) entomopathogenic fungus after 2, 3, 5 and 7days were illustrated in Table (1).

Data in Table (1) showed that a significant increase in proteases, chitinases and α &β-esterases in haemolymph of 4th instar larvae of red palm weevil, *R. ferrugineus* treated by entomopathogenic fungus compared with the control larvae.

Results indicated that a significant increase (p<0.05) in the activity of protease after 2, 3, 5 and 7 days as compared with control. On the other hand, the activity of protease was increased significant (p<0.05) after 3, 5 and 7 days in

comparison with 2 days. While after 3 and 5 days the activity of haemolymph protease in experimental larvae had no significant increased. Meanwhile, after 7days the activity of protease in haemolymph of fourth instar larvae had increased significantly compared with control and remained at a level higher than that of 2, 3 and 5 days.

Table 1. Enzyme activities of 4th instar larvae of red palm weevil as affected by *B. bassiana*-9894 treatment.

Days after treatment	Protease (μg tyrosine/gm body weight/min)	Chitinase (μg N-acetylglucosamine /gm body weight/min)	α-esterase (μg α-naphthol /g body weight/min)	β-esterase ((μg β-naphthol /g body weight/min)
2days	44.63 a,c,d,e± 1.87	40.17 d,e± 2.98	388.81 a,c,d,e± 16.48	243.23e± 16.93
3days	51.99 a,b,e± 1.49	42.01 d,e± 2.01	450.87 a,b,d,e± 21.70	267.16 a,e± 12.56
5days	54.77 a,b,e± 1.92	48.89 a,b,c,e ± 0.75	466.21 a,b,e ±21.48	293.47 a,e ± 22.69
7days	64.63 a,b,c,d ± 2.44	70.88 a,b,c,d ± 3.15	632.56 a,b,c,d ± 16.32	586.89 a,b,c,d ± 38.98
control	38.69 ± 1.01	36.26 ± 1.52	338.54 ± 6.72	207.16±18.58

All data are expressed as means± SE.a. Significant different versus control at p<0.05, b. Significant different versus after 2days at p<0.05, c. Significant different versus after 3days at p<0.05, d. Significant different versus after 5days at p<0.05, e. Significant different versus after 7days at p<0.05

The activity of chitinase was increased significantly (p<0.05) after 5 and 7 days compared with control (Table 1). Meanwhile, after 5 and 7 days the activity of haemolymph chitinase in 4th instar larvae had increased significantly and remained at a level higher than that of the control. But after 2 and 3 days no significantly increase observed in activity of haemolymph chitinase concentration compared with control.

Results in table (1) shown a significantly increased in α-esterase activity in haemolymph of 4th instar larvae of red palm weevil treated with *B. bassiana*-9894 after 2, 3, 5 and 7 days compared with control. While β-esterase obtained a significantly increased in haemolymph after 3, 5 and 7 days compared with control. From aforementioned results, it is obvious that the fungus, *B. bassiana*-9894 caused a significant increase after 2, 3, 5 and 7 days in activity of protease, chitinase, α-esterase and β-esterase.

2. Effect on total protein and total lipids.

Data in table (2) showed that the total protein content in haemolymph of 4th instar larvae decreased from 3, 5 and 7 days after treatment. The results showed a significant decrease (p<0.05) in the total protein concentration after 5 and 7 days compared with control. The changes in the total lipids concentration of 4th instar larvae of red palm weevil treated by entomopathogenic fungus as demonstrated in

table (2). Results obtained revealed that a significant increased in total lipids concentration after 3, 5 and 7 days compared with untreated larvae. But, after 5 days of treatment no significant increased was obtained compared with 2 and 3 days. Meanwhile, results shown a significantly increased in total lipids concentration in haemolymph of 4th instar larvae of red palm weevil after 7 days compared with control and 2, 3 and 5 days.

Table 2. Total protein and total lipids of 4th instar larvae of red palm weevil as affected by *B. bassiana* treatment.

Treatment	Total protein (mg/ml)	Total lipids (µg/g body weight)
2days	$1.28^{a,c,d} \pm 0.09$	$130.99^{e} \pm 9.91$
3days	$1.04^{b,d} \pm 0.06$	$180.69^{a,e} \pm 9.11$
5days	$0.80^{a,b} \pm 0.06$	$240.48^{a,e} \pm 7.11$
7days	$0.51^{a,b,c} \pm 0.06$	$268.76^{a,b,c,d} \pm 11.87$
Control	1.14 ± 0.09	73.78 ± 5.14

All data are expressed as means± SE.a. Significant different versus control at $p<0.05$, b. Significant different versus after 2days at $p<0.05$, c. Significant different versus after 3days at $p<0.05$, d. Significant different versus after 5days at $p<0.05$, e. Significant different versus after 7days at $p<0.05$

At the present study, *Beauveria bassiana*-9894 severely deplete the protein concentration. The entomopathogenic fungi (*B. bassiana* and *M.* anisopliae) siginficanly decreased the immune enzyme activity and protein concentration (Gabarty *et al.*, 2013). Insect infected with the fungi was declined its total protein concentration, it was dramatically declined in desert locust treated by Metarhizium anisopliae (Elbanna *et al.*, 2012). reduction in the total protein content of the adult haemolymph of *Schistocerca gregaria* reported during the course of infection with the *M. anisopliae* (Gillespie *et al.*, 2000). The reduction in total protein and loss of soluble protein from the host haemolymph during parasitism may be due to proteolytic enzymes which were secreted into the haemoceol of the insect and hydrolyze the host protein (Gillespie *et al.*, 2000). Decreasing the total protein content may lead to the death of treated insects and this may be one of the reasons of insect mortality. The reduction or the lack of protein leads to retardation of many physiological processes in insects, adult insect requires protein to promote ovulation and egg development supports this conclusion (House, 1963).

CONCLUSION

The results indicated a significant (P≤ 0.05) increase in the protease, chitinase and α &β-esterases in 4th instar larvae of *R. ferrugineus*, this increase was greatly remarkable in 7 days after treatment. The results indicated a significant (P≤ 0.05) decrease in total protein content of 4th instar larvae of *R. ferrugineus* resulted from treated larvae with LC50 of entomopathogenic fungus, *B. bassiana*-9894.

On the other hand, the results showed a significant (P≤ 0.05) increase on total lipids in the 4th instar larvae which treated with LC50 of *B. bassiana*-9894.

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التـــأثيـــــرات البيوكيميائية لفطــر البــوفــاريــا باســيانا
علــى ســوســـة النخيل الحمراء
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تم أختيار العمر اليرقى الرابع لسوسة النخيل الحمراء المعامل بالفطر بوفاريا باسيانا لتقييم النشاط البيوكيميائى (البروتييز والشيتينيز والأستيرزس) تحت الظروف المعملية. وأشارت التحاليل البيوكيميائية الى ان الفطر بوفاريا باسيانا ادى الى زيادة معنوية فى نشاط الإنزيمات الحشرية البروتييز والشيتينيز والأستيرزس عند معاملة العمر اليرقى الرابع مقارنة بالكنترول، وحدث نقص معنوى فى تركيز البروتين الكلى فى الحشرات المعاملة مقارنة بالكنترول وعلى العكس من ذلك كانت هناك زيادة معنوية فى تركيز الليبيدات الكلية مقارنة بالكنترول.