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Toxicological and Biochemical Effects of *Beauveria bassiana* (Bals.) on Peach Fruit Fly, *Bactrocera zonata* (Saunders) Immature Stages

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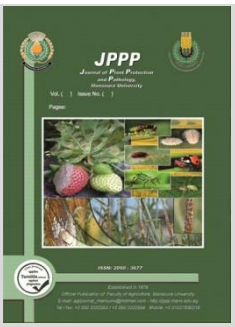

ABSTRACT

Peach fruit fly, *Bactrocera zonata* (Saunders) is a destructive pest of horticultural crops in Egypt. The aim of the study is to find a natural eco-friendly control bioagents of this insect. The entomopathogenic fungus, *Beauveria bassiana* (Bals.) infected the immature stages of this insect and viewed concentration dependent patterns. Flies were the most susceptible stage of the fungal infection. Enzymatic activity measures of *B. bassiana* endorsed chitinase, protease and lipase activities. Biochemical analysis of infected larvae showed increases and decreases and decreased in total proteins, carbohydrates and lipids. An extreme increase in phenoloxidase level was recorded after 24 and 48 hours from infection. Acetylcholinesterase showed a decrease after 24 hours from infection followed by an increase after 48 hours. *B. bassiana* could be included in IPM programs as a control agent against *B. zonata*.

Keywords: *Bactrocera zonata*, *Beauveria bassiana*, pathogenicity, biochemical analysis

INTRODUCTION

Peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) is a destructive polyphagous pest that infest most of the horticultural fruit (Allwood *et al*., 1999). Efflaton, in (1924) recorded the pest in Egypt for the first time supported with description then it disappeared. The pest restored its activity at early1990s and established in Egypt lately at the same decade (El-Minshawy *et al*., 1999).The pest causes a direct loss to different horticultural crops affecting the agricultural Egyptian economy. Producers use chemical pesticides to protect fruit crops from this pest infestation. Pesticides residues act as an obstacle in front of fresh fruit export as many importing countries refuse the consignments according to their public health standards. The use of chemical pesticides not only affect the natural environmental balance negatively but also it harm human health. On the same point of view, increase of agricultural pests threatens food industry and human safety, so, there is a tendency to control these pests by their biological enemies. Fungal diseases in insects are common and widespread and often decimate insect populations in spectacular epizootics. Virtually, all insect orders are susceptible to fungal diseases. Entomopathogenic fungi are associated with insects living in diverse habitats, including fresh water, soil, soil surfaces, and aerial locations. *Beauveria bassiana* (Bals.) is a natural inhabitant of the soil, an obligate parasite of several insect species (Zimmermann, 2007b) and one of the most virulent biological agents infecting dipteran insects at their different stages(Castillo *et al.,* 2000; Quesada-Moraga *et al.*,2006; Ibrahim *et al.,* 2014 and Soliman *et al*., 2014). The cuticle is penetrated by a combination of mechanism pressure from the appressoria and the action of cuticle degrading enzymes, such as lipase, protease and chitinase (St-Leger, 1987 and St-Leger *et al.,* 1995). As fungal conidia reach the haemocoel, the host is killed by some combination of mechanical damage produced by fungal growth, nutrient exhaustion and toxicosis (Gillespie and Claydon, 1989). The aim of this work is to figure out the effect of *B. bassiana* on the apparent immature *B. zonata* stages (full-grown larvae, pupae and flies). In addition, estimation of *B. bassiana* enzymatic activity to penetrate the larvae followed by studying the biochemical changes occurred due to fungal infection.

MATERRIALS AND METHODS

Peach fruit fly, *Bactrocera zonata* (Saunders)

Peach fruit fly, *B. zonata* full-grown larvae, pupae and flies were obtained from the laboratory colony reared in the Horticulture Insects Research Department (HIRD), Plant Protection Research Institute (PPRI), Dokki, Giza, Egypt. The insect larvae reared using artificial larval rearing medium according to the technique of Tanaka *et al*., (1969**)**.The flies were fed on regular diet (sugar and enzymatic yeast hydrolysate in ratio 3:1, respectively in addition of water source) according to El-Sayed (1979).

Entomopathogenic fungus, *Beauveria bassiana* (Bals.)

The fungus *B. bassiana* (Bals.) was isolated from the white fly, *Bemisia tabaci* in Sharqiya governorate, eastern delta, Egypt (Ibrahim, 2006).

Fungal cultures

Blastospores cultures of entomopathogenic fungus, *B. bassiana* produced by inoculating conidia into Sabouraud dextrose broth according to Gabarty *et al*., (2013). A series of concentrations of 2.3×10^4, 2.3×10^5, 2.3×10^6, 2.3×10^7 and 2.3×10^8 conidia/ml were prepared to investigate their effect on *B. zonata* tested stages. Suspensions were held overnight on ice at 4°C to prevent germination of conidia before use in experiments. Suspensions checked before use in bioassays (Yeo *et al*., 2003).

Effect of *B. bassiana* on full-grown larvae, pupae and flies of *B. zonata*

Full-grown larvae and pupae

Mortality of full-grown larvae of *B. zonata* tested in sterilized cups measured 5×10×3 cm containing 50g of fine sand sieved through 2 mm sieve. *B. bassiana* conidial

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concentrations (2.3×10^4, 2.3×10^5, 2.3×10^6, $2.3\times10^7$and 2.3×10^8 conidia/ml) were incorporated with the sand using small sprayer. The sand was mixed with spatula to guarantee a well conidial distribution. Ten full-grown larvae of *B. zonata* freshly popped from the larval medium placed on the surface of the treated sand in each cup allowing them to burrow naturally into it. The cups closed with pieces of muslin and rubber bands. Each concentration replicated five times and other five replicates without conidia ran in parallel as control treatment. After 48 hours, the treated individuals were picked up from sand, washed with distilled water and kept in non-treated cups until flies' emergence. Unemerged individuals were kept in sterilized Petri dishes (9cm) accompanied with a piece of moistened cotton at 25℃ in complete darkness for mycosis confirmation (Ekesi *et al*., 2002). The same steps were done for testing one-day old pupae except that treated sand was divided into two portions; the first acted as a cradle for laying pupae on it and the other was a cover.

Flies

One-day old flies of *B. zonata* were subjected to test with five conidial concentrations, 2.3×10^4, 2.3×10^5, 2.3×10^6, 2.3×10^7 and 2.3×10^8 conidia/ml of *B. bassiana*. Sterilized transparent plastic jars measured 20 cm in diameter and 30 cm height were used for this experiment. The jars sprayed with concentrations separately and left for an hour for complete dryness before entering flies' regular diet to avoid food fungal contamination. Twenty-five flies regardless the sex were transferred gently using aspirator in each jar allowing them to move on the sprayed dried walls. The jars were closed with pieces of muslin and rubber bands. Each conidial concentration was replicated five times and other five replicates sprayed with water only ran in parallel as control treatment. Fungal treatments were kept at 25 ± 2 ˚C and 75 % RH cabinet. The control treatments were kept under the same conditions in another cabinet. Dead flies were counted and collected daily for mycosis confirmation.

Estimation of *B. bassiana* extracellular enzymatic activities

The extracellular enzymes, chitinase, protease and lipase activities of *B. bassiana* were determined by plate assay method (Hankin and Anagnostakis, 1975). Enzymatic rate or Enzymatic activity index of chitinase, protease and lipase were calculated determined according to (St-Leger *et al*., 1997) with this formula:

$$\text{Enzymatic Activity index} = \frac{(\text{Colony diameter} + \text{halo diameter})}{\text{Colony diameter}}$$

Enzymatic index value of >1.0 indicates enzymatic activity.

Biochemical effects of *B. bassiana* on full-grown *B. zonata* larvae

The concentration, 2.3×10^8 of *B. bassiana* was used for studying its effect on certain biochemical aspects (total protein, total carbohydrates and total lipids contents). In addition, the activity of the enzymes, acetylcholinesterase and phenoloxidase on full-grown *B. zonata* larvae were measured.

Biochemical assays

Estimation of the total protein, carbohydrate and lipid contents

Total protein content of *B. zonata* larvae was determined spectrophotometrically according to Bradford (1976). Protein content was expressed as mg protein/gm fresh body weight. Total carbohydrate content in *B. zonata* larvae was quantitatively determined according to Dubois *et al*., (1956). Total carbohydrate was expressed as mg glucose/gm fresh body weight. Total lipids of *B. zonata* larvae were determined according to Knight *et al*., (1972). Lipids content was expressed as mg lipids /gm fresh body weight.

Insect Enzymes assays (Phenoloxidase and Acetylcholine esterase)

Phenoloxidase (PO) assay was estimated by the method of Cotter and Wilson (2002) with certain modifications. A 100µl sample and 400µl of PBS (Phosphate Buffer Saline) mixture was added to 100µl of 20mM L-Dopa. The increase in absorbance was measured at 475nm with 1min interval for 30 minutes. The amount of PO in the sample was calculated where one unit is the amount of enzyme required to increase the absorbance by 0.001 per minute.

The acetylcholine assay was carried out according to Ellman *et al*., (1960) as follows: The insect sample prepared in phosphate buffer (pH 8.0, 0.1 M) (10µl sample into 100 µl buffer). The suspension was pipetted into a cuvette. 25 µl of 0.01 M the reagent dithiobis-2-nitrobenzoic acid (DTNB) were added {39.6 mg of DTNB were dissolved in 10 ml phosphate buffer pH 7.0 (0.1M) and 15 mg of sodium bicarbonate}. Finally, 20 µl of the substrate acetylcholine bromide (3mM) were added to this cuvette. Changes in absorbance at 412 nm were recorded for at least 10 min.

The enzyme activity was calculated by using the following formula according to Bali and Kaur (2013):

$$\text{Enzyme activity} = \left(\frac{\Delta A \times V}{E \times d \times v \times \Delta t}\right) \times d.f$$

Where:
ΔA = Change in absorbance. E = Extinction coefficient ($3.6 mM^{-1}cm^{-1}$).
V= Total volume of assay mixture (in µl). d = Light path.
Δt = Time for which change was observed (in minutes).
***v* = Volume of Sample (in µl). d.f = Dilution factor.**

Statistical analysis:

Mortalities were corrected using Abbot's formula (1925) then subjected to ANOVA using MAXStatPro v.3.6 statistics software (2015).Toxicity values the of the entomopathogenic fungus, *B. bassiana* were determined by Bakr E. LDP line software (2005).

RESULTS AND DISCUSSION

Effect of *B. bassiana* on full-grown larvae, pupae and flies of *B. zonata*

Data obtained were significant at significance level 95% and $P<0.05$. Mortality percentages showed that *B. bassiana* different conidial concentrations were able to cause death to all tested *B. zonata* (larval, pupal and flies stages) (Table1).

Table 1. Effect of *B. bassiana* conidial concentrations on *B. zonata* different stages

B. bassiana conidia/ml				
2.3×10^4	2.3×10^5	2.3×10^6	2.3×10^7	2.3×10^8
Mean % of full-grown larval mortality ± SE				
40.11±0.32	44.90±0.25	56.10±0.41	78.61±0.20	89.71±0.24
F=99.82, P<0.0001, df=5,29				
Mean % of one-day old pupal mortality± SE				
30.61±0.44	40.23±0.23	42.86±0.34	55.10±0.37	71.43±0.25
F=64.65 , P<0.0001, df=5,29				
Mean % of one-day old flies mortality± SE				
58.33±0.33	81.39±0.29	83.50±0.23	87.35±0.57	93.81±0.41
F=155.57, P<0.0001, df=5,29				

***Means are significant at the significance level 95%, *P*<0.05.**

Mortality percentages were concentration dependent. Toxicity values of *B. bassiana* based on LC_{50} and LC_{90} revealed its ability of to cause death to all *B. zonata* stages specially flies that were the most susceptible stage to

infection followed by full-grown larvae and one-day old pupae (Table 2). The highest mortality pattern appeared in *B. zonata* treated flies of with *B. bassiana*.

Table 2. Toxicity of *B. bassiana* to different stages of *B. zonata*

Stage	LC_{50}	LC_{90}	slope±SE	$\chi 2$	*P*
Full-grown larvae	8.2×10^{5}	1.1×10^{10}	0.251±0.04	5.94	0.1148
One-day old pupae	3.6×10^{6}	5.1×10^{11}	0.250±0.06	1.24	0.7925
One-day old flies	3.5×10^{2}	7.0×10^{7}	0.248±0.18	2.18	0.5345

Beauveria bassiana different conidial concentrations were able to cause death to all tested *B. zonata* stages (larval, pupal and flies). These results coincide with those of Ibrahim *et al*., (2014) and Hussein *et al*., (2018) who tested susceptibility of Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) and peach fruit fly, *B. zonata* larvae and pupae to different concentrations of *B. bassiana* that resulted in varied concentration dependent mortality patterns. Entomopathogenic fungi penetrate its insect host via cuticle that covered by a thin layer of lipids usually composed of a mixture of very-long chain hydrocarbons together with variable amounts of fatty alcohols and fatty acids. These lipids act as a protection for insects from desiccation, penetration of chemical or biological insecticides (Juárez, 1994). The infecting fungus has ability to degenerate cuticular hydrocarbons by using them as a source of carbon for energy production and fusion into fungal components (Napolitano and Juárez, 1997). A host-pathogen interactions provide somewhat an explanation into the dynamics of the highly attacking and resistant co-evolutionary arms that race between entomopathogenic fungi and their pest hosts. However, the host enhances its capabilities via excluding the pathogen or bandage the wounds caused by the damage inflicted while the pathogen responds with immune evasion and utilization of host resources. *B. bassiana* showed pathogenic activity against tested immature stages (full-grown larvae, pupae and flies) of *B. zonata* with different levels. The degree of host resistance depends on combine effects of the cuticle's thickness (David, 1969), the tensile strength imparted to the cuticle by the system of chitin lamellae and the degree of cuticle hardening by sclerotization (Hassan and Charnley, 1989). Insects have heavily sclerotized body segments are usually invaded via arthrodial membranes or spiracles (Charnley, 1989).Percentages of larval and pupal mortality reflected that *B. bassiana* was able to overcome the resistance of some treated individuals. Tsakas and Marmaras, (1990), studied larvae and pupae of *C. capitata* and demonstrated that three haemocyte polypeptides in the larval integument may be involved in cuticle sclerotization that may prevent infection. This point may explain the variation of *B. zonata* larval and pupal mortality. The variation in susceptibility of *B. zonata* larvae and pupae to *B. bassiana* infection may be due to individual differences related to larval feeding or even genetics. Percentages of mortality seemed to be influenced by fungal concentration. It could be expected that when the number of adhered conidia increase, they could produce more amounts of cuticle degrading enzymes and overcome the defense action of the host stage followed by penetration and toxins production and finally death of the host. The highest mortality pattern appeared in *B. zonata* treated flies of with *B. bassiana*. These findings are in agreement with Castillo *et al*., (2000) and Quesada-Moraga *et al*., (2006) who reported 100 % mortality in flies of *C. capitata* and other tephritids by *B. bassiana* infection. No doubt, the topographical features of each treated stages have played an important role in enabling *B. bassiana* conidia to adhere to the epicuticle. Flies body that covered with a large number of setae and hairs varied in thickness and length that may keep the conidia of *B. bassiana* adhered to the epicuticle. Gomez *et al*., 1997 examined the conidial attachment of *Metarhizium anisopliae* on the southern green stinkbug, *Nezara viridula* nymphal stage and found that conidia were stucked in areas covered with large number of setae (antennal tips, apical portions of tibia and tarsi). Both topography and chemistry of the cuticle affected adhesion and germination of the conidia. Galhoum (2017) studied *B. zonata* flies cuticular hydrocarbons that formed from alkenes, alkanes, monocyclic hydrocarbons and alkynes. Pedrini *et al*., (2013) stated that *B. bassiana* is able to grow on straight chain alkanes up to n-C33 as a single source of carbon and energy. The fungus use monooxygenases to degrade insect hydrocarbons alkanes using terminal oxidation to alcohols, which are further, oxidized by alcohol and aldehyde dehydrogenases, whose production can enter β-oxidation pathways. In conclusion, differences in induced mortality rates among *B. zonata* stages may be due to differences in the conidial attachments onto the insect cuticle, germination or to the suppression of the host's defense system Chandler *et al*., (1993).

Estimation of *B. bassiana* extracellular enzyme activity

The entomopathogenic fungi activity depends on the ability of its enzymatic equipment consisting of lipases, proteases and chitinases, which are in charge of breaking down the insect's integument. *B. bassiana* extracellular enzyme activity was considered as an important factor for its virulence. *B. bassiana* enzymatic index values for the three extracellular enzymes were 42.0mm for chitinases production followed by proteases with value of 30.6mm and 25mm for lipases production (Table 3).

Table 3. Estimation of the three extracellular enzymes produced by *B. bassiana*.

Extracellular Enzymes	Enzymatic Index (mm)
Chitinases	42.00
Proteases	30.60
Lipases	25.00

Beauveria bassiana extracellular enzyme activity was considered as an important factor for its virulence. The presented results are in agreement with the findings of Dhawan and Joshi, *(*2017) who mentioned that *B. Bassiana* showed highest extracellular chitinase activity and was more pathogenic against their hosts. Chitinase is the most important enzyme to degrade the chitin polymer of the insect cuticle that could be correlated with the virulence of *B. bassiana*. Protease was considered as an important enzyme in the infective process of entomopathogenic fungus (Mustafa and Kaur, 2009). *B. bassiana* produced large quantities of protease that degraded the proteinaceous material and the solubilized proteins that then degraded by amino peptidases and exopeptidases to form amino acids served nutrients needs for the fungus. Extracellular proteases produced by *B. bassiana* may be involved in the pathogenesis, thus, proteases may act as an important key factor governs virulence in *B. bassiana* against host (Dhawan and Joshi, 2017). Li *et al.*, (2009) proved that lipases are the first enzymes synthesized by the entomopathogenic fungi that hydrolyze the ester bonds of lipoproteins, fats and waxes at the interior of the insect integument and significantly contribute to the cuticle penetration and initial release of nutrients. Hence, lipases may assist *B. bassiana* and support the role of proteases to degrade the proteinaceous material and finally, chitinases degrade the network of chitin the main structural component of the

exoskeleton in the walls, so that the cuticle lose rigidity and facilitating penetration**.**

Biochemical effects of *B. bassiana* on *B. zonata* full-grown larvae

In the present study, biochemical changes occurred in the larval total protein, carbohydrate and lipid contents during the course of *B. bassiana* infection were presented in Table (4). The total protein content of the treated larvae increased as compared to control treatment after 24 hours and 48 hours. The highest increase in total protein content was recorded after 48 hours days post infection where it was about 1.4 folds more than control. A significant decrease in means larval total carbohydrate content was observed at 24 and 48 hours after infection compared to control treatment. The values told that the decrease of carbohydrate content was directly proportional with exposure time (Table 4). The larval total lipids content increased significantly at the use of *B. bassiana* as compared to the control treatment. The recorded total lipids content after 24 hours increased by 3.5 folds than control and was higher than that recorded at 48 hours of full-grown larvae exposure (Table 4).

Effect of *B. bassiana* on full-gown larvae detoxification enzymes of *B. zonata*:

The obtained data presented in (Table 5) showed that the activity of phenoloxidase in the treated larvae increased significantly by 1.5 folds than control after 24 hours of exposure to *B. bassiana* while its highest activity recorded after 48 hours by 12 folds as compared to control. Data in (Table 5) revealed that, a significant decrease in acetylcholine esterase activity in full-grown larvae after 24 hours of exposure to *B. bassiana* as compared to control treatment while increased after 48 hours of exposure. There was a significant difference in acetylcholine esterase activity.

Table 4. Effect of *B. bassiana* 2.3×10^8 conidial concentration on the B. zonata full grown larvae metabolites

Period after exposure	Mean biomolecule content ± SE (mg/g fresh body weight)					
	Control	Total proteins	Control	Total lipids	Control	Total carbohydrates
24 hours	81.90±1.35	85.93±1.74	1.93±0.07	6.67 ± 0.233	22.40±1.102	13.1±0.702
38 hours	92.01± 0.20	119.67±5.24	1.93±0.07	3.47± 0.173	22.40±1.102	16.23±0.633

Biochemical changes of the treated larvae occurred in the total protein, carbohydrate and lipid contents during the course of *B. bassiana* infection. The increase of total protein content is confirmed with the findings of Silva *et al*., (2005)who studied the larvicidal effect of *M. anisopliae* isolates against *Aedes aegypti* L. that showed a high variability of total protein production after 24, 48 and 72 hours of treatment. El-Badawy *et al.,* (2018) stated that only fungus *B. bassiana* caused increase in haemolymph protein content of the treated *S. littoralis* 5th instar from 1st day to 4th day compared to control. On contrary, Nada, (2015) reported that the total protein level of adult *N. viridula* treated with *M. anisopliae* were significantly decreased than the treatment with *B. bassiana*, after 24, 48 and 72hrs.Thus, the increase of protein synthesis of treated *B. zonata* larvae may indicate the possibility of forming a humoral immune system to detoxify the fungal toxins (Wilkinson, 1976). The decrease of carbohydrate content was directly proportional with exposure time. Nirupama, (2015) mentioned that total carbohydrates content level in the haemolymph of *B. bassiana* treated *Bomyx mori* 5th instar larvae showed normal value in the 1st day and decreased gradually towards the end of 5th day in inoculated batches. Also, El-Badawy *et al.*,(2018) cleared that a significant decrease was recorded in 2nd,3rdand 4thday of fungal infection of *S. littoralis* 5thinstar with *B. bassiana* compared to control. The decrease in total carbohydrate content in larvae treated with *B. bassiana* may be referred to a state of physiological starvation. The decrease in the total carbohydrates of larvae treated with *B. bassiana* may be considered as energy reserves required for defense reactions and other vital processes. Levels of carbohydrates decreased in haemolymph and attributed to carbohydrate excessive utilization that required for fungus development and growth (Mallikarjuna *et al.,* 2002).

The recorded total lipids content after 24 hours was higher than that recorded at 48 hours of full-grown larvae exposure. The results are agreement with the results of Gabarty, (2011) who cleared a significant increase in the total content of lipids in the greasy cutworm, *A.ipsilon* larvae treated with the two pathogenic fungi, *B. bassiana* and *M. anisopliae* as compared with control. El-Badawy *et al.,* (2018) confirmed that a significant increase in the total content of lipids in the larvae of *Spodoptera littoralis* (Bois.) treated with four entomopathogenic fungi (*M. anisopliae, P. lilacinus, L. antillanum* and *B. bassiana*) compared to untreated larvae. In contrast to results of the present study, Nada, (2015) showed that total lipids decreased significantly when adults of *N. viridula* treated with *M. anisopliae* during 24, 48, 72 hours. Changes in the amounts of lipids could be due to the upset of the homeostatic mechanism in insects by fungus (Oguri and Steele, 2007). Lipids are fundamental for structural components of cells and serve as a source of metabolic energy. The infection progress by a pathogen in the host tissue can be monitored by studying the degree of variation in metabolic constituents (Rajitha and Savithri, 2014).The increase and decrease in the proteins, carbohydrates and lipids contents of treated larvae after inoculation due to infection by pathogen of fungi have an important role in *B. zonata* development. Infections of *B. zonata* with *B. bassiana* tend to its stimulated protein, carbohydrate and lipid utilization in order to meet requirements of toxic stress. Several biochemical and physiological alterations caused in insect tissues owing to pathogenic infections (Shigemitsu and Noguchi, 1969). The present findings support that infection with *B. bassiana* had different effects on *B. zonata* larvae, particularly as they changed haemolymph nutrients contents (e.g. carbohydrates, lipids and proteins).Under fungi infection there are correlation between the changes in the concentration of these bio-molecules and the degree of their absorption, inter conversion and utilization and the high toxicity of the *B. zonata* larvae. The results indicated fungi infection caused physiological and biochemical changes in the *B. zonata*. Metabolic changes play an important role in understanding the interaction between the host and pathogen as a part of a survival strategy and this tend to dramatically changes in the composition of insect haemolymph, thus abnormal development of insect caused.

Table 5. Effect of *B. bassiana* 2.3×10^8 conidial concentration on the specific activities of phenoloxidase and acetylcholinesterase of *B. zonata* full-grown larvae.

Period after exposure	Mean specific enzyme activities (IU/gm fresh body weight)± SE			
	Control	Phenoloxidase	Control	Acetylcholinesterase
24 hours	1280.00 ± 105.99	1783.33 ± 72.65	159.00 ± 2.65	149.67
48 hours	1280.00 ± 105.99	14166.00 ± 902.56	159.00 ± 2.65	164.33 ± 2.96

#: IU: International unit (the amount of enzyme that under defined assay conditions will catalyze the conversion of 1 μ mole of substrate /min.)
Acetylcholine esterase enzyme activity (Moles/µl hemolymph/min)±SE , Phenoloxidase activity (Unit/µl hemolymph/min)±SE

There was a very high significance between phenoloxidase activity at 24 and 48 hours measuring activity periods. Results of Ali *et al.,* (2016) indicated a significant increase in the activity of phenoloxidase of larvae of *S. littoralis* with all fungal isolates after 1,2, 3 and 4 days, as compared with controls. Other studies supported and confirmed the present results, Gabarty *et al*., (2013) found that injecting of LC_{50} concentration of both *B. bassiana* (0.4×10^5 spores/µl) and *M. anisopliae* (8×10^8spores/µl) to the last 5th instar larvae of *S. littoralis* significantly increased phenoloxidase, prophenoloxidase activities after one day. The results of Bali and Kaur, (2013) in haemolymph of 3rd, 4th and 5th instar larvae of *S. littoralis* showed significant increase in PO level after 24 hours of infection with 4.0×10^6 and 2.0×10^7 spores/ml compared to control. Zibaee *et al*., (2011) suggested that *B. bassiana* strongly affect the cellular immune reaction and phenoloxidase activity of the sun pest, *Eurygaster intergriceps.* The defensive responses to fungal infection lead to elevated levels of phenoloxidase and other enzymes (James *et al*., 2003). Moreover, Vergas-Albores and Plascencia (2000) found that upon fungal infection, a complex compound induces degranulation and the activation of prophenoloxidase. The increase in levels of PO may help to suppress microbial infection during the time interval (Hagstrum, 1983). The increasing of acetylcholine esterase activities after 48 hours when *B. zonata* larvae were treated with *B. bassiana* may be due to increased secretion of the fungus toxins in hemolymph of *B. zonata* larvae. This result coincides with those obtained by El-Gendy *et al*., (2014) when tested Bio-fly (*B. bassiana* biopesticide) on *B. zonata* flies. Our results also come in agreement with Zibaee *et al*., (2009) who reported a decrease in acetylcholine esterase activity after treating sun pest *E. griceps* larvae with *B. bassiana*. Also, Ali *et al*., (2016) found that in comparison to the injected larvae by the different isolates of fungi (*B. bassiana*, *L. mantillanum*, *P. lilacinus* and *M. anisopliae*) caused greater acetylcholine esterase activity of larvae of *S. littoralis* than the larvae in control treatment after 1, 2 and 3 days. Inhibition of AChE causes accumulation of Ach (acetylcholine) at the synapses, so that the post-synaptic membranes remain in a state of permanent stimulation, which results in paralysis, ataxia, general lack of coordination in the neuromuscular system and eventual death (Singh and Singh, 2000). The activities of AChE were increased after the fungal infection of insects' larvae. The increase of AChE activity in the case of *B. bassiana* may be due to increase the secretion of the fungus toxins in hemolymph of insect larvae. The enzymatic defense in insects, detoxification enzymes play significant roles in eliminating exotic compounds and maintaining normal physiological functions (Li *et al*., 2007). Thus, insect infection with entomopathogenic fungi sharply affect enzyme activities mediating degradation and detoxification of xenobiotics of different origin as a defense mechanism. Overall, enzymes have many functions and can mediate repair processes, detoxification of pathogenic products, and/or metabolism of biologically active compounds. Hence, changes in their activity can have an impact on biological and physiological processes (Serebrov *et al*., 2006). The defensive responses to *B. bassiana* infection lead to activation of enzymes. This may be caused by an impact on the role of enzymes in insect resistance to entomopathogenic fungi or they succeeded to suppress fungal infection and fungi failed to complete their life cycle within the insect.

CONCLUSION

The results of this study clearly indicated that *B. bassiana* could infect *B. zonata* different apparent immature stages and flies. Mortality of treated stages showed concentration dependent patterns. Biochemical tests assessed that *B. bassiana* caused a sharp disturbance in the protein, carbohydrates and lipids contents. Consequently, *B. bassiana* can use as an ecofriendly alternative of the synthetic chemical insecticides against *B. zonata*. The larval metabolite disturbance caused by *B. bassiana* could cause physiological imbalances in the host that lead to changes in enzyme activities and a reduction in haemolymph protein, carbohydrates and lipid contents. This may refer to that *B. bassiana* kill its host via depletion of energy reserves. The increase in phenoloxidase level after the exposure to *B. bassiana* reflects the larval resistance facing the fungal infection. Acetylcholine esterase decreased and increased expressing a disturbance in the physiological processes due to fungal infection. However, more researches needed to investigate the *in vivo* and *in vitro* determination of virulence factors of entomopathogenic fungi.

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REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, 18: 265-267.

AIi, S.S., El-Badawy, S. S., EI-Hefny, A.A., & Marwa, A. (2016).Vital Enzymatic Responses in Haemolymph of *Spodoptera littorulis* Boisd.) (Lepidoptera:Noctuidae) to Entomopathogenic Fungi Infection. *Egyptian Journal of Biological Pest Control*, 26 (2), 277-282.

Allwood, A. J., Chiajariyawng, A., Kritsaneepaiboon, S., Drew, R.A.L., Hamacok,E. L., Hancock, D.L., Hengsawad, C., Jipanin, J. C., Leong, C. T.S., & Vijaysegaran, S. (1999). Host plant records for fruit flies in southeast Asia. *Raffeles Bulletin Zoology Supplement*.No.7.

Bakr, E.M. (2005). http://www.ehabsoft.com/ldpline.

Bali, G. K., & Kaur, S. (2013). Phenoloxidase activity in haemolymph of *Spodoptera litura* (Fabricius) mediating immune responses challenge with entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillmin. *Journal of Entomology and Zoology Studies*. 1 (6): 118-123.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Annals of Biochemistry*, 72: 248-254.

Castillo, M.A., Moya, P., Hernandez, E., & Primo-Yufera, E. (2000). Susceptibility of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) to entomopathogenic fungi and their extracts. *Biological Control*, 19: 274-282.

Chandler, D., Heale, J.K., & Gillespie, A.T. (1993). Competitive Interaction between Strains of *Verticillium lecanii* on Two Insect Hosts. *Annals of Applied Biology*, 122, 435-440.

Charnley, A.K. (1989). Mechanisms of fungal pathogenesis in insects. In The Biotechnology of Fungi for Improving Plant Growth. eds. J.M Whipps, RD Lumsden. pp. 85-125. London: Cambridge University Press

Cotter, S.C., & Wilson, K. (2002). Heralibility of immune function in the caterpillar *Spodoptera littoralis*. *Heredity*, 88: 229-234.

Crompton, M., & Birt, L.M. (1967). Changes of the amounts of carbohydrates, phosphagen and related compounds during the metamorphosis of the blowfly, *Lucilia cuprina*. *Journal of Insect Physiology*, 13: 1575-1595.

David, W.A.L. (1969). The physiology of the insect integument in relation to the invasion of pathogens. In Insects and Physiology. ed. JWL Beament, JE Treherne, pp. 17-35 . London: Oliver& Boyd

Dhawan M., & Joshi, N. (2017). Enzymatic comparison and mortality of *Beauveria bassiana* against cabbage caterpillar *Pieris brassicae* LINN. *Brazilian journal of microbiology*, 48 (3): 522-529.

Dubios, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956).Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28: 350-356.

Efflatoun, H. C. (1924). A monograph of Egyptian Tryponeidae. *Memoirs of the Society of Royal Entomology*, Egypt 2: 1–132.

Ekesi, S., Maniania, N.K., & Lux, S.A. (2002). Mortality in three African tephritid fruit fly puparia and adults caused by the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. *BioControl Science and Technology*, 12:7-17.

El-Badawy, S. S., Ali, S.S., El-hefny, A. A., & Gamila, A. M. H. (2018). Study of infection impact by entamopathogenic fungi on some biochemical contents in haemolymph of cotton leaf worm, *Spodoptera littoralis* (Bois.), *Journal of Plant Protection and Pathology*, Mansoura University, Vol.9 (9): 605 – 610.

El-Gendy, I. R., Nasr, H. M., Badawy, M. E., & Rabeah, E. I. (2014). Toxic and biochemical effects for certain natural compounds on the peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae). *American Journal of Biochemistry and Molecular Biology*, vol. 4(3):112-121.

Ellman G. L., Diane, K. C., Valentino, J.R., & Robert, M. F. (1960). New and rapid colorimetric determination of acetylcholine esterase activity. *Biochemical Pharmacology*, 7: 88-95.

El-Minshawy, A.M., El-Eryan, M.A., & Awad, A.I. (1999). Biological and morphological studies on the guava fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) found recently in Egypt. 8th National Conference of Pests & Disinfestation of Vegetables & Fruits in Ismailia. 71-82, Egypt

El-Sayed, A.I.H. (1979). Biotic potential of *Ceratitis capitata* (Wied.) as influenced by the type of diet. M. Sc. Thesis, Faculty of Agriculture, Zagazig University.

Gabarty, A. (2011). Combined effect of gamma radiation and some fungal control agents on the greasy cutworm *Agrotis ipsilon* (Huf.) Ph.D. Thesis, *Faculty of Science for Girls Al- Azhar University Cairo- Egypt.*

Gabarty, A., El-Sonbaty, S. M., & Ibrahim, A. A. (2013). Synergistic effect of gamma radiation and entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* on the humoral immune enzyme response in cotton leaf worm *Spodoptera littolaris* (Boisd).*Egyptian Academic Journal of Biological Science*,6 (3):1-10.

Galhoum, A.H. (2017). Taxonomic studies on two tephritid species, *Bactrocera oleae* and *B. zonata*, using the cuticular hydrocarbons profile. *Al-Azhar Bulletin of Science*,vol.28(1-c):45-54.

Gillespie, A.T., & Claydon, N. (1989). The use of entomogenous fungi for pest control and role of toxins in pathogenesis. *Pest Science*, 27:203-215.

Gomez, D.R.S., Boucias, D.G., & Nation, J.L. (1997). Attachment of *Metarhizium anisopliae* to the southern green stink bug, *Nezara viridula* cuticle and fungistatic effect of cuticular lipids and aldehydes. *Journal of Invertebrate Pathology*, vol.69(1): 31-39.

Hagstrum, D. W. (1983). Self-provisioning with paralyzed hosts and age, density, and concealment of hosts as factors influencing parasitization of *Ephestia cautella* (Walker) (Lepidoptera: Pyralidae) by *Bracon hebetor* Say (Hymenoptera: Braconidae). *Environmental Entomology* 12:1727–1732.

Hankin, L., & Anagnostakis, S. L. (1975). The use of solid media for detection of enzyme production by fungi. *Mycologia*. 67: 597-607.

Hassan, A.E.M., & Charnley, A.K. (1989).Ultrastructural study of the penetration by *Metarhizium anisopliae* through Dimilin-affected cuticle of *Manduca sexta*. *Journal of Invertebrate Pathology*, 54: 1 17-24

Hussein, M.A., Khaled, A.S., Ibrahim, A.A., Soliman, N.A., & Attia, S.H. (2018). Evaluation of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* on peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera:Tephritidae). *Egyptian Academic Journal of Biological Science*, 10(1): 59-68.

Ibrahim, A. A., Soliman, N. A., Shams El-Deen, M. M., Ramadan, N. F., & Farag, S. R. (2014). Susceptibility of the peach fruit fly, *Bactrocera zonata* (Saunders) and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) adults to the entomopathogenic fungi: *Metarhizium anisopliae* (Met.) and *Beauveria bassiana* (Bals.). *Egyptian Journal of Biological Pest Control*, 24(2), 2014, 491-495.

Ibrahim, A.A. (2006). Action of certain microbes on cotton leaf worm. Ph. D. Thesis, Faculty of Science, Al-Azar University, 171 pp.

James, R. R., Buckner, J. S., & Freeman, T. P. (2003). Cuticular lipids and silver leaf whitefly stage affect conidial germination of *Beauveria bassiana* and *Paecilomyces fumosoroseus*. *Journal of Invertebrate Pathology*, 84:67–74

Jua´rez, M.P. (1994): Inhibition of insect surface lipid synthesis and insect survival. Arch. Insect Biochem. Physiol. 25, 177–191.

Knight, J.A., Anderson, S., & Rawle, J.M. (1972). Chemical basis of the sulfophospho-vanillin reaction for estimating total serum lipids. *Clinical Chemistry*, 18: 199-202.

Li, S., Huang, Z., & Ren, S.X. (2009). Production and extraction of extracellular lipase from the entomopathogenic fungus *Isaria fumosoroseus* (Cordycipitaceae: Hypocreales). *BioControl Science and Technology*, 19:81–89.

Mallikarjuna, M., Balavenkatasubbaiah, M., Nataraju, B., & Thiagrajan, V. (2002). Effect of systematic fungicide on total haemocyte count and haemolymph biochemical changes in silkworm *Bombyx mori* L. infected with *Beauveria bassiana*. *International Journal of Industrial Entomology* 5: 189-194.

MaxStat 2015: MaxStat. Pro v3.6, statistics, Jevery, Germany.

Mustafa, U., & Kaur, G. (2009). Extracellular enzyme production in *Metarhizium anisopliae* isolates. *Folia Microbiology*, 54(6):499–504.

Nada, M. S. (2015). Response of green stinkbug *Nezara viridula* (linnaeus), to the activity of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* Journal of Plant Protection and Pathology, Mansoura University, vol.6 (12): 1633– 1644.

Napolitano, R., & Jua´rez, M.P.(1997). Entomopathogenous fungi degrade epicuticular hydrocarbons of *Triatoma infestans*. *Archives of Biochemistry and Biophysics*, 344: 208–214.

Nirupama, R. (2015). Biochemical studies on total protein, carbohydrate and lipids content level during the infection by fungi white muscardine disease of silkworm, *Bombyx mori* L. *Munis Entomology and Zoology*, vol. 10, No. 2.

Oguri, E., & Steele, J.E. (2007). A comparative study of the metabolic effects of hypertrehalosemic hormone and 1,2,3,4,5,6- hexachlorocyclohexane (γ-HCH) in the American cockroach, *Periplaneta americana*. *Pesticide Biochemistry and Physiology*, 87: 196–203.

Pedrini, N., Ortiz, A., Bonnet, C., Zhang, S., & Keyhani, N. (2013). Targeting of insect cuticular lipids by entomopathogenic fungus, *Beauvaria bassiana*: hydrocarbons oxidation within context of host pathogen interaction. *Microbiological Chemistry*, 4:1-18.

Quesada-Moraga, E., Ruiz-Garcia, A., & Santiago-Alvarez, C. (2006). Laboratory evaluation of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against puparia and adults of *Ceratitis capitata* (Dipter: Tephritidae). *Journal of Economic Entomology* 99 (6): 1955-1966.

Rajitha, K., & Savithri, G. (2014). Amino acid profiles in the haemolymph of silkworm, *Bombyx mori* L. infected with fungal pathogen, *Beauveria bassiana* (Bals) vuill. *International Journal of Applied Biology and Pharmaceutical Technology*, 5(1): 163-166.

Serebrov, V.V., Gerber, O.N., Malyarchuk, A.A., Martemyanov, V.V., Alekseev, A.A., & Glupov, V.V. (2006). Effect of entomopathogenic fungi on detoxification enzyme activity in greater wax moth *Galleria mellonella* l. (Lepidoptera, Pyralidae) and role of detoxification enzymes in development of insect resistance to entomopathogenic fungi. *Biological Bulletin*, 33: 581-586.

Shigematsu, H., & Noguhi, A. (1969). Biochemical studies on the multiplication of a nuclear polyhedrosis virus in the silkworm, *Bombyx mori*. II. Protein synthesis in the larval tissue after infection. *Journal of Invertebrate Pathology*, 14: 301-307.

Silva, R.O., Silva, H. H. G., Uihoa, C. J., & Luz, C. (2005). Is there a relationship between *N,acetyl-β-D-glucosaminidase* activity of *Metarhizium anisopliae* (Metsch.) Sorokin (Hyphomycetes) isolates from peridomestic areas in Central Brazil and larvicidal effect on *Aedes aegypti* L. (Diptera: Culicidae), *Journal of Applied Entomol*ogy, 129: 158-164.

Singh, K., & Singh, D. K. (2000). Toxicity to the snail *Limnaea acuminata* of plant-derived molluscicides in combination with synergists. *Pest Management Science*, 56, 889–898.

Soliman, N.A., Ibrahim, A.A., Sham El-Deen, M.M., Ramadan, N.F., & Farag, S.R. (2014). Entomopathogenic nematodes and fungi as BioControl agents for the peach fruit fly, *Bactrocera zonata* (Saunders) and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) soil-borne stages. *Egyptian Journal of Biological Pest Control*, 24(2): 497-502.

St.Leger, R. J., Joshi, L., & Roberts, D. W. (1997). Adaptation of proteases and carbohydrates of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. Microbiology, 143: 1983-1992.

St-Leger, R.J. (1995). The role of cuticle-degrading proteases in fungal pathogenesis of insects. *Canadian Journal of Botany*, 73, s1119–s1125.

St-Leger, R.J., Cooper, R. M., & Charnley, A. K. (1987). Characterization of chitinase and chitobiase produced by the entomopathogenic fungus, *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, 58(3): 415-426.

Tanaka, N., Steiner, L.F., Ohinata, K., & Okamoto, R. (1969). Low cost larval rearing medium for mass rearing production of oriental and Mediterranean fruit flies. *Journal of Economic Entomology*, 62: 967-968.

Tsakas, S., & Marmaras, V. (1990). Detection of haemocyte protein in the integument of developing Mediterranean fruitfly, *Ceratitis capitata*. Roux's *Archives of Developmental Biol*ogy, 199:281-288.

Vergas-Albores, F., & Plascencia, G.Y. (2000). Beta glucan binding protein and its role in shrimp immune response. *Aquaculture* 191: 13-21.

Wilkinson, F. (1976). Insecticide biochemistry and physiology. *Plenum Press, New York*, USA.

Yeo, H., Pell, J.K., Alderson, P.G., Clark, S.J., & Pye, B.J. (2003). Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. *Pest Management Science*, 59: 156-165.

Zibaee, A. , Bandani, A. R., & Malagoli, D. (2009). Identification of *Beauveria bassiana* extracellular protease as a virulence factor in pathogenicity toward the migratory grasshopper, *Melanoplus sanguinipes*. *Biochemistry and Molecular Biology*, 158 (1): 17-123.

Zibaee, A., Bandani, A. R. ,Louei, H. R. T., & Malagoli, D. (2011). Cellular immune reactions of the sun pest, *Eurygaster integriceps* to the entomopathogenic fungus, *Beauveria bassiana* and its secondary metabolites. *Journal of Insect Science*, 38: 1-16.

Zimmermann, G. (2007b). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *BioControl Science and Technology*, 17: 553-596.

التأثيرات السمية والكميائية الحيوية *Beauveria bassiana* على الأطوار الغير كاملة لذبابة ثمار الخوخ *Bactrocera zonata*

نهاد عبد الحميد سليمان*

معهد بحوث وقاية النباتات - 7 ش نادى الصيد , الدقى – جيزة

تعتبر ذبابة ثمار الخوخ باكتروسيرا زوناتا حشرة بالغة الضرر للحاصلات البستانية في مصر وأثبتت الدراسة أن الفطر الممرض للحشرات بوفيريا باسيانا استطاع أن يصيب الأطوار الغير كاملة للحشرة معتمدا على التركيزات المستخدمة وقد كانت الحشرات الطائرة الغير بالغة هي أكثر الأطوار حساسية للإصابة بالفطر . وقد أثبت النشاط الإنزيمى للفطر قدرته على استخدام الإنزيمات خارج الخلية الكايتينيز والبروتييز والليبيز . أما الإختبارات الكيميائية الحيوية فقد أثبتت زيادة ونقصان في البروتين والكربوهيدرات والدهون الكلية لليرقات المصابة بالفطر كما كانت هناك زيادة هائلة في مستوى الفينول اوكسديز بعد 24 و 48 ساعة من العدوى ؛ كما أثبتت الدراسة حدوث نقص في مستوى أسيتايل كولين استيريز بعد 24 ساعة و زيادة بعد مرور 48 ساعة من العدوى .