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Virulence of entomopathogenic fungi *Beauveria bassiana* against the seed bug *Graptostethus servus* (Hemiptera : Lygaeidae) and its immune response

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Abstract:

The pathogenicity of native isolate of entomopathogenic fungi *Beauveria bassiana* against seed bugs *Graptostethus servus* (Fabricius) (Hemiptera: Lygaeidae) was investigation for the first time in laboratory bioassay. The results obtained that *G. servus* was highly susceptible to *B. bassiana.* The calculated LC_{50} was$2.9x10^6$ spores/ml and the LT_{50} values was 4.16 days. The mechanisms of *G. servu*s immune defenses against *B. bassiana* was studied. Result revealed that both of total protein, total carbohydrates, Esterase and alkaline phosphatase activities were significant decreased after 48-72hrs. post treatment than control. There was significant decreased in Glutathione S-transferase (GST) after 24 -72 hrs. posttreatment. Both of phenoloxidases and peroxidase activity were significantly increased after 24hrs. and recorded non-significant decreased after 48 and 72hrs. posttreatment than control.

Introduction

The seed bugs *Graptostethus servus* (Fabricius) (Hemiptera: Lygaeidae) belong to order Hemiptera and suborder, Heteroptera which included family Lygaeidae are recognized as seed bugs (Magsi *et al*., 2018). These seed bugs is distributed in Japan, except for Hokkaido island (Yasunaga *et al.*, 1993) and in Africa, Europe, Asia, and the south Pacific Island (Hussain *et al*., 2014; Kment *et al*., 2005 and Pericart, 2001). Chin *et al.* (2018) reported that, during the dry season there are various swarms of native sucking bugs *G. servus* appear in Darwin, Palmerston, Katherine and rural areas in China. In Egypt, Ibrahim and Elshewy (2020) recorded a swarms of both seed bugs *G. servus* and *Spilostethus pandurus* (Scopoli) (Hemiptera : Lygaeidae) during June 2017, 2018 and 2019 and early winter seasons 2020 and in Giza Governorate, Egypt, on oil crops such as *Sesamum indicum* (Pedaliaceae), sorghum crops (*Sorghum bicolor*) and milkweed plants (*Convolvulus arvensis* L.).

The seed bugs *G. servus* as sap - sucking bugs are known as a pest of not only Convolvulaceous crops such as *Ipomoea tuberosa* (L.) but also other families of crops such as *Gossypium* spp., sorghum bicolor (L.) Moench, in various regions (Khaing *et al*., 2002; Kment *et al*., 2005 and Yeates, 2001). Suzaki and Okada (2016) suggested that *G. servus* have a serious damage to sunflower, *Helianthus annuus* L. because *G. servus* can grow on sunflower seed in the rearing laboratory (Gamberale-Stille and Tullberg, 1999 and 2001). Chin *et al.* (2018) recorded that huge swarm of *G. servus*

aggregated on fruit trees, ornamentals, vegetables and native tre es. The bugs are attracted to moisture from irrigation, and rain. They also make large groups to aggregate and mate. They occasionally cause indirect damage on fruit or vegetable crops, as pin –prick spots by feeding on moisture found on fruit, shoots or flowers, and cause physical damage by breaking off stems and making scratch marks on leaves, flowers or fruit by moving on the plants in a large groups. There is not much information on using biological control for controlling seed bugs *G. servus* in literature. These findings may be considered the first investigation of the susceptibility of seed bugs *G. servus* adult to infection by entomopathogenic fungi *Beauveria bassiana* as promising biocontrol agent.

The aim of this study to evaluate the virulence of native isolate of entomopathogenic fungi *B. bassiana* against seed bugs *G. servus* and recording the physiological reactions to infection.

Material and methods

1. Rearing of *seed* bugs *Graptostethus servus* :

Individuals of the seed bugs adults and nymphs was collected from faculty of Agriculture Experimental Station, Cairo University, and Giza, Egypt. Seed bugs were transported to the laboratory and placed in plastic cages supplemented with pods of milkweed plant for feeding, which renewed every two day intervals. Sheets papers were added to prevent cannibalism and a saturated piece of cotton will hanged in all cages. All cages were covered with muslin for ventilation. Adult were injecting there egg out the muslin caver, Eggs were collected over the muslin caver and incubated in petri dish supplemented with a pieces of moistened cotton wool until hatching. All hatches were transferred to new cages for rearing as pervious methods.

2. Entomopathogenic fungi culture:

The entomopathogenic fungi *B. bassiana* (Hypocreales : cordycipitaceae) isolate used in these experiments was isolated from Soil which collected from East Owainat at New valley Governorate , Egypt, during 2018 . Using larvae of the greater wax moth, *Galleria mellonella* L. for trapping of entomopathogenic fungi methods According to Zimmermann (1986). *B. bassiana* was cultured on autoclaved Sabourad dexstrose yeast agar media (SDAY), at 25 ± 1 ºC for 14 days (Nada 2015).

3. Bioassays procedure:

Spores were collected from the culture media by rinsing with sterilized aqueous solution of 0.02% Tween 80, and then filtered through cheese cloth to reduce mycelium clumping. By using Haemocytometer (Neubauer improved HBG, Germany 0.100 mm X 0.0025mm^2) concentrations of spores were counted. For bioassay, the suspensions were prepared at concentrations of 10^6, 10^7, $5x10^7$ and 10^8 spores/ml (Nada, 2006).

Adult of *G. servus* were treated with dipping methods technique for 10 seconds in each concentration and the residual suspension was then filtered out with filter paper. In control adults were treated with sterile aqueous solution of 0.02% tween 80. A total of 25 insect were used for each concentration and control. The treated insect and control were maintained individual in transparent plastic boxes (d=6cm, h=3cm) with holes in the cover for ventilation, and supplement with saturated filter paper. All plastic boxes were supplemented with pods of milkweed plant for feeding which renewed every two days. Then all boxes were incubated in 25±ºC. Mortality was monitored daily for 7 days after treatment.

4. Biochemical studies:

Adults were treated with $1x10^7$ spores/ml of *B. bassiana* as previous methods. Sterile aqueous solution of 0.02% tween 80 was used as control. After 24, 48 and 72 hrs. the surviving larvae were used for the biochemical analysis using methods of Gholamzadeh- Chitgar *et al*. (2015).

4.1. Sample preparation:

Adults of *G. servus* were collected after 24, 48 and 72 post treatment (n=20 per treatment for each enzymes). Samples were homogenized separately in 250 µl of 0.2 M phosphate buffer (pH 7.0) containing 0.05 % triton X- 100 using a plastic pestle. Then, the homogenate was centrifuged at 12000g for 10 min at 4ºC. The supernatant was used as an enzyme solution for assessing the total protein, total carbohydrates, Alpha esterases, Alkalin phosphatase, glutathione S-transferase (GST), phenoloxidases and peroxidases.

4.2. Biochemical analysis:

4.2.1. Determination of total proteins:

Protein reagent was prepared by dissolving 100mg of Coomassie Brilliant blue G-250 in 50ml 95% ethanol. To this solution 100ml 85% (W/V) phosphoric acid were added. The resulting solution was diluted to a final volume of 1 liter. Sample solution (50µl) or for the preparation of standard curve 50µl of serial concentrations containing 10 to 100µg bovine serum albumin was pipetted into test tubes. The volume in the test tube was adjusted to 1 ml with phosphate buffer (0.1M, pH 6.6). Five millimetres of protein reagent were added to test tube and the contents were mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min and before 1 hrs. against blank prepared from 1 ml of phosphate buffer and 5 ml protein reagent (Bradford, I976).

4.2.2. Determination of total carbohydrates:

Total carbohydrates were estimated in acid extracts of a samples by the phenol-sulphuric acid reaction of DuBois *et al*. (1956). Total carbohydrates were extracted and prepared for assay according to Crompton and Birt (1987). Sample (1 gm) was homogenized in 0.3N HClO4(5 ml) at 0˚C for 1 min. The homogenate was kept in ice for further 10 min. Insoluble matter was removed by centrifugation for 3 min.at 2000 r.p.m and washed twice in ice cold HClO4 (5ml) by redispertion and centrifugation. The three-supernatant combined into acid extract. Hundred microliters of the acid extract were added into a colorimetric tube to 0.5 ml of phenol (20 present w/v). Then 5 ml of concentrated sulphuric acid were added rapidly with shaking. The tubes were allowed to stand 10 min, and then they were shaken and placed for 10-20 min in water bath at 25 to 30 ˚c before readings. Blanks were prepared by substituting distilled water for the sugar solution. The absorbance of characteristic yellow orange color is measured at 490 nm against blank. Total carbohydrate is expressed as: µg glucose / gm fresh weight.

4.2.3. Determination alpha esterases:

Alpha esterases (α-esterases) was determined according to Van Asperen (I962) using α-naphthyl acetate as substrates, respectively. The reaction mixture consisted of 5ml substrate solution (3x10-4M α-or β-naphthylacetate, 1% acetone and 0.1M phosphate buffer, pH7) and 20µl of larval homogenate. The mixture was incubated for exactly 15 min at 27˚C, then 1 ml of diazoblue color reagent (Prepared by mixing 2 parts of 1%diazoblue B and 5 parts of 5% sodium lauryl sulphate) was added. The developed color was read at 600 or 555 nm for α- naphthol produced from

hydrolysis of the substrate. α- naphthol standard curves was prepared by dissolving 20 mg α- naphthol in 100ml phosphate buffer, pH7 (stock solution). Ten millilitres of stock solution were diluted up to 100ml by the buffer. Aliquots of 0.1, 0.2, 0.4, 0.8 and 1.6 ml of diluted solution (equal to 2, 4, 8, 16 and 32 µg naphthol) were pippeted into test tubes and completed to 5 ml by phosphate buffer. One millilitre of diazoblue reagent was added and the developed color was measured as mentioned before.

4.2.4. Determination of alkalin phosphatase:

Acid and alkaline phosphatases were determined according to the method described by Powell and Smith (1954). In this method, the phenol released by enzymatic hydrolysis of disodium phenylphosphate reacts with 4-aminoantipyrine, and by the addition of potassium ferricyanide, the characteristic brown color is produced. The reaction mixture consisted of 1 ml carbonate buffer (pH 10.4) for alkaline phosphatase or 1 ml citric buffer (pH 4.9) for acid phosphatase, 1 ml of 0.01 M disodium phenylphosphate (Substrate), and 0.1 ml sample. Mix and incubate for exactly 30 min at 37 C0. At the end of incubation period .0.8 ml of 0.5 N Na0H was added to stop the reaction. Then add 1.2 ml of 0.5 N NaHC03, followed by the addition of 1 ml of 4-aminoantipyrine solution (1%) and 1 ml potassium ferricyanide (0.5 %) . The produced color was measured immediately at 510 nm. The enzyme activity is expressed by unit (U), where 1 unit will hydrolyze 1.0 u mole of p-nitopheny l phosphate per minute at 37 Co, and pH 10.4 and 4.8 for alkaline and acid phosphatases, respectively.

4.2.5. Determination of Glutathione S- transferase (GST):

Glutathione S-transferase (GST) catalyzes the conjugation of reduced glutathione (GSH) with 1-chloro 2,4-dinitrobenzene (CDNB) via the -SH group of glutathione. The conjugate , S-(2,4-dinitro-phenyl)-L-glutathione could be detected as described by the method of Habig *et al*.(I974).The reaction mixture consisted of 1 ml of the potassium salt of phosphate buffer (pH6.5),100µl of GSH and 200µl of larval homogenate. The reaction started by the addition of 25µl of the substrate CDNB solution. The concentration of both GSH and CDNB was adjusted to be 5mM and 1mM, respectively. Enzyme and reagents were incubated at 30˚C for 5 min. The increment in absorbance at 340 nm was recorded against blank containing everything except the enzyme to determine the nanomole substrate conjugated/min/larva using a molar extinction coefficient of 9.6/m M/cm.

4.2.6. Determination of phenoloxidases:

Phenoloxidase activity was determined according to a modification of Ishaaya (I971), in a reaction mixture consisting of 0.5 ml phosphate buffer (0.1 M, pH 7), 200 µl enzyme solution and 200 µl catechol solution (2%). Prior to the initiation of the reaction, the substrate and other ingredients of the reaction mixture were separately incubated at the optimum temperature of the reaction (25 ^{o}c). Enzyme reaction was initiated by adding catechol solution. Then after exactly 1 min, the optical density was determined. Zero adjustment was against sample blank. The phenol oxidase activity was determined as O.D. units ×10 3 at an absorbancy of 405 nm.

4.2.7. Determination of peroxidases:

Peroxidase activity was determined according the procedure given by Hammerschmidt *et al*. (1982). To a spectrophotometer sample cuvette, 1.5 ml of pyrogallol (0.05 M) and 100 UL enzyme extract were added. The readings were adjusted to zero at 420

NM. To initiate the reaction, 100 UL of hydrogen peroxide (1%) was added to the sample cuvette. The enzyme activity was expressed as change in absorbance /min/ g sample.

5. Statistical analysis:

The corrected mortality percentages were statistically computed according to Finney (1971), to determine the LC_{50} and LT_{50} values, using "Ldp" software by Bakr (2000). All data of Biochemical analysis was expressed as the mean ± standard error. Statistical analysis Obtained data was analyzed as one way ANOVA, using Proc ANOVA in SAS (Anonymous. 2003), and means were compared by Tukey's HSD (*P*= 0.05 level) in the same program.

Results and discussion

1. Virulence of *Beauveria bassiana* for *Graptostethus servus*:

The obtained results showed that *G. servus* adults are highly susceptible to infection with entomopathogenic fungi *B. bassiana* and demonstrated the first report documenting the pathogenicity of *B. bassiana* to *G. servus*. The correspondents LC_{25}, LC_{50} and LC_{90} values were $3x10^5$, 2.9 $x10^6$ and $2.08x10^8$ spores/ml, respectively (chi^2= 0.407, slope= 0.689±018, P<0.816) (Figure 1). Mortality was observed from 4 days after treatment. Mean lethal time LT_{25}, LT_{50} and LT_{90} values were 3.12, 4.16 and 7.2 days, respectively (chi2= 0.79, slope= 5.38±1.008, P<0.851) (Figure 2). After death insect were incubated in high humidity at 25±ºC and observed the emerged of the growth mycelium and spores from insect body to prove the pathogenicity (Figure 3).

B. bassiana was used by Steinhaus (1975) to monitor (Hemiptera : Lygaeidae). Reports of naturally occurring epizootics induced by *B. bassiana* in populations of *B. leucopterus* support the idea of using Entomopathogenic fungi against chinch bugs.

Also, Krueger *et al*. (1991) reported that Adults of chinch bug, *B. leucopterus* are highly susceptible to *B. bassiana* under laboratory conditions. Entomopathogenic fungi isolates of *B. bassiana* and *Metarhizium anisopliae* were tested for pathogenicity and virulence in *B. leucopterus* eggs in a laboratory analysis. According to Samuels *et al.* (2002) all entomopathogenic fungi treated isolates were virulent to chinch bug eggs.

Adult chinch bugs can be killed by *B. bassiana* at high conidial concentrations, according to Boyle and Cutler (2012), who also found that early instars were more vulnerable to infection than nymphs and adults. Sahayaraj and Borgio (2010) announced for the first time that cotton seed bugs *Oxycarenus hyalinipennis* (Costa) (Hemiptera: Lygaeidae) are susceptible to *M. anisopliae* in an in vitro bioassay, with LC_{50} values of $1.93x\ 10^5$ spores/ml.

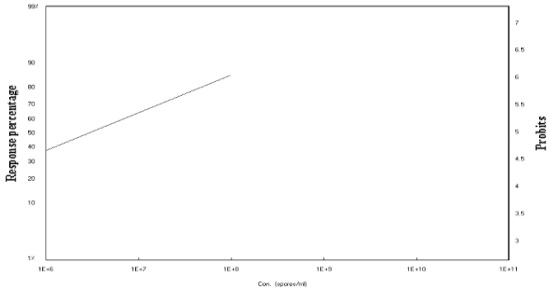

Figure (1): Percentage mortality regression line of *Graptostethus servus* treated with *Beauveria bassiana* .

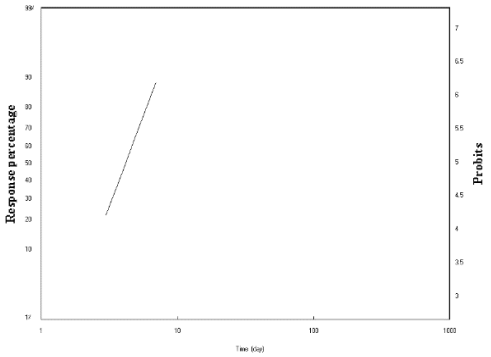

Figure (2): Time-mortality regression line of *Beauveria bassiana* at concentration10^8 spores/ml against *Graptostethus servus* .

Figure (3): (A): Healthy *Graptostethus servus* and (B): Sporulation process of *Beauveria bassiana* in *Graptostethus servus* cadavers after death .

2. Biochemical analysis:

2.1. Determination of total proteins:

Proteins are integral components of living cells and are made up of a variety of substances such as enzymes, hormones, and antibodies, all of which are important for an organism's proper functioning (Fagan *et al*., 2002). Data in Table (1) showed that total protein activity of adult was decreased significant during 48 and 72 hrs. after fungal infection than control (16.97, 12.3, 28.7 and 23.3 mg/g. b. wt., respectively). Our results match what was found by Nada (2015) who reported that Total protein activity in green bug *Nezara viridula* (L.) (Hemiptera: *Pentatomidae)* treated with *Metarhizium anisopliae* and *B. bassiana* were significantly decreased. Vidhya *et al.* (2016) reported a significant reduction of total protein on arm worm *Spodoptera litura* treated with three entomopathogenic fungi *Verticillium lecanii* , *B. bassiana* and *M. anisopliae* at fourth day after infection compared with control. El-Sayed and Yousef (2021) explained that when *Spodoptera littoralis* (Boisd) (Lepidoptera : Noctudiae) treated with *Purpureocillium lilacinum* (*Paecilomyces lilacinus*) this prompted a sharp decreased in total protein in cadavers after death .

Table (1): Effect of *Beauveria bassiana* on total protein in *Graptostethus servus*.

Hours after treatment	Total protein (mg/g.b.wt)		F	P	M.S.D
	Control± SE	*Beauveria bassiana* ± SE			
24	22.3±0.4[a]	21.1±1.06[a]	0.94	0.39	3.15
48	28.7±0.73[a]	16.97±0.5[b]	170.64	0.0002	2.49
72	23.3±0.93[a]	12.3±1.17[b]	54.37	0.0018	4.142

[a]Mean, within a row, bearing different subscripts are significantly different.

2.2. Determination of total carbohydrates:

Carbohydrates are the primary source of energy for the formation and growth of living cells, and they also act as fundamental basic elements for cells and components of a variety of metabolic intermediates. The current investigation proved that in the first 24h, the total carbohydrates activity was slight decreased than control (84.3 and 86.2, respectively). After that, there is a sharp significant decreased after 48, 72 h than control (77.7, 59.8, 91.7 and 84.97 mg/g.b.wt, respectively) (Table 2). Our results match what found by Elbanna *et al.* (2012) who reported that total carbohydrates contains in 5th instars of *Schistocerca gregaria* Forskål (Orthoptera: Acrididae), treated with *M. anisopliae* was actual decreased after 24h than control. Nada (2015) mentioned that there are significant decreased of total carbohydrates activity when adults of *N. viridula* treated with both entomopathogenic fungi *M. anisoplae* and *B. bassiana* In contrast El-Sayed and Yousef (2021) reported that there is a significant increase in total carbohydrates in haemolymph of *S. littoralis* treated with *P. lilacinum* than control.

Table (2): Effect of *Beauveria bassiana* on total carbohydrates in *Graptostethus servus*.

Hours after treatment	Total carbohydrates (mg/g.b.wt)		F	P	M.S.D
	Control ±SE	*Beauveria bassiana* ±SE			
24	86.2±0.92[a]	84.3±1.7[a]	0.99	0.38	5.314
48	91.7±1.4[a]	77.7±0.9[b]	69.45	0.001	4.64
72	84.97±1.9[a]	59.8±1.08[b]	132.56	0.0003	6.069

[a]Mean, within a row, bearing different subscripts are significantly different.

2.3. Determination of Alkaline phosphatase:

Under the name of dephosphorylation, alkaline phosphatase is a hydrolytic enzyme that removes phosphate groups from a variety of molecules, including nucleotides, proteins, and alkaloids, in alkaline conditions (Zibaee *et al*., 2009 and 2011). Data represented in Table (3) revealed that after 24hrs. the activity of Alkaline phosphatase was slight decreased than control (83.3 and 92.67 mU/g.b.wt, respectively). There are a significant deceased after 48 and 72hrs. compared with control (59.3, 66, 136 and 170.67 mU/g.b.wt, respectively). These results are in agreement with Gad and Nada (2020) which recorded a highly significant reduction of Alkaline phosphatase activity on *N. viridula* treated with *B. bassiana* than control during 24hrs. post treatment.

Table(3): Effect of *Beauveria bas siana* on alkaline phosphatase activity in *Graptostethus servus*.

Hours after treatment	Alkaline phosphatase (mU/g.b.wt)		F	P	M.S.D
	Control ±SE	*Beauveria bassiana* ±SE			
24	92.67±3.7[a]	83.3±4.37[a]	2.65	0.179	15.92
48	136±7.2[a]	59.3±6.67[b]	61.80	0.0014	27.077
72	170.67±6.67[a]	66±4.62[b]	169.99	0.0002	22.289

[a] Mean, within a row, bearing different subscripts are significantly different.

2.4. Determination of alpha esterases activity (ESTs):

Esterases (ESTs) play an important role in the insect's life by catabolizing the esters of higher fatty acids, which stimulates activity in the flight muscles and allows insects to fly by mobilizing lipids, including those found in the fat body, and degrading inert metabolic esters including various xenobiotic (Roslavtseva *et al.*,1993). Table (4) observed that after 48-72hrs. post treatment there are a significant decreased on EST activity than control (396.33, 250, 579.67 and 451.67 Ug α-naphthol/min/g.b.wt, respectively). According to Cao *et al.* (2016), the observed decrease in ESTs activities by *M. anisoplae* IM1330189 isolate can overcome EST immunity by inhibiting EST transcription on the hemocytes of *Locusta migratoria*, while IBC200614 isolate has no apparent effect on ESTs activities, implying that the insects are resistant to IBC200614 due to the stability of ESTs activities.

Table (4): Effect of *Beauveria bassiana* on alpha esterases activity in *Graptostethus servus*.

Hours after treatment	Alpha esterases (Ug α- naphthol/min/g.b.wt)		F	P	M.S.D
	Control ±SE	*Beauveria bassiana* ±SE			
24	373±6.7[a]	392.6±3.7[a]	6.66	0.0613	21.165
48	579.67±11.84[a]	396.33±7.9[b]	166.21	0.0002	39.48
72	451.67±14.66[a]	250±5.8[b]	163.92	0.0002	43.73

[a]Mean, within a row, bearing different subscripts are significantly different.

2.5. Determination of Glutathione-S-transferase activity:

Glutathione-S- transferase (GST) is involved in metabolite removal as well as tissue protection from free radical damage, and increase in insecticide-resistant insects, according to Papadopoulos *et al.* (2000). Data represented in Table (5) revealed that GST was significant decreased stalely during 24, 48 and 72hrs. after treatment than control . In insects of different orders, increased expression of genes for detoxifying enzymes developing resistance to various xenobiotics was observed. During the progression of infection, the activity of esterases and GST in the degradation of toxic molecules may play a significant role in protecting insects from pathogens (Dubovskiy *et al.*, 2012). Gillespie *et al.* (2000) explained that reduced Esterase and GST activity during the acute phase of mycosis may be linked to successful entomopathogenic fungi inhibition of the host defense systems.

Table(5): Effect of *Beauveria bassiana* on Glutathione-S- transferase activity in *Graptostethus servus*.

Hours after treatment	GST (mmol sub.conjugated/min/g.b.wt)		F	P	M.S.D
	Control ±SE	*B. bassiana* ±SE			
24	44±2.03[a]	33±1.61[b]	20.33	0.0107	7.184
48	41 ±1.15[a]	26.9±0.59[b]	133.32	0.0003	3.575
72	43±1.17[a]	30±1.097[b]	73.36	0.0010	4.4519

[a]Mean, within a row, bearing different subscripts are significantly different.

2.6. Determination of phenoloxidases activity:

Phenoloxidases are play a key role in nodule formation and encapsulation. The hydroxylation of mono and diphenols to quinone intermediates is catalysed by Phenoloxidases, which are located in the host cuticle. Phenoloxidases products are involved in a participant in a variety of critical processes such as defense, wound healing, cuticle sclerotisation, and foreign particle identification and melanization. The results in Table (6) showed that there was a significant increase of phenoloxidases activity in the first 24h after treatment than control (5.03 and 3.83 O.D./min/g.b.wt , respectively), whoever it decreased after 48 and 27 h than control (3.6, 3.4, 4.07 and 3.73 O.D./min/g.b.wt, respectively). Our result matches what was found by Gad and Nada (2020), who reported that, phenoloxidates activity in the haemolymph of *N. viridula* was significant increased during 24hrs. post infection than control, then decreased . Serebrov *et al.* (2006) reported that inhibiting detoxification enzymes dramatically increases the death rate of insects from fungal infection, suggesting that detoxification enzymes are involved in the creation of insect resistance to Entomopathogenic fungi and opening new avenues for the production of highly effective combined biological products based on Entomopathogenic fungi and detoxification enzyme inhibitors.

Table (6): Effect of *Beauveria bassiana* on phenoloxidases activity in *Graptostethus servus*.

Hours after treatment	Phenoloxidases (O.D./min/g.b.wt)		F	P	M.S.D
	Control ±SE	*Beauveria bassiana* ±SE			
24	3.83±0.12[b]	5.03±0.15[a]	40.50	0.0003	0.524
48	4.07 ±0.15[a]	3.6±0.12[a]	6.32	0.066	0.515
72	3.73±0.13[a]	3.4±0.06[a]	5.26	0.0835	0.403

[a]Mean, within a row, bearing different subscripts are significantly different.

2.7. Determination of peroxidases activity:

Peroxidases as antioxidant enzymes play a pivotal role of defense against pathogens and insecticides (Jia *et al.,* 2016). Table (7) obtained that there is a significant increase in peroxidases activity during 24hrs post treatment than control (8.6 and 6.2 O.D./min/g.b.wt, respectively), while the activity of peroxidases was stable and not significant decreased after 48 and 72 hrs post treatment . Result revealed that seed bugs *G. servus* were highly susceptibility to infection by entomopathogenic fungi *B. bassiana* and represent as the first record documenting the pathogenicity of *B. bassiana* on *G. servus* . When living spores come into contact with an

insect's cuticle, they germinate, and then penetrate the insect's cuticle and start to expand within its host' s body, reducing the insect's ability to feed and movement (Elbanna *et al*., 2012). Trudeau *et al*. (2001) confirmed that invasion of the insect body and haemolymph occurs conidia bind to the cuticle of a suitable host and germinate, triggering a series of recognition and enzyme activation reactions in both the host and the fungal parasite. The activation of the phenoloxidates and other cascade enzymes occurs as a result of protective responses to fungi infection. The activities of glutathione s transferases, nonspecific esterases, and alkaline phosphatases were altered by fungi, which is thought to be a nonspecific body reaction to integument harm or fungal toxin. Fungi also evolved strategies to defeat the immune systems of insects over time. The first mechanism involves modifying the morphology and formation of their cell surface in order to evade the host immune system(Cao *et al*., 2016). Entomopathogenic fungi, for example, have cells with various morophologies, such as no cell wall or cells that are almost in the host hemolymph; these types help in the dispersion and clustering of entomopathogenic fungi in the hemocoel and may increase the surface area to consume further nutrients (Cao *et al*., 2016).

Table (7): Effect of *Beauveria bassiana* on peroxidases activity in *Graptostethus servus*..

Hours after treatment	Peroxidases (O.D./min/g.b.wt)		F	P	M.S.D
	Control ±SE	*Beauveria bassiana* ±SE			
24	6.2±0.25[b]	8.6±0.31[a]	36.77	0.0037	1.098
48	4.9 ±0.06[a]	4.6±0.12[a]	5.40	0.0808	0.358
72	6.83±0.09[a]	3.87±0.15[a]	304.65	< 0.0001	0.472

[a]Mean, within a row, bearing different subscripts are significantly different.

In this study there is inhibition of detoxification enzymes sharply increases insect death rate from fungal infection which indirectly confirms the involvement of detoxification enzymes in the formation of insect resistance to entomopathogenic fungi. *B. bassiana* was found to be severely deficient in total protein after 48 hours in the current research. According to Gillespie *et al*. (2000) , a decrease in the total protein content of adult *S. gregaria* haemolymph during infection with *M. anisopliae* was reported. During parasitism, the parasite may secrete proteolytic enzymes into the insect's haemocoel, which hydrolyze the host protein, causing the host haemolymph to lose soluble protein. Furthermore, after 24 hours of care, the total carbohydrates content of the infected decreased. This result agrees with Elbanna *et al*. (2012) who reported a decreased in total protein and total carbohydrates contents after 24h of infected *S. gregaria* with *M. anisopliae*.

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