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Effect of entomopathogenic fungi *Beauveria bassiana* on the cellular immunity and biochemistry of green bug *Nezara viridula* L.

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ABSTRACT

Entomopathogenic fungi (EPF) are considered effective methods for investigating insects' immunity in response to infection. Insects have a variety of defense mechanisms against infectious diseases, namely phenoloxidase, hemocytes, detoxification and antioxidant enzymes. The instinctive immune system of insects is highly advanced and depends on humoral and cellular elements. In this study, five haemocytes types were found in fifth nymphal instar and adults of green bug, *Nezara viridula* (Heteroptera: Pentatomidae) namely; prohaemocyte (pr), granulocyte (gr), spherulocyte (sp), plasmatocyte (pl), coagulate cells (co) and oenocytoid (oe). Infection with *Beauveria bassiana* significantly decreased the different haemocytes count in both fifth nymphal instar and adults, especially after 72h post-infection. The haemocytes' response against fungi was also investigated. A significant increase in Alpha esterase (EST), Acid phosphatise (ACP) and Alkaline phosphatise (ALP) were observed after 72h post infection in the haemolymph of *N. viridula* adults. In contrast, phenoloxidase activity significantly decreased in adults' hemolymph after the same period of infection. Results indicated that infection with *B. bassania* affects the number of circulating haemocytes and decreases phenoloxidase activity.

Keywords: *Beauveria bassiana*, *Nezara viridula*, Alpha esterases, Acid phosphatase, Phenoloxidase and Alkaline phosphatase, haemocyte response.

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INTRODUCTION

The green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae) is considered as one of the most important pests that is distinguished by a wide host range and a global distribution. This insect species has the ability to damage more than 30 plant types affiliated to different families. *N. viridula* can attack field crops, vegetables, fruit trees and ornamental plants (Pushnya *et al*., 2020). It feeds by inserting its piercing sucking mouth parts into plant tissues and introducing salivary digestive enzymes, making holes which allow microorganisms entrance causing many plant diseases (Nada, 2015). Medrano *et al.* (2009) mentioned that *N. viridula* can transmit *Pantoea agglomerans* bacteria (Ewing and Fife) into green cotton bolls.

Biological control is an alternative method for controlling *N. viridula* other than using insecticides to avoid their hazardous effect on human health and environment (Permadi *et al*., 2019). Gupta and Dikshit (2010) demonstrated that biopesticides based on target pest native pathogenic microorganisms provide an environmentally sound control method.

Entomopathogenic fungi are recognized as promising insects biological control agents. There are over 700 reported species of entomopathogenic fungi (Rabindra and Ramanujam, 2007), however, some species have been effectively utilized on a wide scale as microbial control agents such as *Beauveria*

bassiana, that attack a numerous numbers of pests' species. The fungus *B. bassiana* (Hyphocreales, Ascomycota) is an optional saprophytic fungus that infects and consumes insects and other arthropods. Furthermore, Srinivasan (2012) ensured that entomopathogenic fungi play a crucial role in insect pests control in humid tropics, *B. bassiana* and *Metarhizium anisoplae* accounting for about 68 percent of entomopathogenic fungi dependent on microbial pesticides. Many previous studies reported the effectiveness of *B. bassiana* against *N. viridula* (Hasnah *et al*., 2012; Nada 2015; Permadi *et al*., 2019; Pushnya, 2020). The mode of action of entomopathogenic fungi differs from pathogenic bacteria and viruses, by breaching host cuticle and passing through the integument to reach insect hemocoel by mechanical and enzymatic processes. It continuously produces blastospores throughout the infection process (Mirhaghparast *et al.*, 2013).

On the other hand, insects have many effective ways to control microbial pathogens. Infection stimulates host cellular defenses causing blood clotting and activation of a prophenoloxidase activity leading to melanization. Cellular immunity includes haemocytes as phagocytosis defenses, nodulation and encapsulation of pathogens. Haemocytes play an essential role in insects' immune responses against different pathogens (Russo *et al.*, 2001). Insects have different types of hemocytes that are determined by their morphological and functional characteristics (Gupta, 1985). There are five major haemocytes types that were reported in most insect species, i.e. prohemocytes, oenocytoids, plasmatocytes, granulocytes, and spherule cells. Nodulation is a complex behavior that begins rapidly following a microbial infection. The development of the nodules starts with the micro-aggregation of hemocytes that capture many numbers of microorganisms. These micro-aggregates increase in size by gathering sufficient haemocytes. Ultimately, nodulation process comes to end and a dark nodule is developed by melanization, where they are attached to cuticle and to various internal organs (Ratcliffe *et al.*, 1985). Phenoloxidase (PO) is a melanizing enzyme which is produced as a result of insect infection by activated hemocytes (Abou Taleb *et al.*, 2015). PO activity induces the formation of melanin coat around the invading pathogen. PO defense requires the creation of quinones (Castrillo and Brook, 1998). Humoral immune include several enzymes and antimicrobial proteins (AMP) produced in insect fat bodies and haemocytes which are the main target of bacterial infections (Wang and Leger, 2006; Abdel Haleem *et al*., 2020). Accordingly, the current study aimed to determine the effect of the entomopathogenic fungi, *B. bassiana,* on cellular immunity and the activity of some detoxification enzymes on the green bug *N. viridula.*

MATERIALS AND METHODS

Insect colony

Nymphs and adults of *N. viridula* were collected from an experimental station affiliated to the Faculty of Agriculture, Giza. Green bug was transferred to the laboratory and maintained on potted broad *Vicia fabae* L. that was supplemented by fresh green beans *Phaseolus vulgaris* L. These fresh green beans were renewed every two-day intervals. Pots were covered with cylindrical glass. Egg-masses were collected daily and placed in petri dishes containing pieces of moistened cotton wool, supplemented with fresh green beans until hatching. Finally, adult and 5^{th} nymphal instar were collected and used for the following experiments (Nada, 2006).

Entomological fungi isolated

A *Beauveria bassania* strain was originally isolated from Dakahalia Governorate's soil in Egypt and according to the method described by Nada (2006). The fungus was cultured on autoclaved Sabroud Dexstrose Agar with Yeast

(SDAY), containing 1% peptone, 0.2% yeast extracts, 4% dextrose and, 1.5% agar dissolved in 1L distilled water and incubated for two weeks at 25±1ºC.

Bioassay procedure

Spores were harvested by rinsing with sterilized aqueous solution of 0.02% Tween 80, then filtered through cheesecloth to reduced mycelium clumping. Spores were counted in the suspension using a haemocytometer (Neubauer improved HBG, Germany 0.100 mm x 0.0025 mm^2). A concentration of $1x10^7$ spores/ ml of isolated spores was prepared. An aqueous solution of 0.02% Tween 80 was used as a control (Nada, 2015).

Biochemical analysis

Adults were treated with $1x10^7$ spores/ml of *B. bassiana* for enzyme analysis. Surviving adults were used to assess the function of enzymes using the methods of Gholamzadeh-Chitgar *et al*. (2015).

Sample preparation

Adults of *N. viridula* were collected after 24, 48 and 72h post infection (n = 20 per treatment for each enzyme). 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 12000 g for 10 min at 4°C. The supernatant was used as an enzyme solution for assessing the phenoloxidase, alkaline phosphatase, Acid phosphatase and α-esterase.

Enzymes assay

PO activity measured as described by Söderhäll (1984). Activity of alpha esterase (α-esterase) was estimated using Van Asperen (1962) method. Acid and alkaline phosphatases were determined according to the method described by Powell and Smith (1954).

Haemolymph collection and haemocyte count

To examine the difference in haemocytes count (DHC), one hundred cells were identified to their typical haemocytes type after staining a smear of hemolymph with Wright's stain under oil immersion and phase contrast using Leitz microscope. (Mahmood and Yousuf, 1985; Gad and El-DaKheel, 2009).

Statistical analysis

All data was expressed as the mean ± standard error. Statistical analyses were performed with one-way analysis of variance followed by unpaired two-tailed Student's t-test. A p-value < 0.05 was considered to be significant (Steel and Torrie, 1980).

Results and Discussion

***N. viridula* cellular immunity**

In the present study, we used *N. viridula* as a model to test the pathogenesis of entomopathogenic fungi *B. bassania*. Five morphological forms of circulating hemocytes have been detected in *N. viridula* haemolymph: prohaemocytes (PRs), granulocytes (GRs), plasmatocytes (PLs), oenocytes (OEs) and spherulocytes (SPs) (Fig. 1).

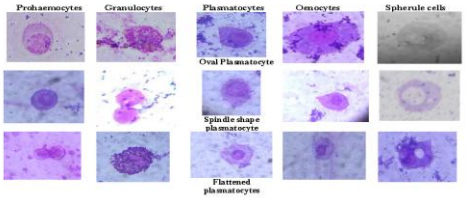

Figure 1: Fresh smear of haemolymph (*in vitro*) from healthy 5th nymphal instar and adult of *N. viridula*.

Moreover, three morphological subtypes of PLs are investigated: oval PLs, spindle shape PLs and flattened PLs. All five types of haemocytes have been found in the haemolymph of normally developing fifth nymphal instar and adult stage: PRs, PLs, GRs, SPs and OEs (Fig. 1). Number of haemocytes fluctuated during the development of the 5th nymphal instar to adult stage. GRs dominated, with a concentration ranging from 41.5 to 52.7 cells during 24-72hrs of nymphal haemolymph. GRs number was twice more than PLs, ranging from 22.5 to 29.8 cells of haemolymph (Fig. 2). Uninfected nymphs, on the other hand, showed lower numbers of OEs and SPs than that of GRs, ranging from 8.5 to

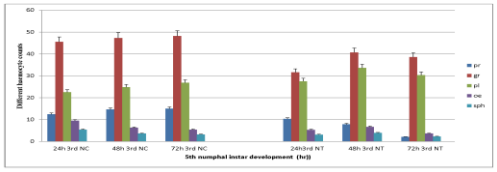

Figure 2: Effect of fungal infection on the different haemocytes counts of *N. viridula* 5th numphal instar

7.4 in the number of OEs, whereas numbers of SPs ranging from 3.5 to 2.8 cells of haemolymph (Fig. 2). The effect of fungal infection on the different hemocyte counts of *N. viridula* 5th nymphal instar and adult stage was also investigated. After fungal infection, different haemocyte count significantly decreased in 5th nymphal instar especially after 72h by about 70.6% in PRs, 19.9% in GRs, 33.3% in OEs and 28% in SPs compared to the control group, while PLs significantly increased by about 13.05% compared to control (Fig. 2).

Furthermore, the same trend was observed in adult's different haemocyte counts after fungal infection (Fig.3). Fungal infection caused significant reduction in adult's different haemocyte counts, especially after 72hrs by about 70.6% in PRs, 41.8% in GRs, 25.8% in OEs and 28% in SPs compared to control group, while the number of PL increased about 11.6% (Fig.3).

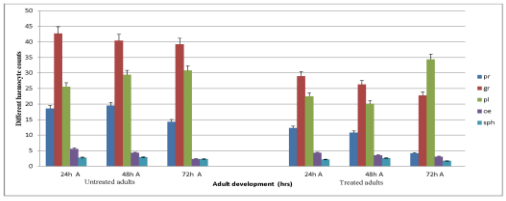

Figure 3: Effect of fungal infection on the haemocytes of *N. viridula* adult's stage.

Results of the present investigation proved to be in agreement with those of Bogu's, *et al.* (2017) who studied the effect of *Conidiobolus coronatus* strain number 3491, isolated from *Dendrolaelaps* spp. on *G. mellonella* and reported haemolymph THC and DHC significantly decrease in the L7 larvae post fungal infection (1d and 5d). This reduction could be attributed to insect immunity responses against fungal infection i.e. encapsulation process (Rivers *et al.*, 2002; Andrade *et al.*, 1984). Furthermore, significant reduction was noticed also in the number of different haemocytes in larvae of *Bombyx mori* and *Spodoptera exigua* infected by *B. bassiana* as stated by Hung *et al.* (1993) and Rajitha *et al*. (2013). On the other hand, Noskov *et al.* (2019) suggested that immune and detoxifying systems responses disruption during combined therapy and the production of opportunistic bacteria may be among the causes of the synergistic impact.

In contrast, response to pathogen infection that induced hematopoesis and haemocyte mitosis, some studies recorded a significant increase in TCH in insect haemolymph (Ratcliffe, 1993; Russo *et al.*, 2001; Chen *et al.*, 2002; Kumar *et al.*, 2011). Other studies were also not in accordance with our results, i.e. Sapna *et al.* (2015) demonstrated that the total hemocyte counts (THCs) showed an initial increase (from 6 to 9 hours) in *B. bassiana*-infected housefly, followed by a subsequent decrease (9-12 h) with an increase in infection time. The THCs in infected flies were significantly greater than non-infected ones. Differences in results might be attributed to the difference in host response.

N. viridula haemocytes morphology and behavior

During the first day post fungal infection, cell appearance began to change (Fig.4). The OEs adhered to the Pl and Gr cellular network (A_1). This network became more clustered after 24 and 48 h post infection ($A_{2,3}$). It was difficult to identify the component cells and nodule (A_4). The elongated PLs developed a pseudopodial shaped and pushed towards other haemocytes (B_1). The cultured haemocytes form a cellular network consisting primarily from PLs and GRs and formed capsule (B_2).

Melanization of clustered cells was frequently observed in cultures after 48hrs post infection (Fig. B3).

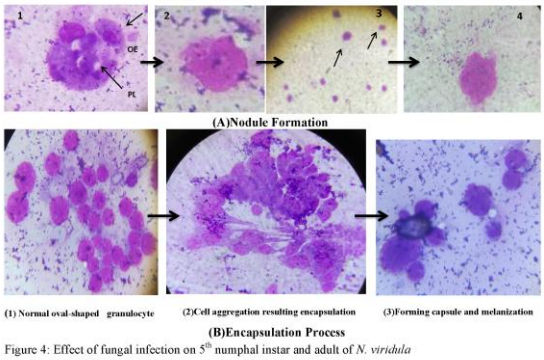

Figure 4: Effect of fungal infection on 5th numphal instar and adult of *N. viridula*

Similar findings were obtained by (Sato *et al.*, 1976) and Schmit and Ratcliffe (1977) as they observed that granular cells accompanied by plasmatocytes were the first to bind to foreign surfaces. Encapsulation occurs when granular cells bind several layers of plasmatocytes to the external target and capsule termination is accomplished by the creation of a peripheral monolayer of granular cells. Our results also proved that after fungal infection, oenocyte cell had irregular cell boundary with cytolysis (Fig.5a).

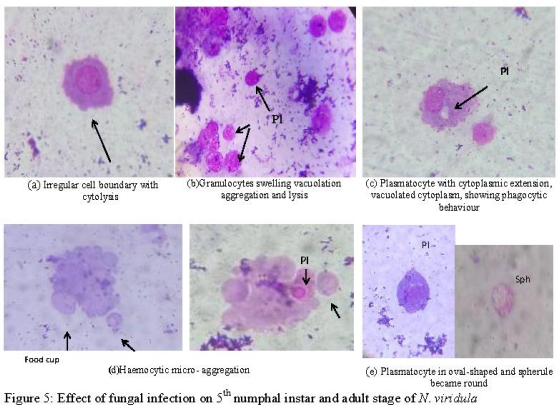

Figure 5: Effect of fungal infection on 5th numphal instar and adult stage of *N. viridula*

Despite that, granulocytes apparently lost granules, since cells from infected adults had fewer granules than those from uninfected, some granulocytes swell, vacuolate, aggregate and lysis (Fig.5b). Morphological changes were also shown by plasmatocytes of the fungal infected adults showing vacuoles in their cytoplasmic extension and phagocytic activity (Fig. 5c). In addition, many micro-aggregation haemocytes and nodules were observed in the haemolymph of 5th nymphal instar and adult stages of *N. viridula* (Fig. 5d) 72hrs post infection.

These results are in agreement with Masaya *et al*. (2019). They noticed that not only granular cells were independently bound to the flller in foreign targets and distributed to them but also plasmatocytes. Moreover, plasmatocytes themselves may appear extraneousness. The SPs looked natural and were round and smaller in fresh hemolymph than the SPs (Fig. 5e). After 72hrs post infection, the number of oval-shaped PLs began to be more visible (Fig. 5e). PLs dominance suggests that these cells play a significant role in the immune system of insects. Mitotic division may be due to the increased number of PLs found in infected *N. viridula* 5th nymphal instar and adults because in -vitro cultures multiple dividing oval PLs are observed. It was also observed in the present study that *N. viridula* PLs are spread extensively 24 hrs post infection, and elongated to be larger than normal cells. Dean *et al.* (2004) noticed similar changes in PLs after infection of *Manduca sexta* with *B. bassiana*. These cells are hyper-spreading haemocytes' and it is reported to be involved in nodulation and invader trapping.

Enzyme activities

Our results on PO activities match those reported by Elbanna *et al*. (2012) who found that this enzyme fluctuated between decreasing and increasing at different doses. Mullen and Goldsworthy (2006) noticed a temporary increase in phenoloxidase activity that temporary raised it in the haemolymph in locusts fifth instar nymphs and adults after

Table 1. Effect of *Beauveria bassiana* infection on the enzymes profile of *Nezara viridula* adults.

Samples	Phenol oxidase (O.D units X10^3/min/g.b.wt)	Alpha esterase (µg α-naphthol/min/g.b.wt)	Alkaline phosphatise (U X 10^3 /g.b.wt)	Acid phosphatise (U X 10^3 /g.b.wt)
B24	220±6.7[a]	256.3±20.9[c]	480. 3±10.5[d]	174.33±8.9[d]
B48	201.7±3.2[b]	315±15.09[b]	507. 7±9.07[c]	204.33±9.3[c]
B72	199±3.0[b]	453.7±38.9[a]	533. 7±14.8[b]	228.67±10.3[b]
Control	204.3±9.3[b]	353. 3±47.3[b]	703. 3±24.8[a]	253.33±10.3[a]

- Mean in same column followed by the same letters is not significant. -Probability level at 0.05.

injecting blastospores with Met 189. *Locusta migratoria* nymphs treated with *Metarhizium anisopliae* showed high level of PO enzyme during the early period but decreased later (Jia *et al*., 2016). Cao *et al.* (2016) explained that when two isolates of *M. anisopliae* were assayed for the above enzyme's activities in locust from 1st to 7th days after application with a single concentration of IM1330189 or IBC200614 isolates, no obvious impact was noticed on PO activity treated with IM1330189 strain which might overcome the immunity provided by PO, yet, a significant increase in PO activity was recorded by IBC200614 strain suggesting that insects are immunized by PO. Also, Wang *et al.* (2012) indicated that disruption of the synthesis of destruxin in *M. roberstii* led to the inability of silkworm larvae to inhibit melanisation development.

Nonspecific esterase Activity (EST)

The current investigation proved there were significant differences ($P \leq 0.05$) in *N. viridula* esterase activity after 24, 48, and 72hrs of fungal infection. Infection inhibited esterase activation after 24 and 48h post infection (256.33 and 315 µg α-naphthol/ min/ g.b.wt), respectively. In contrast, we observed a significant increase in EST activity after 72 hrs post infection than control (Table 1). Our results matches what was found by Petlamul *et al*. (2019) who reported that EST activity in the haemolymph of *Helicoverpa armigera* larvae treated with *B. bassiana* was significantly induced. The highest activities were observed after 72h post treatment and were higher than control. After 72 -120 h EST was significantly higher than control and its secondary metabolites increased ESTs and GSTs activities in the haemolymph of infected and treated adults of *E. integriceps* (Zibaee *et al*., 2009). Cao *et al.* (2016) reported a reduction in ESTs activities by *M. anisoplae* isolate IM1330189. They assumed that immunity of ESTs could be overcome by inhibiting the transcription of ESTs while the uncertain effect on the activity of ESTs by IBC200614 indicated that the insect resistance to IBC200614 is due to the stability of ESTs activities.

Acid and alkaline phosphatase

Acid and alkaline phosphatase are the hydrolytic enzymes responsible for removing phosphate groups from several molecules form, including nucleotides, proteins and alkaloids in alkaline and acidic conditions, under the name of dephosphorylation (Zibaee *et al*., 2009). The enzymes are also involved in lipid hydrolysis in many tissues such as midgut, hemolymph and fat bodies (Zibaee *et al*., 2009).

There was a highly significant reduction in ALP activity during 24- 48 and 72 h post treatment than control (Table1). Results presented in Table (1) indicate a high reduction in APL activity during 24 -48 and 72h post treatment than control. After 24h, APS activity decreased dramatically as compared to control (174.33 and 253.33 UX10^3/g. B. wt), respectively.

Whereas, 48-72hrs post-treatment have caused a huge increase in APS activity (204.33 and 228.67 UX10^3/g. B. wt), respectively. This excessive increase could be attributed to the secretion of this enzyme by the pathogenic fungus as highlighted by Castrillo and Brooks (1998). This explanation is confirmed by the fact that alkaline phosphatase is considered one of the major biomarkers to differentiate between *B. bassiana* different isolates found in the dark beetle, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae).

It is concluded that fungus infection with *B. bassania* increased detoxification enzyme as a protective mechanism in insects and also influenced the number of circulating haemocytes as a result of fungal infection.

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